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(54) **Titre : MEDICAMENT THERAPEUTIQUE INDUISANT DES LESIONS CELLULAIRES A UTILISER DANS LE TRAITEMENT DU
CANCER**
(54) **Title: CELL INJURY INDUCING THERAPEUTIC DRUG FOR USE IN CANCER THERAPY**

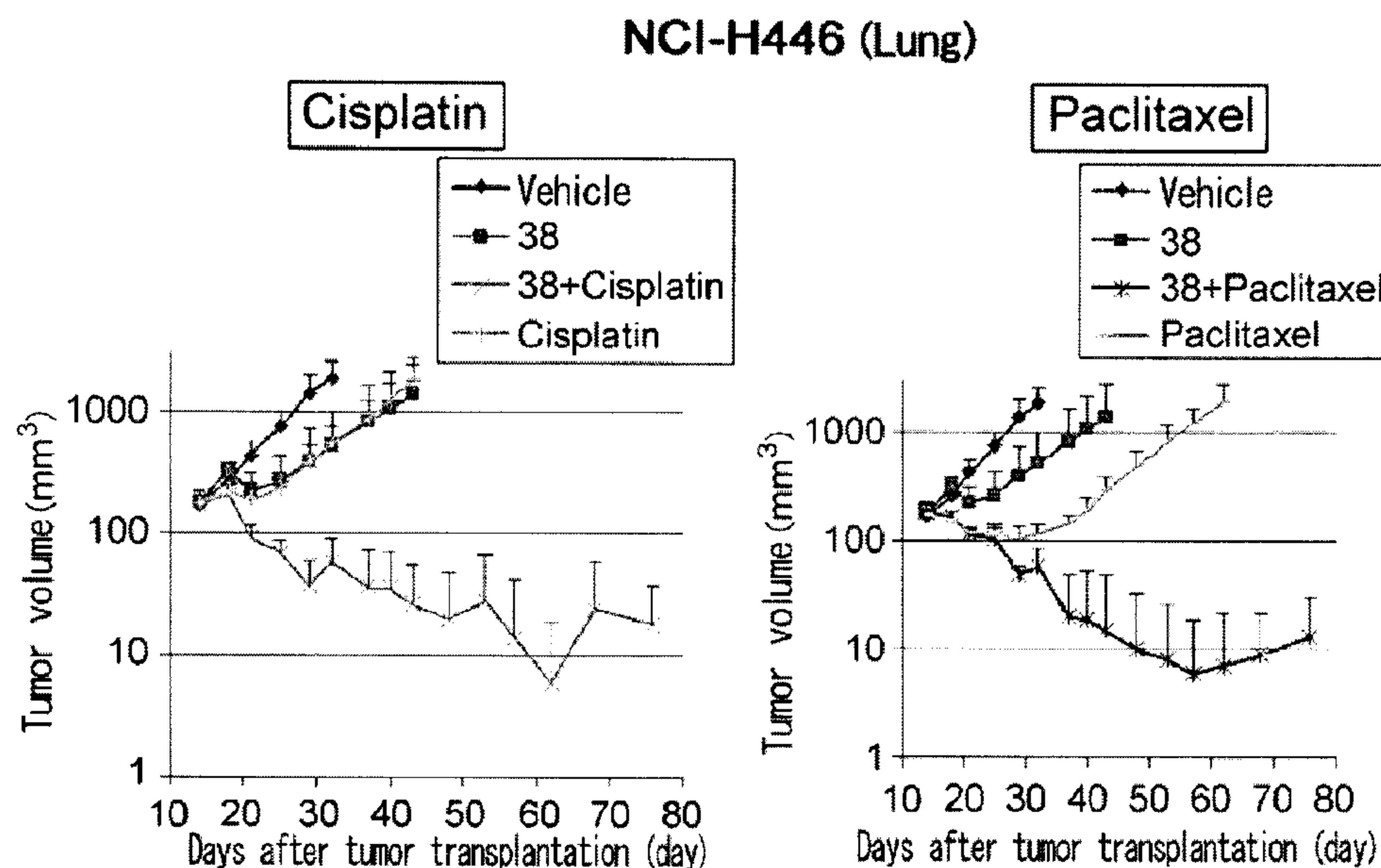


FIG. 8

(57) **Abrégé/Abstract:**

The present invention provides: an anticancer drug having, as an active ingredient, a bispecific antibody that contains, in a domain including an antibody variable region that binds to glypican-3 and a domain including an antibody variable region that binds to a T-

(57) Abrégé(suite)/Abstract(continued):

cell receptor complex, a common L chain capable of improving affinity for both antigens; and a pharmaceutical composition that includes the bispecific antibody as an active ingredient and that is for concomitant use with another anticancer drug. The bispecific antibody is a novel and highly-safe molecule having high production efficiency, as well as strong antitumor activity and excellent pharmacokinetics, which is expected to be applied to various cancers.

ABSTRACT

The present invention provides anticancer agents comprising as an active ingredient a bispecific antibody that comprises a domain comprising a glypican 3-binding antibody variable
5 region, a domain comprising a T cell receptor complex-binding antibody variable region, and common L chains that can enhance the affinity for the two antigens, as well as pharmaceutical compositions comprising the bispecific antibody as an active ingredient, the compositions being for use in combination with other anticancer agents. The bispecific antibodies are novel molecules which are produced with high efficiency and have strong anti-tumor activity as well as
10 safety and excellent pharmacodynamics. The bispecific antibodies can be expected to be applied to various cancers.

DESCRIPTION

CELL INJURY INDUCING THERAPEUTIC DRUG FOR USE IN CANCER THERAPY

5 Technical Field

The present invention relates to multispecific antigen-binding molecules, uses thereof, and such.

Background Art

10 Antibodies are drawing attention as pharmaceuticals because of their high stability in plasma and few adverse reactions (Non-patent Documents 1 and 2). Antibodies are known to induce not only an antigen-binding action, an agonistic action, and an antagonistic action, but also effector-mediated cytotoxic activities (also called effector functions) such as antibody-dependent cellular cytotoxicity (ADCC), antibody dependent cell phagocytosis
15 (ADCP), and complement-dependent cytotoxicity (CDC), and exhibit antitumor effects against cancer cells (Non-patent Document 3). ADCC is a cytotoxicity exhibited by effector cells against antibody-bound target cancer cells via binding of the antibody Fc region to an Fc receptor present on effector cells such as NK cells and macrophages. A complement complex binds to the complement-binding site present in an antibody structure. CDC is a cytotoxicity
20 that results from cell destruction where an influx of water and ions into cells is promoted by pore formation on the cell membrane of the antibody-bound cells by complement components present in the complex. A number of therapeutic antibodies showing excellent anti-tumor effects have been developed as pharmaceuticals for cancer treatment (Non-patent Document 4); and while existing therapeutic antibodies have shown excellent actions, the therapeutic outcome achieved
25 by administration of these antibodies is still not satisfactory.

For an antibody to show ADCC, ADCP, and CDC, it is necessary for the antibody Fc region, the antibody receptor (FcγR) present on effector cells such as NK cells and macrophages, and various complement components to bind. In humans, isoforms of FcγRIa, FcγRIIa, FcγRIIb, FcγRIIIa, and FcγRIIIb have been reported as the FcγR protein family, and the
30 respective allotypes have been reported as well (Non-patent Document 5). Among these isoforms, FcγRIa, FcγRIIa, and FcγRIIIa carry a domain called the Immunoreceptor Tyrosine-based Activation Motif (ITAM) in the intracellular domain, and transmit activation signals. On the other hand, only FcγRIIb carries a domain called the Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) in the intracellular domain, and transmits inhibitory
35 signals. Every one of the FcγRs is known to transmit signals via crosslinking by immune complexes and such (Non-patent Document 6). When antibodies actually exert an effector

function on cancer cells, FcγRs on the effector cell membrane form clusters at the Fc regions of several antibodies bound on the cancer cell membrane, and activation signals are transmitted by effector cells. A cytotoxic effect is exerted as a result, but since FcγRs are crosslinked only in effector cells present near cancer cells this time, activation of immunity is shown to occur locally
5 in cancer cells (Non-patent Document 7).

Naturally-occurring immunoglobulins bind to antigens at their variable regions, and bind to receptors such as FcγR, FcRn, FcαR, and FcεR, and complements at their constant regions. FcRn is one of the binding molecules that interact at the IgG Fc region, and since each of the antibody heavy chains binds one molecule of FcRn, two molecules of FcRn have been
10 reported to bind one IgG-type antibody molecule. However, unlike FcRn and such, FcγR interacts at the antibody hinge region and CH2 domain, and only one molecule of FcγR binds to one molecule of IgG-type antibody (Non-patent Document 8). Furthermore, a common naturally-occurring IgG-type antibody recognizes and binds a single epitope via its variable region (Fab); therefore, it can bind to only one antigen. On the other hand, many types of
15 proteins are known to be involved in cancer and inflammation, and there may be crosstalk among the proteins. For example, several inflammatory cytokines (TNF, IL1, and IL6) are known to be involved in immunological diseases (Non-patent Document 9). Furthermore, activation of other receptors is known as one of the mechanisms of cancer in acquiring drug resistance (Non-patent Document 10). In such cases, common antibodies that recognize a
20 single epitope would be unable to inhibit multiple proteins.

Antibodies (bispecific antibodies) that bind to two or more types of antigens with one molecule are being studied as molecules that inhibit multiple targets. It is possible to confer binding activities to two different antigens (a first antigen and a second antigen) by modifying naturally-occurring IgG-type antibodies (Non-patent Document 11). Accordingly, there will
25 not only be neutralization of two or more types of antigens by a single molecule, but also enhancement of antitumor activity due to crosslinks between cells having cytotoxic activity and cancer cells. As molecular forms of a bispecific antibody, a molecule comprising an antigen-binding site added to the N or C terminus of an antibody (DVD-Ig and scFv-IgG), a molecule having different sequences for the two Fab regions of an antibody (common L-chain
30 bispecific antibody and hybrid hybridoma), a molecule in which one Fab region recognizes two antigens (two-in-one IgG), and a molecule having a CH3 region loop site as a new antigen-binding site (Fcab) have been reported so far (Non-patent Documents 12 and 13). Since all bispecific antibodies interact at their Fc regions with FcγR, antibody effector functions are preserved. Thus, the bispecific antibody binds to any antigen that it recognizes and at the
35 same time binds to FcγR, and exhibits ADCC activity against cells expressing the antigen.

If all the antigens recognized by the bispecific antibody are antigens specifically

expressed in cancer, the bispecific antibody exhibits cytotoxic activity to cancer cells when it binds to any of the antigens. Therefore, in comparison to a conventional antibody pharmaceutical that recognizes one antigen, a more efficient antitumor effect can be expected from such an antibody. However, in the case where any one of the antigens recognized by the bispecific antibody is expressed in normal tissues or cells expressed on immunocytes, damage on normal tissues or release of cytokines occurs due to crosslinking with FcγR (Non-patent Document 14). As a result, strong adverse reactions are induced.

A T-cell redirecting antibody that employs cytotoxicity mobilizing T cells as effector cells as the mechanism for its antitumor effect has been known from the 1980s as a bispecific antibody (Non-patent Documents 15, 16, and 17). Unlike antibodies that employ ADCC mobilizing NK cells or macrophages as effector cells as the mechanism for their antitumor effects, a T-cell redirecting antibody is an antibody against any one of the subunits constituting the T-cell receptor (TCR) complex on T cells, and is specifically a bi-specific antibody comprising an antibody that binds to the CD3 epsilon chain and an antibody that binds to an antigen on the target cancer cell. T cells come close to cancer cells via simultaneous binding of the CD3 epsilon chain and a cancer antigen by a T-cell redirecting antibody. As a result, antitumor effects against cancer cells are considered to be exerted through the cytotoxic activity possessed by T cells.

Catumaxomab, which is known as a T-cell redirecting antibody, binds at two Fabs each to a cancer antigen (EpCAM) and to a CD3ε (CD3 epsilon) chain expressed on T cells. Catumaxomab induces T cell-mediated cytotoxic activity by binding to the cancer antigen and the CD3ε at the same time, and induces cytotoxic activity mediated by antigen-presenting cells such as NK cells and macrophages, by binding to the cancer antigen and FcγR at the same time. By use of these two cytotoxic activities, catumaxomab exhibits a high therapeutic effect on malignant ascites by intraperitoneal administration and has thus been approved in Europe (Non-patent Document 18). In addition, there are cases where the administration of catumaxomab reportedly yields cancer cell-reactive antibodies, which clearly shows that acquired immunity is induced (Non-patent Document 19). From this result, antibodies having both T cell-mediated cytotoxic activity and the FcγR-mediated activities by cells such as NK cells or macrophages (these antibodies are particularly referred to as trifunctional antibodies) have received attention because a strong antitumor effect and induction of acquired immunity can be expected.

The trifunctional antibodies, however, bind to CD3ε and FcγR at the same time even in the absence of a cancer antigen and therefore crosslink CD3ε-expressing T cells with FcγR-expressing cells even in a cancer cell-absent environment, leading to production of various cytokines in large amounts. Such cancer antigen-independent induction of production of

various cytokines restricts the current administration of the trifunctional antibodies to an intraperitoneal route (Non-patent Document 20). The trifunctional antibodies are very difficult to administer systemically due to severe cytokine storm-like adverse reactions. In fact, in the Phase I clinical trial of administering catumaxomab systemically to non-small-cell lung cancer patients, a very low dose of 5 µg/body is the maximum tolerable dose, and administration of a larger dose has been reported to cause various serious adverse reactions (Non-patent Document 21).

As such, bispecific antibodies by conventional techniques may bind to both antigens, the first antigen being the cancer antigen (EpCAM) and the second antigen being CD3ε, at the same time when they bind to FcγR; and therefore, in view of their molecular structure it is impossible to avoid adverse reactions caused by the simultaneous binding to FcγR and the second antigen CD3ε.

Meanwhile, unlike catumaxomab, BiTE has no Fcγ receptor-binding site, and therefore it does not cross-link the receptors expressed on T cells and cells such as NK cells and macrophages in a cancer antigen-independent manner. Thus, it has been demonstrated that BiTE does not cause cancer antigen-independent cytokine induction which is observed when catumaxomab is administered. However, since BiTE is a modified low-molecular-weight antibody molecule without an Fc region, the problem is that its blood half-life after administration to a patient is significantly shorter than IgG-type antibodies conventionally used as therapeutic antibodies. In fact, the blood half-life of BiTE administered *in vivo* has been reported to be about several hours (Non-patent Documents 22 and 23). In the clinical trials of blinatumomab, it is administered by continuous intravenous infusion using a minipump. This administration method is not only extremely inconvenient for patients but also has the potential risk of medical accidents due to device malfunction or the like. Thus, it cannot be said that such an administration method is desirable.

In recent years, use of an Fc region with reduced FcγR-binding activity has enabled maintenance of the strong antitumor activity possessed by BiTE and the excellent safety property of not inducing a cytokine storm in a cancer antigen-independent manner, and has provided novel polypeptide assemblies that have long half-lives in blood (Patent Document 1).

On the other hand, when expressing a bispecific antibody by conventional techniques, since two types of H chains and two types of L chains are expressed, ten combinations are conceivable. Among them, only one of the produced combinations has the binding specificity of interest. Therefore, to obtain the bispecific antibody of interest, the single antibody of interest must be purified from the ten types of antibodies, which is very inefficient and difficult.

A method of preferentially secreting IgGs with a heterodimeric combination of H chains, for example, a combination of an H chain against antigen A and an H chain against antigen B, by

introducing amino acid substitutions into the IgG H-chain CH3 region has been reported as a method for solving this problem (Patent Documents 2, 3, 4, 5, 6, 7, and Non-patent Documents 24 and 25). A method that utilizes physical disturbance, i.e., “knob” and “hole”, and a method that utilizes electric charge repulsion have been reported as such methods.

5 To obtain the molecule of interest with better efficiency, methods using L chains that can bind to two different antigens even though the L chains have the same amino acid sequence have been reported (Patent Documents 8 and 9). However, the antigen affinity may decrease greatly with the use of common L chains, and it is difficult to find common L chains that maintain antigen affinity.

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Blinatumomab as a BiTE has been approved for acute lymphoblastic leukemia (ALL) and is also being tested in clinical trials for blood cancer such as non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL). Clinical trials of AMG330, which is a BiTE targeting CD33, have also been started to test it for acute myelocytic leukemia (AML).

15 Furthermore, BiTEs against solid tumors are under development. AMG211, which is a BiTE targeting CEA, AMG110, which is a BiTE targeting EpCAM, AMG212, which is a BiTE targeting PSMA, and such are undergoing in clinical trials. However, the responsiveness of BiTEs against solid tumors remains unproved (Non-patent Document 26).

20 In recent years, immunotherapeutic agents, of which representatives are inhibitors targeting immune checkpoint molecules such as CTLA-4, PD-1, and PD-L1, have been demonstrated to have drug efficacy in clinical settings. However, these pharmaceutical agents are not effective in every patient, and there is demand for further enhancement of the drug efficacy. With respect to combined use of multiple immunotherapies, combined use of Nivolumab and Ipilimumab has been found to achieve the enhanced drug efficacy against
25 melanoma as compared to Ipilimumab alone (Non-patent Document 27).

Citation List

[Patent Documents]

[Patent Document 1] WO2012/073985

30 [Patent Document 2] WO96/27011

[Patent Document 3] WO2006/106905

[Patent Document 4] WO2007/147901

[Patent Document 5] WO2009/089004

[Patent Document 6] WO2010/129304

35 [Patent Document 7] WO2013/065708

[Patent Document 8] WO98/050431

[Patent Document 9] WO2006/109592

[Non-patent Documents]

- [Non-patent Document 1] Nat. Biotechnol. (2005) 23, 1073-1078
- 5 [Non-patent Document 2] Eur J Pharm Biopharm. (2005) 59 (3), 389-396
- [Non-patent Document 3] Drug Des Devel Ther (2009) 3, 7-16
- [Non-patent Document 4] Clin Cancer Res. (2010) 16 (1), 11-20
- [Non-patent Document 5] Immunol. Lett. (2002) 82, 57-65
- [Non-patent Document 6] Nat. Rev. Immunol. (2008) 8, 34-47
- 10 [Non-patent Document 7] Ann. Rev. Immunol. (1988). 6. 251-81
- [Non-patent Document 8] J. Bio. Chem., (20001) 276, 16469-16477
- [Non-patent Document 9] Nat. Biotech., (2011) 28, 502-10
- [Non-patent Document 10] Endocr Relat Cancer (2006) 13, 45-51
- [Non-patent Document 11] MAbs. (2012) Mar 1, 4(2)
- 15 [Non-patent Document 12] Nat. Rev. (2010) 10, 301-316
- [Non-patent Document 13] Peds (2010), 23(4), 289-297
- [Non-patent Document 14] J. Immunol. (1999) Aug 1, 163(3), 1246-52
- [Non-patent Document 15] Nature (1985) 314 (6012), 628-31
- [Non-patent Document 16] Int J Cancer (1988) 41 (4), 609-15.
- 20 [Non-patent Document 17] Proc Natl Acad Sci USA (1986) 83 (5), 1453-7
- [Non-patent Document 18] Cancer Treat Rev. (2010) Oct 36(6), 458-67
- [Non-patent Document 19] Future Oncol. (2012) Jan 8(1), 73-85
- [Non-patent Document 20] Cancer Immunol Immunother. (2007) 56(9), 1397-406
- [Non-patent Document 21] Cancer Immunol Immunother. (2007) 56 (10), 1637-44
- 25 [Non-patent Document 22] Cancer Immunol Immunother. (2006) 55(5), 503-14
- [Non-patent Document 23] Cancer Immunol Immunother. (2009) 58(1), 95-109
- [Non-patent Document 24] Protein Engineering. (1996) vol.9, p.617-621
- [Non-patent Document 25] Nature Biotechnology. (1998) vol.16, p.677-681
- [Non-patent Document 26] Immunological Reviews. (2016) vol.270, p.193-208
- 30 [Non-patent Document 27] N Eng J Med (2015) vol.373, p.23-34

Summary of the Invention

[Problems to be Solved by the Invention]

- The present invention was achieved in view of the above circumstances. An objective
- 35 of the present invention is to provide anticancer agents comprising as an active ingredient multispecific antigen-binding molecules that bring T cells close to the target cancer cells, and can

treat cancer through the cytotoxic activity of T cells against target cancer tissues containing glypican 3-expressing cells, and are molecular forms that can be produced with high efficiency. Another objective of the present inventions is to provide combination therapies using the multispecific antigen-binding molecules and other pharmaceutical agents.

5

[Means for Solving the Problems]

The present inventors discovered an L chain common to a domain comprising a glypican 3-binding antibody variable region, and a domain comprising a T-cell receptor complex-binding antibody variable region, where the common L chain is capable of improving affinity to both antigens. This allows preparation of molecular forms that can be produced with high efficiency, and further discovery of novel multispecific antigen-binding molecules that maintain the strong antitumor activity possessed by T-cell redirecting antibodies such as BiTE and the excellent safety property of not inducing a cytokine storm in a cancer antigen-independent manner, and also have long half-lives in blood. Furthermore, the present inventors discovered that the multispecific antigen-binding molecules comprising common L chains target glypican 3-expressing cancer cells and cause cytotoxicity. Based on this discovery, the present inventors elucidated that the multispecific antigen-binding molecules of the present invention cause injury to cancer tissues containing glypican 3-expressing cancer cells. The present inventors revealed anticancer agents that comprise as an active ingredient the multispecific antigen-binding molecule; methods for treating or preventing cancer by combined use of the multispecific antigen-binding molecule and another anticancer agent; multispecific antigen-binding molecules, anticancer agents, or pharmaceutical compositions comprising a combination of a multispecific antigen-binding molecule and an anticancer agent, each of which is used in combination therapies.

25

Specifically, the present invention provides the following:

- [1] An anticancer agent comprising as an active ingredient a bispecific antibody of any one of (a) to (c) below that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity:
- 30 (a) a bispecific antibody in which CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences

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having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody in which the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody which has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[2] An anticancer agent comprising as an active ingredient a bispecific antibody that comprises the antibody H chain of SEQ ID NO: 385 having glypican 3-binding activity, the antibody H chain of SEQ ID NO: 402 having CD3-binding activity, and the common L chains of SEQ ID NO: 410.

[3] The anticancer agent of [1] or [2], wherein the cancer is a glypican 3-positive cancer.

[4] The anticancer agent of [3], wherein the glypican 3-positive cancer is a cancer in which the number of glypican 3 antigens on cell surface per cell is 100 or more.

[5] The anticancer agent of any one of [1] to [4], wherein the cancer is any cancer selected from the group consisting of gastric cancer, head and neck cancer, esophageal cancer, lung cancer, liver cancer, ovary cancer, breast cancer, colon cancer, kidney cancer, skin cancer, muscle tumor, pancreas cancer, prostate cancer, testis cancer, uterine cancer, cholangiocarcinoma, Merkel cell carcinoma, bladder cancer, thyroid cancer, schwannoma, adrenal cancer, anus cancer, central nervous system tumor, neuroendocrine tissue tumor, penis cancer, pleura tumor, salivary gland tumor, vulva cancer, thymoma, and childhood cancer.

[6] The anticancer agent of any one of [1] to [5], which is for treating a patient having cancer that is refractory to treatment with an immune checkpoint inhibitor.

[7] A pharmaceutical composition for use in combination with another anticancer agent, the pharmaceutical composition comprising as an active ingredient a bispecific antibody of any one of (a) to (c) below that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity:

(a) a bispecific antibody in which CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206,

respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences
 5 having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody in which the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80%
 10 identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody which has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H
 15 chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[8] A pharmaceutical composition comprising as an active ingredient a bispecific antibody that has an antibody H chain of SEQ ID NO: 385 having glypican 3-binding activity, an antibody H
 20 chain of SEQ ID NO: 402 having CD3-binding activity, and common antibody L chains of SEQ ID NO: 410, wherein the pharmaceutical composition is for use in combination with another anticancer agent.

[9] The pharmaceutical composition of [7] or [8], wherein the bispecific antibody is administered simultaneously with said another anticancer agent.

25 [10] The pharmaceutical composition of [7] or [8], wherein the bispecific antibody is administered before or after administration of said another anticancer agent.

[11] The pharmaceutical composition of any one of [7] to [10], wherein said another anticancer agent is a chemotherapeutic agent, a T cell-activating agonist agent, an immune checkpoint inhibitor, or an angiogenic inhibitor.

30 [12] The pharmaceutical composition of [11], wherein the chemotherapeutic agent is an antimetabolite, a plant alkaloid, or a platinum compound.

[13] The pharmaceutical composition of [11], wherein the T cell-activating agonist agent is an agonist antibody against TNFRSF.

35 [14] The pharmaceutical composition of [11], wherein the immune checkpoint inhibitor is a PD1 antibody, a PDL1 antibody, a TIM3 antibody, or an LAG3 antibody.

[15] The pharmaceutical composition of [11], wherein the angiogenic inhibitor is a VEGFR2

antibody.

[16] An agent for inducing cytotoxicity, an agent for suppressing cell proliferation, an agent for inhibiting cell proliferation, an agent for activating immune response, an agent for treating cancer, or an agent for preventing cancer, which comprises the pharmaceutical composition of any one of [7] to [15].

[17] The anticancer agent of [1], wherein CDR1, CDR2, and CDR3 are CDR1, CDR2, and CDR3 regions based on Kabat numbering.

The following inventions are also provided:

[2-1] An anticancer agent comprising as an active ingredient a multispecific antigen-binding molecule that comprises:

(1) a domain comprising an antibody variable region having glypican 3-binding activity,
(2) a domain comprising an antibody variable region having T-cell receptor complex-binding activity, and

(3) a domain comprising an Fc region with reduced binding activity towards an Fcγ receptor, wherein the L chain variable regions comprised in the variable region of (1) and the variable region of (2) have a common amino acid sequence; wherein the multispecific antigen-binding molecule has a cytotoxic activity equivalent to or greater than that of the bispecific antibody GPC3_ERY22_rCE115 comprising a glypican 3-binding domain comprising SEQ ID NOs: 47 and 48, and a T-cell receptor complex-binding domain comprising SEQ ID NOs: 49 and 50.

[2-2] The anticancer agent of [2-1], wherein the cytotoxic activity is T-cell-dependent cytotoxic activity.

[2-3] The anticancer agent of [2-1] or [2-2], wherein the T-cell receptor complex-binding activity is binding activity towards a T-cell receptor.

[2-4] The anticancer agent of any one of [2-1] to [2-3], wherein the T-cell receptor complex-binding activity is binding activity towards a CD3ε chain.

[2-5] The anticancer agent of any one of [2-1] to [2-4], wherein the antibody variable region of (1) in [1] is an antibody variable region that comprises any one of the combinations of H-chain CDR1, CDR2, and CDR3 selected from (a1) to (a5) below, or an antibody variable region functionally equivalent thereto:

(a1) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 40;

(a2) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 197;

(a3) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206;

- (a4) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 211; and
 - (a5) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 215.
- 5 [2-6] The anticancer agent of any one of [2-1] to [2-4], wherein the antibody variable region of (2) in [2-1] is an antibody variable region that comprises any one of the combinations of H-chain CDR1, CDR2, and CDR3 amino acid sequences selected from (b1) to (b15) below, or an antibody variable region functionally equivalent thereto:
- (b1) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 52;
 - (b2) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 103;
 - (b3) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 122;
 - 15 (b4) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 128;
 - (b5) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 129;
 - (b6) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 132;
 - 20 (b7) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 142;
 - (b8) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 144;
 - 25 (b9) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 164;
 - (b10) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168;
 - (b11) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 421;
 - 30 (b12) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 424;
 - (b13) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 426;
 - 35 (b14) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 429; and

(b15) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 430.

[2-7] The anticancer agent of any one of [2-1] to [2-4], wherein the antibody variable regions of (1) and (2) in [2-1] are antibody variable regions comprising any one of the combinations of H-chain CDR1, CDR2, and CDR3 selected from the following (c1) to (c19), or antibody variable regions functionally equivalent thereto:

(c1) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 40; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 52;

(c2) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 40; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 421;

(c3) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 40; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 426;

(c4) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 40; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 429;

(c5) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 40; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 430;

(c6) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 197; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 128;

- (c7) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions
5 comprised in SEQ ID NO: 142;
- (c8) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions
10 comprised in SEQ ID NO: 144;
- (c9) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions
15 comprised in SEQ ID NO: 164;
- (c10) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions
20 comprised in SEQ ID NO: 168;
- (c11) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 211; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions
25 comprised in SEQ ID NO: 142;
- (c12) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 211; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions
30 comprised in SEQ ID NO: 144;
- (c13) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 211; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions
35 comprised in SEQ ID NO: 164;
- (c14) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and

- identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 211; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168;
- 5 (c15) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 215; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 103;
- 10 (c16) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 215; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 122;
- 15 (c17) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 215; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 129;
- 20 (c18) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 215; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 132; and
- 25 (c19) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 215; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 424.
- 30 [2-8] The anticancer agent of any one of [2-5] to [2-7], wherein CDR1, CDR2, and CDR3 are CDR1, CDR2, and CDR3 regions based on Kabat numbering.
- [2-9] The anticancer agent of any one of [2-1] to [2-4], wherein the antibody variable region of (1) in [2-1] is an antibody variable region comprising any one of the H-chain variable regions selected from (a1) to (a5) below, or an antibody variable region functionally equivalent thereto:
- 35 (a1) an H-chain variable region having the amino acid sequence of SEQ ID NO: 40;
- (a2) an H-chain variable region having the amino acid sequence of SEQ ID NO: 197;

- (a3) an H-chain variable region having the amino acid sequence of SEQ ID NO: 206;
- (a4) an H-chain variable region having the amino acid sequence of SEQ ID NO: 211; and
- (a5) an H-chain variable region having the amino acid sequence of SEQ ID NO: 215.

5 [2-10] The anticancer agent of any one of [2-1] to [2-4], wherein the antibody variable region of (2) in [2-1] is an antibody variable region comprising any one of the H-chain variable regions selected from (b1) to (b15) below, or an antibody variable region functionally equivalent thereto:

- (b1) an H-chain variable region having the amino acid sequence of SEQ ID NO: 52;
- (b2) an H-chain variable region having the amino acid sequence of SEQ ID NO: 103;
- (b3) an H-chain variable region having the amino acid sequence of SEQ ID NO: 122;
- 10 (b4) an H-chain variable region having the amino acid sequence of SEQ ID NO: 128;
- (b5) an H-chain variable region having the amino acid sequence of SEQ ID NO: 129;
- (b6) an H-chain variable region having the amino acid sequence of SEQ ID NO: 132;
- (b7) an H-chain variable region having the amino acid sequence of SEQ ID NO: 142;
- (b8) an H-chain variable region having the amino acid sequence of SEQ ID NO: 144;
- 15 (b9) an H-chain variable region having the amino acid sequence of SEQ ID NO: 164;
- (b10) an H-chain variable region having the amino acid sequence of SEQ ID NO: 168;
- (b11) an H-chain variable region having the amino acid sequence of SEQ ID NO: 421;
- (b12) an H-chain variable region having the amino acid sequence of SEQ ID NO: 424;
- (b13) an H-chain variable region having the amino acid sequence of SEQ ID NO: 426;
- 20 (b14) an H-chain variable region having the amino acid sequence of SEQ ID NO: 429; and
- (b15) an H-chain variable region having the amino acid sequence of SEQ ID NO: 430.

25 [2-11] The anticancer agent of any one of [2-1] to [2-4], wherein the antibody variable regions of (1) and (2) in [2-1] are antibody variable regions comprising any one of the combinations of H-chain variable regions selected from (c1) to (c19) below, or antibody variable regions functionally equivalent thereto:

- (c1) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 40; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of SEQ ID NO: 52;
- 30 (c2) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 40; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of SEQ ID NO: 421;
- (c3) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 40; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
- 35

SEQ ID NO: 426;

(c4) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 40; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
5 SEQ ID NO: 429;

(c5) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 40; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
10 SEQ ID NO: 430;

(c6) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 197; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
15 SEQ ID NO: 128;

(c7) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 206; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
20 SEQ ID NO: 142;

(c8) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 206; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
25 SEQ ID NO: 144;

(c9) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 206; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
30 SEQ ID NO: 164;

(c10) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 206; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
35 SEQ ID NO: 168;

(c11) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 211; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
SEQ ID NO: 142;

(c12) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 211; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of

SEQ ID NO: 144;

(c13) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 211; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
5 SEQ ID NO: 164;

(c14) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 211; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
10 SEQ ID NO: 168;

(c15) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 215; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
15 SEQ ID NO: 103;

(c16) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 215; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
20 SEQ ID NO: 122;

(c17) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 215; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
25 SEQ ID NO: 129;

(c18) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 215; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
30 SEQ ID NO: 132; and

(c19) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] which has the amino acid sequence of SEQ ID NO: 215; and an H-chain variable region comprised in the antibody variable region of (2) in [2-1] which has the amino acid sequence of
35 SEQ ID NO: 424.

[2-12] The anticancer agent of any one of [2-1] to [2-11], wherein the common L chain of [2-1] is a common L chain comprising any one of the combinations of CDR1, CDR2, and CDR3 selected from (d1) to (d11) below, or a common L chain functionally equivalent thereto:

(d1) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 53;

(d2) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223;

- (d3) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 299;
- (d4) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 301;
- 5 (d5) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 302;
- (d6) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 304;
- (d7) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 306;
- 10 (d8) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 307;
- (d9) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 309;
- 15 (d10) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 310; and
- (d11) CDR1, CDR2, and CDR3 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 319.
- [2-13] The anticancer agent of any one of [2-1] to [2-11], wherein the L chain variable region of [2-1] is a variable region of any one of the L chain amino acid sequences selected from (d1) to (d11) below:
- 20 (d1) an L chain comprising the amino acid sequence of SEQ ID NO: 53;
- (d2) an L chain comprising the amino acid sequence of SEQ ID NO: 223;
- (d3) an L chain comprising the amino acid sequence of SEQ ID NO: 299;
- 25 (d4) an L chain comprising the amino acid sequence of SEQ ID NO: 301;
- (d5) an L chain comprising the amino acid sequence of SEQ ID NO: 302;
- (d6) an L chain comprising the amino acid sequence of SEQ ID NO: 304;
- (d7) an L chain comprising the amino acid sequence of SEQ ID NO: 306;
- (d8) an L chain comprising the amino acid sequence of SEQ ID NO: 307;
- 30 (d9) an L chain comprising the amino acid sequence of SEQ ID NO: 309;
- (d10) an L chain comprising the amino acid sequence of SEQ ID NO: 310; and
- (d11) an L chain comprising the amino acid sequence of SEQ ID NO: 319.
- [2-14] The anticancer agent of any one of [2-1] to [2-4], wherein the antibody variable regions of (1) and (2) of [2-1] and the common L chain variable region are antibody variable regions comprising any one of the combinations of H-chain CDR1, CDR2, and CDR3 and L-chain CDR1, CDR2, and CDR3 selected from (e1) to (e25) below, or antibody variable regions
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functionally equivalent thereto:

- (e1) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 197; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 128; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 53;
- (e2) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 197; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 128; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 299;
- (e3) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 197; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 128; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 310;
- (e4) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 197; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 128; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 319;
- (e5) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 142; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223;

(e6) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in
5 SEQ ID NO: 144; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223;

(e7) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ
10 ID NO: 206; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 164; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223;

(e8) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ
15 ID NO: 206; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3
20 regions comprised in SEQ ID NO: 223;

(e9) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ
ID NO: 211; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1]
25 and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 142; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223;

(e10) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and
30 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 211; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 142; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3
35 regions comprised in SEQ ID NO: 299;

(e11) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and

[illegible]

and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 103; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 309;

5 (e22) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 215; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 122; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of
10 the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 53;

(e23) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 215; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1]
15 and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 129; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 53;

(e24) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and
20 identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 215; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 132; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3
25 regions comprised in SEQ ID NO: 53; and

(e25) CDR1, CDR2, and CDR3 comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 215; CDR1, CDR2, and CDR3 comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in
30 SEQ ID NO: 424; and CDR1, CDR2, and CDR3 comprised in the antibody variable region of the common L chain and identical to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 53.

[2-15] The anticancer agent of any one of [2-1] to [2-4], wherein the antibody variable regions of (1) and (2) of [2-1] and the common L chain variable region are antibody variable regions
35 comprising any one of the combinations of variable regions selected from (f1) to (f26) below, or antibody variable regions functionally equivalent thereto:

identical to the amino acid sequence of SEQ ID NO: 206; an H-chain variable region comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of the common L chain identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 223;

5 (f9) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 211; an H-chain variable region comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 142; and an antibody variable region of the common L chain identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 223;

10 (f10) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 211; an H-chain variable region comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 142; and an antibody variable region of the common L chain identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 299;

15 (f11) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 211; an H-chain variable region comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 144; and an antibody variable region of the common L chain identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 223;

(f12) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 211; an H-chain variable region comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 164; and an antibody variable region of the common L chain identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 223;

25 (f13) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 211; an H-chain variable region comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of the common L chain identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 223;

30 (f14) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 215; an H-chain variable region comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 103; and an antibody variable region of the common L chain identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 53;

35 (f15) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 215; an H-chain variable region comprised

ID NO: 122; and an antibody variable region of the common L chain identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 53;

(f23) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 215; an H-chain variable region comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 129; and an antibody variable region of the common L chain and identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 53;

(f24) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 215; an H-chain variable region comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 132; and an antibody variable region of the common L chain and identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 53;

(f25) an H-chain variable region comprised in the antibody variable region of (1) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 215; an H-chain variable region comprised in the antibody variable region of (2) in [2-1] and identical to the amino acid sequence of SEQ ID NO: 424; and an antibody variable region of the common L chain and identical to the amino acid sequence of the variable region comprised in SEQ ID NO: 53; and

(f26) multispecific antigen-binding molecule that binds to an epitope overlapping with each of the epitopes on glypican 3 and T-cell receptor complex bound by the multispecific antigen-binding molecule of any one of (f1) to (f25), and which has a common L chain.

[2-16] The anticancer agent of any one of [2-1] to [2-15], wherein the Fc region of (3) in [2-1] is an Fc region with an amino acid mutation at any of the Fc region-constituting amino acids of SEQ ID NOs: 23 to 26 (IgG1 to IgG4).

[2-17] The anticancer agent of [2-16], wherein the Fc region of (3) in [2-1] is an Fc region with mutation of at least one amino acid selected from the following amino acid positions specified by EU numbering:

position 220, position 226, position 229, position 231, position 232, position 233, position 234, position 235, position 236, position 237, position 238, position 239, position 240, position 264, position 265, position 266, position 267, position 269, position 270, position 295, position 296, position 297, position 298, position 299, position 300, position 325, position 327, position 328, position 329, position 330, position 331, and position 332.

[2-18] The anticancer agent of [2-16], wherein the Fc region of (3) in [2-1] is an Fc region comprising at least one amino acid selected from the following amino acids specified by EU numbering:

Arg at amino acid position 234, Ala or Arg at amino acid position 235, Lys at amino acid position 239, and Ala at amino acid position 297.

[2-19] The anticancer agent of any one of [2-16] to [2-18], wherein the Fc region of (3) in [2-1] further comprises an amino acid mutation for promoting formation of a heterodimeric Fc region.

[2-20] The anticancer agent of [2-19], wherein the heterodimeric Fc region is the amino acid sequence combination of (g1) or (g2) below:

5 (g1) a combination of an amino acid sequence identical to the Fc region of a constant region comprising the amino acid sequence of SEQ ID NO: 57, and an amino acid sequence identical to the Fc region of a constant region comprising the amino acid sequence of SEQ ID NO: 58; and

(g2) a combination of an amino acid sequence identical to the Fc region of a constant region comprising the amino acid sequence of SEQ ID NO: 60 or 62, and an amino acid sequence
10 identical to the Fc region of a constant region comprising the amino acid sequence of SEQ ID NO: 61.

[2-21] The anticancer agent of any one of [2-1] to [2-20], wherein the multispecific antigen-binding molecule is a bispecific antibody.

[2-22] An anticancer agent comprising as an active ingredient a bispecific antibody of any one
15 of (h1) to (h25) below:

(h1) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain
20 variable region having the amino acid sequence of SEQ ID NO: 424 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 53;

(h2) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H
25 chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 103 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 53;

(h3) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H
30 chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 103 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 299;

- (h4) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 103 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 301;
- (h5) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 103 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 302;
- (h6) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 103 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 304;
- (h7) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 103 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 306;
- (h8) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 103 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 307;
- (h9) a bispecific antibody having an antibody H chain having glypican 3-binding activity that

comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 103 and a constant region
 5 having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 309;

(h10) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H
 10 chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 122 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 53;

(h11) a bispecific antibody having an antibody H chain having glypican 3-binding activity that
 15 comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 129 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain
 20 having the amino acid sequence of SEQ ID NO: 53;

(h12) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 215 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H
 25 chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 132 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 53;

(h13) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO:
 30 197 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 128 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 299;

35 (h14) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO:

197 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 128 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain
 5 having the amino acid sequence of SEQ ID NO: 310;

(h15) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 197 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain
 10 variable region having the amino acid sequence of SEQ ID NO: 128 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 319;

(h16) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 197 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 128 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 53;

(h17) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 211 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 142 and a constant region
 25 having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 299;

(h18) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 211 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 142 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 223;

(h19) a bispecific antibody having an antibody H chain having glypican 3-binding activity that
 35 comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 211 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H

chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 144 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 223;

5 (h20) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 206 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 144 and a constant region
10 having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 223;

(h21) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 206 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H
15 chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 142 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 223;

(h22) a bispecific antibody having an antibody H chain having glypican 3-binding activity that
20 comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 206 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 164 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain
25 having the amino acid sequence of SEQ ID NO: 223;

(h23) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 206 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H
30 chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 168 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 223;

(h24) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO:
35 211 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain

variable region having the amino acid sequence of SEQ ID NO: 164 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 223; and

(h25) a bispecific antibody having an antibody H chain having glypican 3-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 211 and a constant region having the amino acid sequence of SEQ ID NO: 61; an antibody H chain having T-cell receptor complex-binding activity that comprises an antibody H-chain variable region having the amino acid sequence of SEQ ID NO: 168 and a constant region having the amino acid sequence of SEQ ID NO: 60 or 62; and a common antibody L chain having the amino acid sequence of SEQ ID NO: 223.

Furthermore, the present invention relates to an anticancer agent comprising as an active ingredient a multispecific antigen-binding molecule which comprises the following domains:

(3-1) a domain comprising an antibody variable region having glypican 3-binding activity;
 (3-2) a domain comprising an antibody variable region having T-cell receptor complex-binding activity;
 wherein the L-chain variable regions contained in the variable regions of (3-1) and (3-2) have a commonly shared amino acid sequence. The present invention also relates to an anticancer agent comprising as an active ingredient the domain of (3-1), which is more specifically a domain that comprises antibody heavy-chain and/or light-chain variable regions having glypican 3-binding activity, and which is comprised in the multispecific antigen-binding molecule. The present invention additionally relates to an anticancer agent comprising as an active ingredient the domain of (3-2), which is more specifically a domain that comprises an antibody variable region having T-cell receptor complex-binding activity, and which is comprised in the multispecific antigen-binding molecule. Details of the domains of (3-1) and (3-2) may include those described in [2-1] to [2-22] mentioned above. The multispecific antigen-binding molecule may be a bispecific antibody. Furthermore, the multispecific antigen-binding molecule may further comprise a domain comprising an Fc region, and the Fc region may have a reduced Fcγ receptor-binding activity. Details of the domain comprising an Fc region may include those described in [2-1] to [2-22] mentioned above. Furthermore, the present invention relates to an anticancer agent comprising the multispecific antigen-binding molecule and a pharmaceutically acceptable carrier. The anticancer agent may induce cytotoxicity, the cytotoxicity may be T-cell-dependent cellular cytotoxicity, and the agent may be for administration to a patient in need of the multispecific antigen-binding molecule.

The present invention also provides an anticancer agent comprising as an active ingredient a multispecific antigen-binding molecule that binds to epitopes overlapping and/or

competing with epitopes on each of glypican 3 and T-cell receptor complex bound by the multispecific antigen-binding molecule of any one of (e1) to (e25) of [2-14] mentioned above, and a multispecific antigen-binding molecule that binds to epitopes overlapping and/or competing with epitopes on each of glypican 3 and T-cell receptor complex bound by the multispecific antigen-binding molecule of any one of (f1) to (f25) of [2-15].

Regarding (g1) and (g2) of [2-20] mentioned above, of the two Fc regions, the former Fc region may be included in the antibody H chain having glypican 3-binding activity and the latter Fc region may be included in the antibody H chain having T-cell receptor complex-binding activity; or the former Fc region may be included in the antibody H chain having T-cell receptor complex-binding activity and the latter Fc region may be included in the antibody H chain having glypican 3-binding activity.

The present invention also provides anticancer agents comprising as an active ingredient a bispecific antibody that binds to epitopes overlapping and/or competing with epitopes on each of glypican 3 and T-cell receptor complex bound by a bispecific antibody which has the antibody H chain of SEQ ID NO: 385 having glypican 3-binding activity, the antibody H chain of SEQ ID NO: 402 having CD3-binding activity, and the common antibody L chains of SEQ ID NO: 410.

The following inventions are also provided:

[4-1] A pharmaceutical composition comprising another anticancer agent as an active ingredient, the pharmaceutical composition being used in combination with a bispecific antibody of any one of (a) to (c) below:

(a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding

activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-2] The pharmaceutical composition of [4-1], wherein said another anticancer agent is administered simultaneously with the bispecific antibody.

[4-3] The pharmaceutical composition of [4-1], wherein said another anticancer agent is administered before or after administration of the bispecific antibody.

[4-4] A pharmaceutical composition for treating or preventing cancer, comprising a combination of a bispecific antibody of any one of (a) to (c) below and another anticancer agent:

(a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific

antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-5] The pharmaceutical composition of [4-4], which is a combination preparation.

[4-6] The pharmaceutical composition of [4-4], wherein the bispecific antibody and said another anticancer agent are administered separately.

[4-7] The pharmaceutical composition of [4-6], wherein the bispecific antibody and said another anticancer agent are administered simultaneously or sequentially.

[4-8] The pharmaceutical composition of any one of [4-1] to [4-7], wherein said another anticancer agent is a chemotherapeutic agent, a T cell-activating agonist agent, an immune checkpoint inhibitor, or an angiogenic inhibitor.

[4-9] The pharmaceutical composition of any one of [4-1] to [4-8], which is for treating or preventing any cancer selected from the group consisting of gastric cancer, head and neck cancer, esophageal cancer, lung cancer, liver cancer, ovary cancer, breast cancer, colon cancer, kidney cancer, skin cancer, muscle tumor, pancreas cancer, prostate cancer, testis cancer, uterine cancer, cholangiocarcinoma, Merkel cell carcinoma, bladder cancer, thyroid cancer, schwannoma, adrenal cancer, anus cancer, central nervous system tumor, neuroendocrine tissue tumor, penis cancer, pleura tumor, salivary gland tumor, vulva cancer, thymoma, and childhood cancer.

[4-10] An agent for inducing cytotoxicity, an agent for suppressing cell proliferation, an agent for inhibiting cell proliferation, an agent for activating immune response, an agent for treating cancer, or an agent for preventing cancer, which comprises the pharmaceutical composition of any one of [4-1] to [4-9].

The following inventions are also provided:

[4-11] A combination of a bispecific antibody of any one of (a) to (c) below and another anticancer agent:

(a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the

amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

- (b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and
- (c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-12] The combination of [4-11], wherein the bispecific antibody is administered simultaneously with said another anticancer agent.

- [4-13] The combination of [4-11], wherein the bispecific antibody is administered before or after administration of said another anticancer agent.

[4-14] The combination of any one of [4-11] to [4-13], wherein said another anticancer agent is a chemotherapeutic agent, a T cell-activating agonist agent, an immune checkpoint inhibitor, or an angiogenic inhibitor.

- [4-15] The combination of any one of [4-11] to [4-14], which is for treating or preventing any cancer selected from the group consisting of gastric cancer, head and neck cancer, esophageal cancer, lung cancer, liver cancer, ovary cancer, breast cancer, colon cancer, kidney cancer, skin cancer, muscle tumor, pancreas cancer, prostate cancer, testis cancer, uterine cancer, cholangiocarcinoma, Merkel cell carcinoma, bladder cancer, thyroid cancer, schwannoma, adrenal cancer, anus cancer, central nervous system tumor, neuroendocrine tissue tumor, penis cancer, pleura tumor, salivary gland tumor, vulva cancer, thymoma, and childhood cancer.

[4-16] An agent for inducing cytotoxicity, an agent for suppressing cell proliferation, an agent for inhibiting cell proliferation, an agent for activating immune response, an agent for treating cancer, or an agent for preventing cancer, which comprises the combination of any one of [4-11] to [4-15].

- The following inventions are also provided:

[4-17] A method for inducing cytotoxicity, for suppressing cell proliferation, for inhibiting cell

proliferation, for activating immune response, for treating cancer, or for preventing cancer in an individual, comprising administering an effective amount of a bispecific antibody of any one of (a) to (c) below and an effective amount of another anticancer agent:

5 (a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

15 (b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

25 (c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-18] A method for inducing cytotoxicity, for suppressing cell proliferation, for inhibiting cell proliferation, for activating immune response, for treating cancer, or for preventing cancer in an individual with combined use of a bispecific antibody of any one of (a) to (c) below, comprising administering to the individual an effective amount of another anticancer agent:

35 (a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and

CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-19] A method for inducing cytotoxicity, for suppressing cell proliferation, for inhibiting cell proliferation, for activating immune response, for treating cancer, or for preventing cancer in an individual with combined use of another anticancer agent, comprising administering to the individual an effective amount of a bispecific antibody of any one of (a) to (c) below;

(a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-20] A method for enhancing effects of inducing cytotoxicity, suppressing cell proliferation, inhibiting cell proliferation, activating immune response, treating cancer, or preventing cancer in an individual by a bispecific antibody of any one of (a) to (c) below, comprising administering an effective amount of another anticancer agent to the individual:

(a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having
 5 CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-21] A method for enhancing effects of inducing cytotoxicity, suppressing cell proliferation, inhibiting cell proliferation, activating immune response, treating cancer, or preventing cancer in
 10 an individual by another anticancer agent, comprising administering an effective amount of a bispecific antibody of any one of (a) to (c) below:

(a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are
 15 sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an
 20 antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody
 25 variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having
 30 CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-22] The method of any one of [4-17] to [4-21], wherein the bispecific antibody and said another anticancer agent are administrated separately.

[4-23] The method of any one of [4-17] to [4-22], wherein the bispecific antibody and said another anticancer agent are administrated simultaneously or sequentially.

5 [4-24] The method of any one of [4-17] to [4-23], wherein said another anticancer agent is a chemotherapeutic agent, a T cell-activating agonist agent, an immune checkpoint inhibitor, or an angiogenic inhibitor.

[4-25] The method of any one of [4-17] to [4-24], wherein the cancer is any cancer selected from the group consisting of gastric cancer, head and neck cancer, esophageal cancer, lung
10 cancer, liver cancer, ovary cancer, breast cancer, colon cancer, kidney cancer, skin cancer, muscle tumor, pancreas cancer, prostate cancer, testis cancer, uterine cancer, cholangiocarcinoma, Merkel cell carcinoma, bladder cancer, thyroid cancer, schwannoma, adrenal cancer, anus cancer, central nervous system tumor, neuroendocrine tissue tumor, penis cancer, pleura tumor, salivary gland tumor, vulva cancer, thymoma, and childhood cancer.

15 The following inventions are also provided:

[4-26] A kit comprising:

(A) a pharmaceutical composition comprising a bispecific antibody of any one of (a) to (c) below;

(B) a container; and

20 (C) an instruction or a label indicating that the bispecific antibody and at least one type of another anticancer agent are administered in combination to an individual for treating or preventing cancer in the individual;

(a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are
25 sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions
30 comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding
35 activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to

the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

- 5 (c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-27] A kit comprising:

- (A) another anticancer agent;
- (B) a container; and
- 15 (C) an instruction or a label indicating that said another anticancer agent and a pharmaceutical composition comprising at least one type of a bispecific antibody of (a) to (c) below are administered in combination to an individual for treating or preventing cancer in the individual;
- (a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;
- (b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and
- 35 (c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding

activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-28] A kit comprising:

(A) a pharmaceutical composition comprising a bispecific antibody of any one of (a) to (c) below:

(a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410;

(B) a container; and

(C) another anticancer agent.

[4-29] The kit of any one of [4-26] to [4-28], wherein the bispecific antibody is administered simultaneously with said another anticancer agent.

[4-30] The kit of any one of [4-26] to [4-28], wherein the bispecific antibody is administered before or after administration of said another anticancer agent.

5 [4-31] The kit of any one of [4-26] to [4-30], wherein said another anticancer agent is a chemotherapeutic agent, a T cell-activating agonist agent, an immune checkpoint inhibitor, or an angiogenic inhibitor.

[4-32] The kit of any one of [4-26] to [4-31], wherein the cancer is any cancer selected from the group consisting of gastric cancer, head and neck cancer, esophageal cancer, lung cancer,
10 liver cancer, ovary cancer, breast cancer, colon cancer, kidney cancer, skin cancer, muscle tumor, pancreas cancer, prostate cancer, testis cancer, uterine cancer, cholangiocarcinoma, Merkel cell carcinoma, bladder cancer, thyroid cancer, schwannoma, adrenal cancer, anus cancer, central nervous system tumor, neuroendocrine tissue tumor, penis cancer, pleura tumor, salivary gland tumor, vulva cancer, thymoma, and childhood cancer.

15 The following inventions are also provided:

[4-33] A method for inducing damage to a cancer cell or a cancer cell-comprising tumor tissue, or a method for suppressing proliferation of a cancer cell or growth of a cancer cell-comprising tumor tissue, by contacting a cancer cell with a bispecific antibody of any one of (a) to (c) below and another anticancer agent;

20 (a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3
25 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223,
30 respectively;

(b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding
35 activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80%

identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

[4-34] A method for assessing whether a bispecific antibody and another anticancer agent induce damage to a cancer cell or a cancer cell-comprising tumor tissue, or suppress proliferation of a cancer cell or growth of a cancer cell-comprising tumor tissue, by contacting a cancer cell with the bispecific antibody of any one of (a) to (c) below and another anticancer agent;

(a) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity, wherein the bispecific antibody has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and antibody common L chains having at least 80% identity to the amino acid

sequence of SEQ ID NO: 410.

[4-35] The method of [4-33] or [4-34], wherein said another anticancer agent is a chemotherapeutic agent, a T cell-activating agonist agent, an immune checkpoint inhibitor, or an angiogenic inhibitor.

5 [4-36] The method of any one of [4-33] to [4-35], wherein the cancer cell is any cancer cell selected from the group consisting of gastric cancer, head and neck cancer, esophageal cancer, lung cancer, liver cancer, ovary cancer, breast cancer, colon cancer, kidney cancer, skin cancer, muscle tumor, pancreas cancer, prostate cancer, testis cancer, uterine cancer, cholangiocarcinoma, Merkel cell carcinoma, bladder cancer, thyroid cancer, schwannoma, adrenal cancer, anus cancer,
10 central nervous system tumor, neuroendocrine tissue tumor, penis cancer, pleura tumor, salivary gland tumor, vulva cancer, thymoma, and childhood cancer.

The following inventions are also provided:

[5-1] The pharmaceutical composition of any one of [7] to [12] and [4-1] to [4-9], which is for increasing and/or maintaining the expression of CD3 ϵ chain in an individual as compared to
15 administration of the bispecific antibody of any one of [7] (a) to (c) or said another anticancer agent alone.

[5-2] The pharmaceutical composition of any one of [7] to [12] and [4-1] to [4-9], which is for expanding T cell population in an individual as compared to administration of the bispecific antibody of any one of [7] (a) to (c) or said another anticancer agent alone.

20 [5-3] The pharmaceutical composition of [5-2], wherein the T cell population is an activated T cell population.

[5-4] The pharmaceutical composition of any one of [7] to [12] and [4-1] to [4-9], which is for increasing the expression of a cytokine and/or chemokine in an individual as compared to administration of the bispecific antibody of any one of [7] (a) to (c) or said another anticancer
25 agent alone.

[5-5] The pharmaceutical composition of [5-4], wherein the cytokine and/or chemokine is one or more cytokines and/or chemokines selected from the group consisting of IFN γ , IL2, IL6, IL7, IL8, IL10, IL17A, TNF, CXCL9, and CXCL10.

[5-6] The pharmaceutical composition of any one of [7] to [12] and [4-1] to [4-9], which is for increasing the expression of a gene involved in cell death in an individual as compared to administration of the bispecific antibody of any one of [7] (a) to (c) or said another anticancer
30 agent alone.

[5-7] The pharmaceutical composition of [5-6], wherein the gene involved in cell death is one or more genes selected from the group consisting of TNFSF10, FAS, FASL, caspase 8, and
35 caspase 7.

[5-8] The pharmaceutical composition of any one of [7] to [12] and [4-1] to [4-9], which is for

inhibiting a gene involved in cell cycle promotion in an individual as compared to administration of the bispecific antibody of any one of [7] (a) to (c) or said another anticancer agent alone.

5 [5-9] The pharmaceutical composition of [5-8], wherein the gene involved in cell cycle promotion is one or more genes selected from the group consisting of PCNA, CCNA2, and CDK4.

[5-10] The pharmaceutical composition of any one of [7] to [12] and [4-1] to [4-9], which is for increasing the expression of a gene involved in cell cycle suppression in an individual as compared to administration of the bispecific antibody of any one of [7] (a) to (c) or said another anticancer agent alone.

10 [5-11] The pharmaceutical composition of [5-10], wherein the gene involved in cell cycle suppression is p21.

[5-12] The pharmaceutical composition of any one of [7] to [12] and [4-1] to [4-9], which is for increasing a leukocyte marker in an individual as compared to administration of the bispecific antibody of any one of [7] (a) to (c) or said another anticancer agent alone.

15 [5-13] The pharmaceutical composition of [5-12], wherein the leukocyte marker is CD45.

[5-14] The pharmaceutical composition of any one of [7] to [12] and [4-1] to [4-9], which is for increasing a T cell marker and/or T cell activation marker in an individual as compared to administration of the bispecific antibody of any one of [7] (a) to (c) or said another anticancer agent alone.

20 [5-15] The pharmaceutical composition of [5-14], wherein the T cell marker and/or T cell activation marker is one or more T cell markers and/or T cell activation markers selected from the group consisting of CD3, CD4, CD8a, GZB, PRF1, and IFN γ .

25 [5-16] The pharmaceutical composition of any one of [7] to [12] and [4-1] to [4-9], which is for increasing the expression of an immune checkpoint gene in an individual as compared to administration of the bispecific antibody of any one of [7] (a) to (c) or said another anticancer agent alone.

[5-17] The pharmaceutical composition of [5-16], wherein the immune checkpoint gene is one or more immune checkpoint genes selected from the group consisting of PD-L1, PD-1, TIM3, LAG3, and CTLA4.

30 In each item of [4-1] to [4-36] above, “another anticancer agent” refers to an anticancer agent which comprises as an active ingredient a substance that is different from the bispecific antibody recited in each item. Specifically, the term “another anticancer agent” merely indicates that the invention of each item is specified as inventions in which the other/another anticancer agent is an anticancer agent comprising as an active ingredient a substance that is
35 different from the bispecific antibody, and the invention is not limited to inventions in which the bispecific antibody is used as an anticancer agent. For example, even when each invention of

[4-1] to [4-36] recites the term “another anticancer agent”, the invention also includes embodiments where no other anticancer agent than the “another anticancer agent” is used, and in this case, the invention includes embodiments where the bispecific antibody is used as an enhancer, combination drug, excipient, or such for the other anticancer agent.

5

[Effects of the Invention]

The present invention provides novel multispecific antigen-binding molecules with molecular forms that can be produced with high efficiency, which maintain the strong antitumor activity possessed by BiTE and the excellent safety property of not causing cancer antigen-independent induction of a cytokine storm and such, and have long half-lives in blood. Anticancer agents which comprise a multispecific antigen-binding molecule of the present invention as an active ingredient and combination therapies using the multispecific antigen-binding molecule and another anticancer agent target cancer tissues containing glypican 3-expressing cancer cells to cause cytotoxicity, and can treat or prevent various cancers. The invention enables desirable treatment which has not only a high level of safety but also reduced physical burden, and is highly convenient for patients.

15

Brief Description of the Drawings

Fig. 1-1 is graphs showing the cytotoxic activity of antibody-38 when cell lines derived from various cancer types are used as target cells.

20

Fig. 1-2 is graphs showing the cytotoxic activity of antibody-38 when cell lines derived from various cancer types are used as target cells (a continuation of Fig. 1-1).

Fig. 1-3 is graphs showing the cytotoxic activity of antibody-38 when cell lines derived from various cancer types are used as target cells (a continuation of Fig. 1-2).

25

Fig. 2 is graphs showing the anti-tumor activity of antibody-38 against xenograft tumors derived from various cancer types in a human T cell transplantation model.

Fig. 3 is a graph showing the anti-tumor activities of antibody-39 and -40 against PC-10 xenograft tumor in a human T cell transplantation model.

30

Fig. 4 is a graph showing the anti-tumor activities of antibody-30, -31, -32, and -33 against SK-HEP-1/hGPC3 (SK-pca31a) xenograft tumor in a human T cell transplantation model.

Fig. 5 is a graph showing the anti-tumor activity of antibody-38 against PC-10 xenograft tumor in a humanized NOG mouse model.

35

Fig. 6 is a graph showing the anti-tumor activities of antibody-38, and anti-human GPC3 antibody, anti-mouse CTLA-4 antibody, anti-mouse PD-1 antibody, or anti-mouse PD-L1 antibody against LLC1/hGPC3 syngeneic tumor in a human CD3 $\epsilon\delta\gamma$ gene-modified mouse

model.

Fig. 7 is graphs showing the anti-tumor activities resulting from a single or combination of antibody, and capecitabine, cisplatin, or paclitaxel against MKN45 xenograft tumor in a human T cell transplantation model.

5 Fig. 8 is graphs showing the anti-tumor activities resulting from a single or combination of antibody, and cisplatin or paclitaxel against NCI-H446 xenograft tumor in a human T cell transplantation model.

Fig. 9 is graphs showing the anti-tumor activities resulting from a single or combination of antibody-38, and anti-mouseTIM-3 antibody, anti-mouse LAG-3 antibody, anti-mouse CD137
10 antibody, or anti-mouse VEGFR2 antibody against Hepal-6/hGPC3 syngeneic tumor in a human CD3 $\epsilon\delta\gamma$ gene-modified mouse model.

Fig. 10 is a graph showing the anti-tumor activities resulting from a single or combination of antibody-38 and anti-mouse CD137 antibody against LLC1/hGPC3 syngeneic tumor in a human CD3 $\epsilon\delta\gamma$ gene-modified mouse model.

15 Fig. 11 is graphs showing the anti-tumor activities resulting from a single or combination of antibody-38, and anti-mouse PD-1 antibody or anti-mouse PD-L1 antibody against Hepal-6/hGPC3 syngeneic tumor in a human CD3 ϵ gene-modified mouse model.

Fig. 12 shows schematic diagrams of a: ERY22 and b: ERY27.

Fig. 13 is a graph showing the cytotoxic activities of GPC3_ERY22_rCE115 and
20 GPC3_ERY27_hCE115 when NCI-H446 is used as the target cell. The filled diamond (◆) and the filled triangle (▲) indicate the cytotoxic activity of GPC3_ERY22_rCE115 and GPC3_ERY27_hCE115, respectively.

Fig. 14 is a graph showing the cytotoxic activities of GPC3_ERY22_rCE115 and GPC3_ERY27_hCE115 when PC-10 is used as the target cell. The filled diamond (◆) and the
25 filled triangle (▲) indicate the cytotoxic activity of GPC3_ERY22_rCE115 and GPC3_ERY27_hCE115, respectively.

Fig. 15 is a graph showing the cytotoxic activities of the optimized antibodies when NCI-H446 is used as the target cell.

Fig. 16 is a graph showing the cytotoxic activities of the optimized antibodies when
30 NCI-H446 is used as the target cell.

Fig. 17 is a graph showing the cytotoxic activities of the optimized antibodies when NCI-H446 is used as the target cell.

Fig. 18 is a graph showing the cytotoxic activities of the optimized antibodies when NCI-H446 is used as the target cell.

35 Fig. 19 is a graph showing the cytotoxic activities of the optimized antibodies when NCI-H446 is used as the target cell.

Fig. 20 is a graph showing the cytotoxic activities of the optimized antibodies when NCI-H446 is used as the target cell.

Fig. 21 shows the *in vivo* antitumor effects of the optimized antibodies when PC-10 is used as the target cell.

5 Fig. 22 shows the *in vivo* antitumor effects of the optimized antibodies when NCI-H446 is used as the target cell.

Fig. 23 shows the relationship between the amino acid residues constituting the Fc regions of IgG1, IgG2, IgG3, and IgG4, and the Kabat EU numbering system (herein, also referred to as EU INDEX).

10 Fig. 24-1 shows the heavy-chain variable region sequences and their numbering according to Kabat *et al.*

Fig. 24-2 shows the heavy-chain variable region sequences and their numbering according to Kabat *et al.*

15 Fig. 25 shows the light-chain variable region sequences and their numbering according to Kabat *et al.*

Fig. 26A presents the structure of a genomic DNA containing mouse Cd3 ϵ , Cd3 δ , and Cd3 γ genes (1), a mouse Cd3 gene modification vector constructed by modifying a bacterial artificial chromosome (BAC) clone containing the whole gene region (2), the structure of a genomic DNA in which loxP and Rox sequences have been inserted at the target position using the above-mentioned vector (3), and the structure of a Cd3 ϵ , Cd3 δ , and Cd3 γ -gene deficient allele produced by the actions of Cre and Dre recombinases (4).

20

Fig. 26B presents the structures of a BAC clone containing human CD3 ϵ , CD3 δ , and CD3 γ genes (a); 5'-modifying cassette (b) and 3'-modifying cassette (c), both of which are for modifying the BAC clone; and a human CD3 gene region introduction vector constructed through modifications using those above (d).

25

Fig. 27 presents the representative examples of PCR analyses performed for establishing mouse Cd3 gene-modified ES cells.

Fig. 28 presents the representative examples of PCR analyses of genotypes of ES cell clones obtained by introducing into mouse Cd3 gene-modified ES cells the human CD3 gene region introduction vector along with a Cre expression vector and a Dre expression vector. Fig. 28A presents the representative examples of PCR results that detect the deficiency of the mouse Cd3 gene region.

30

Fig. 28B presents the representative examples of PCR results that detect the introduction of the human CD3 gene region.

35 Fig. 29 presents the representative macroscopic photographs of thymuses collected from each of the established lines of human CD3 gene-substituted mice, Cd3 gene-deficient mice,

wild type, and human CD3 ϵ gene-introduced mice. Thymuses extirpated from 12 to 13-week-old males are shown for the respective genotypes.

Fig. 30 presents the results of measuring the tissue weights of the spleens and thymuses collected from each of the established lines of human CD3 gene-substituted mice, Cd3 gene-deficient mice, wild-type, and human CD3 ϵ gene-introduced mice. Ratios of tissue weight per body weight were calculated, and the value obtained for each individual is plotted by a black dot and the mean values are shown by columns.

Fig. 31 presents the results of examining by RT-PCR the expressions of each of the human CD3 molecules and each of the mouse Cd3 genes in each of the established lines of human CD3 gene-substituted mice, Cd3 gene-deficient mice, wild-type mice, and human CD3 ϵ gene-introduced (hCD3 ϵ Tg) mice. Among the established lines of the human CD3 gene-substituted mice, signals specific to hCD3 ϵ , hCD3 δ , and hCD3 γ were detected in line numbers 1C3 and 8I12. The signals were not detected in line numbers 3B1 and 2A4.

Fig. 32 presents the representative examples of immunohistological staining for CD3 performed on the thymus (A) and spleen (B) of each established line of human CD3 gene-substituted mice (1C3, 8I12, and 4HH3). In both tissues, staining was observed only in the T cell zone as in the wild-type mouse. Furthermore, staining was not observed in the Cd3 gene-deficient mice, and this showed that the staining in the human CD3 gene-substituted mice is due to the expression of the introduced human CD3 genes.

Fig. 33 presents the representative results of analyzing by FACS the abundance ratio of mature T cells in the thymus of each established line of human CD3-substituted mice.

Fig. 34 presents the results of measuring the chicken ovalbumin (OVA)-specific IgG1 and IgE serum concentrations in each established line of human CD3-substituted mice immunized with OVA. The OVA-specific serum IgG1 and IgE concentrations for each individual are shown as a bar graph. The numbers below the bar graph indicate the individual identification numbers.

Fig. 35-1 shows a result of comprehensive RNA analysis of tumor tissues when Paclitaxel and antibody-38 are used in combination.

Fig. 35-2 shows a result of comprehensive RNA analysis of tumor tissues when Paclitaxel and antibody-38 are used in combination (a continuation of Fig. 35-1).

Fig. 35-3 shows a result of comprehensive RNA analysis of tumor tissues when Paclitaxel and antibody-38 are used in combination (a continuation of Fig. 35-2).

Fig. 35-4 shows a result of comprehensive RNA analysis of tumor tissues when Capecitabine and antibody-38 are used in combination.

Fig. 35-5 shows a result of comprehensive RNA analysis of tumor tissues when Capecitabine and antibody-38 are used in combination (a continuation of Fig. 35-4).

Fig. 35-6 shows a result of comprehensive RNA analysis of tumor tissues when Capecitabine and antibody-38 are used in combination (a continuation of Fig. 35-5).

Fig. 36-1 shows a result of tumor infiltrating lymphocyte (TIL) analysis of tumor tissues when Paclitaxel and antibody-38 are used in combination.

5 Fig. 36-2 shows a result of tumor infiltrating lymphocyte (TIL) analysis of tumor tissues when Cisplatin and antibody-38 are used in combination.

Mode for Carrying Out the Invention

The definitions below are provided to help understanding of the present invention
10 illustrated herein.

Antibody

Herein, “antibody” refers to a natural immunoglobulin or an immunoglobulin produced by partial or complete synthesis. Antibodies can be isolated from natural sources such as
15 naturally-occurring plasma and serum, or culture supernatants of antibody-producing hybridomas. Alternatively, antibodies can be partially or completely synthesized using techniques such as genetic recombination. Preferred antibodies include, for example, antibodies of an immunoglobulin isotype or subclass belonging thereto. Known human immunoglobulins include antibodies of the following nine classes (isotypes): IgG1, IgG2, IgG3,
20 IgG4, IgA1, IgA2, IgD, IgE, and IgM. Of these isotypes, antibodies of the present invention may include IgG1, IgG2, IgG3, and IgG4.

Methods for producing an antibody with desired binding activity are known to those skilled in the art. Below is an example that describes a method for producing an antibody (anti-GPC3 antibody) that binds to Glypican-3 (hereinafter, also referred to as GPC3), which
25 belongs to the GPI-anchored receptor family (Int J Cancer. (2003) 103(4), 455-65). Antibodies that bind to a T-cell receptor complex can also be produced according to the example described below.

Anti-GPC3 antibodies can be obtained as polyclonal or monoclonal antibodies using known methods. The anti-GPC3 antibodies preferably produced are monoclonal antibodies
30 derived from mammals. Such mammal-derived monoclonal antibodies include antibodies produced by hybridomas or host cells transformed with an expression vector carrying an antibody gene by genetic engineering techniques.

Monoclonal antibody-producing hybridomas can be produced using known techniques, for example, as described below. Specifically, mammals are immunized by conventional
35 immunization methods using a GPC3 protein as a sensitizing antigen. Resulting immune cells are fused with known parental cells by conventional cell fusion methods. Then, hybridomas

producing an anti-GPC3 antibody can be selected by screening for monoclonal antibody-producing cells using conventional screening methods.

Specifically, monoclonal antibodies are prepared as mentioned below. First, the GPC3 gene whose nucleotide sequence is disclosed in RefSeq accession number NM_001164617.1 (SEQ ID NO: 1) can be expressed to produce a GPC3 protein shown in RefSeq accession number NP_001158089.1 (SEQ ID NO: 2), which will be used as a sensitizing antigen for antibody preparation. That is, a gene sequence encoding GPC3 is inserted into a known expression vector, and appropriate host cells are transformed with this vector. The desired human GPC3 protein is purified from the host cells or their culture supernatants by known methods. For example, to prepare soluble GPC3 from culture supernatants, amino acids at positions 564 to 580 that form the hydrophobic region corresponding to the GPI-anchor sequence used to anchor GPC3 on the cell membrane are deleted from the GPC3 polypeptide sequence of SEQ ID NO: 2, and then the resulting protein is expressed instead of the GPC3 protein of SEQ ID NO: 2. Alternatively, it is possible to use a purified natural GPC3 protein as a sensitizing antigen.

The purified GPC3 protein can be used as a sensitizing antigen for use in immunization of mammals. Partial peptides of GPC3 can also be used as sensitizing antigens. In this case, the partial peptides may also be obtained by chemical synthesis from the human GPC3 amino acid sequence. Furthermore, they may also be obtained by incorporating a portion of the GPC3 gene into an expression vector and expressing it. Moreover, they may also be obtained by degrading the GPC3 protein using proteases, but the region and size of the GPC3 peptide used as the partial peptide are not particularly limited to a special embodiment. As the preferred region, any sequence from the amino acid sequence corresponding to the amino acids at positions 524 to 563, or more preferably any sequence from the amino acid sequence corresponding to the amino acids at positions 537 to 563 in the amino acid sequence of SEQ ID NO: 2 may be selected. Preferably, any sequence may be selected from the amino acid sequence of the region not containing the amino acid sequence corresponding to amino acids at positions 550 to 663 in the amino acid sequence of SEQ ID NO: 2. Preferably, any sequence may be selected from the amino acid sequence corresponding to positions 544 to 553, and more preferably, any sequence may be selected from the amino acid sequence corresponding to positions 546 to 551 in the amino acid sequence of SEQ ID NO: 2. The number of amino acids constituting a peptide to be used as the sensitizing antigen is at least five or more, or preferably for example, six or more, or seven or more. More specifically, peptides consisting of 8 to 50 residues or preferably 10 to 30 residues may be used as the sensitizing antigen.

For sensitizing antigen, alternatively it is possible to use a fusion protein prepared by fusing a desired partial polypeptide or peptide of the GPC3 protein with a different polypeptide.

For example, antibody Fc fragments and peptide tags are preferably used to produce fusion proteins to be used as sensitizing antigens. Vectors for expression of such fusion proteins can be constructed by fusing in frame genes encoding two or more desired polypeptide fragments and inserting the fusion gene into an expression vector as described above. Methods for
5 producing fusion proteins are described in Molecular Cloning 2nd ed. (Sambrook, J *et al.*, Molecular Cloning 2nd ed., 9.47-9.58 (1989) Cold Spring Harbor Lab. Press). Methods for preparing GPC3 to be used as a sensitizing antigen, and immunization methods using GPC3 are specifically described in WO 2003/000883, WO 2004/022754, and WO 2006/006693.

There is no particular limitation on the mammals to be immunized with the sensitizing
10 antigen. However, it is preferable to select the mammals by considering their compatibility with the parent cells to be used for cell fusion. In general, rodents such as mice, rats, and hamsters, rabbits, and monkeys are preferably used.

The above animals are immunized with a sensitizing antigen by known methods. Generally performed immunization methods include, for example, intraperitoneal or
15 subcutaneous injection of a sensitizing antigen into mammals. Specifically, a sensitizing antigen is appropriately diluted with PBS (Phosphate-Buffered Saline), physiological saline, or the like. If desired, a conventional adjuvant such as Freund's complete adjuvant is mixed with the antigen, and the mixture is emulsified. Then, the sensitizing antigen is administered to a mammal several times at 4- to 21-day intervals. Appropriate carriers may be used in
20 immunization with the sensitizing antigen. In particular, when a low-molecular-weight partial peptide is used as the sensitizing antigen, it is sometimes desirable to couple the sensitizing antigen peptide to a carrier protein such as albumin or keyhole limpet hemocyanin for immunization.

Alternatively, hybridomas producing a desired antibody can be prepared using DNA
25 immunization as mentioned below. DNA immunization is an immunization method that confers immunostimulation by expressing a sensitizing antigen in an animal immunized as a result of administering a vector DNA constructed to allow expression of an antigen protein-encoding gene in the animal. As compared to conventional immunization methods in which a protein antigen is administered to animals to be immunized, DNA immunization is
30 expected to be superior in that:

- immunostimulation can be provided while retaining the structure of a membrane protein such as GPC3; and
- there is no need to purify the antigen for immunization.

In order to prepare a monoclonal antibody of the present invention using DNA
35 immunization, first, a DNA expressing a GPC3 protein is administered to an animal to be immunized. The GPC3-encoding DNA can be synthesized by known methods such as PCR.

The obtained DNA is inserted into an appropriate expression vector, and then this is administered to an animal to be immunized. Preferably used expression vectors include, for example, commercially-available expression vectors such as pcDNA3.1. Vectors can be administered to an organism using conventional methods. For example, DNA immunization is performed by
 5 using a gene gun to introduce expression vector-coated gold particles into cells in the body of an animal to be immunized. Antibodies that recognized GPC3 can also be produced by the methods described in WO 2003/104453.

After immunizing a mammal as described above, an increase in the titer of a GPC3-binding antibody is confirmed in the serum. Then, immune cells are collected from the
 10 mammal, and then subjected to cell fusion. In particular, splenocytes are preferably used as immune cells.

A mammalian myeloma cell is used as a cell to be fused with the above-mentioned immunocyte. The myeloma cells preferably comprise a suitable selection marker for screening. A selection marker confers characteristics to cells for their survival (or death) under a specific
 15 culture condition. Hypoxanthine-guanine phosphoribosyltransferase deficiency (hereinafter abbreviated as HGPRT deficiency) and thymidine kinase deficiency (hereinafter abbreviated as TK deficiency) are known as selection markers. Cells with HGPRT or TK deficiency have hypoxanthine-aminopterin-thymidine sensitivity (hereinafter abbreviated as HAT sensitivity). HAT-sensitive cells cannot synthesize DNA in a HAT selection medium, and are thus killed.
 20 However, when the cells are fused with normal cells, they can continue DNA synthesis using the salvage pathway of the normal cells, and therefore they can grow even in the HAT selection medium.

HGPRT-deficient and TK-deficient cells can be selected in a medium containing 6-thioguanine, 8-azaguanine (hereinafter abbreviated as 8AG), or 5'-bromodeoxyuridine,
 25 respectively. Normal cells are killed because they incorporate these pyrimidine analogs into their DNA. Meanwhile, cells that are deficient in these enzymes can survive in the selection medium, since they cannot incorporate these pyrimidine analogs. In addition, a selection marker referred to as G418 resistance provided by the neomycin-resistant gene confers resistance to 2-deoxystreptamine antibiotics (gentamycin analogs). Various types of myeloma cells that
 30 are suitable for cell fusion are known.

For example, myeloma cells including the following cells can be preferably used:
 P3(P3x63Ag8.653) (J. Immunol. (1979) 123 (4), 1548-1550);
 P3x63Ag8U.1 (Current Topics in Microbiology and Immunology (1978)81, 1-7);
 NS-1 (C. Eur. J. Immunol. (1976)6 (7), 511-519);
 35 MPC-11 (Cell (1976) 8 (3), 405-415);
 SP2/0 (Nature (1978) 276 (5685), 269-270);

FO (J. Immunol. Methods (1980) 35 (1-2), 1-21);
 S194/5.XX0.BU.1 (J. Exp. Med. (1978) 148 (1), 313-323);
 R210 (Nature (1979) 277 (5692), 131-133), etc.

Cell fusions between the immunocytes and myeloma cells are essentially carried out
 5 using known methods, for example, a method by Kohler and Milstein *et al.* (Methods Enzymol.
 (1981) 73: 3-46).

More specifically, cell fusion can be carried out, for example, in a conventional culture
 medium in the presence of a cell fusion-promoting agent. The fusion-promoting agents include,
 for example, polyethylene glycol (PEG) and Sendai virus (HVJ). If required, an auxiliary
 10 substance such as dimethyl sulfoxide is also added to improve fusion efficiency.

The ratio of immunocytes to myeloma cells may be determined at one's own discretion,
 preferably, for example, one myeloma cell for every one to ten immunocytes. Culture media to
 be used for cell fusions include, for example, media that are suitable for the growth of myeloma
 cell lines, such as RPMI1640 medium and MEM medium, and other conventional culture
 15 medium used for this type of cell culture. In addition, serum supplements such as fetal calf
 serum (FCS) may be preferably added to the culture medium.

For cell fusion, predetermined amounts of the above immune cells and myeloma cells
 are mixed well in the above culture medium. Then, a PEG solution (for example, the average
 molecular weight is about 1,000 to 6,000) prewarmed to about 37°C is added thereto at a
 20 concentration of generally 30% to 60% (w/v). This is gently mixed to produce desired fusion
 cells (hybridomas). Then, an appropriate culture medium mentioned above is gradually added
 to the cells, and this is repeatedly centrifuged to remove the supernatant. Thus, cell fusion
 agents and such which are unfavorable to hybridoma growth can be removed.

The hybridomas thus obtained can be selected by culture using a conventional selective
 25 medium, for example, HAT medium (a culture medium containing hypoxanthine, aminopterin,
 and thymidine). Cells other than the desired hybridomas (non-fused cells) can be killed by
 continuing culture in the above HAT medium for a sufficient period of time. Typically, the
 period is several days to several weeks. Then, hybridomas producing the desired antibody are
 screened and singly cloned by conventional limiting dilution methods.

30 The hybridomas thus obtained can be selected using a selection medium based on the
 selection marker possessed by the myeloma used for cell fusion. For example, HGPRT- or
 TK-deficient cells can be selected by culture using the HAT medium (a culture medium
 containing hypoxanthine, aminopterin, and thymidine). Specifically, when HAT-sensitive
 myeloma cells are used for cell fusion, cells successfully fused with normal cells can selectively
 35 proliferate in the HAT medium. Cells other than the desired hybridomas (non-fused cells) can
 be killed by continuing culture in the above HAT medium for a sufficient period of time.

Specifically, desired hybridomas can be selected by culture for generally several days to several weeks. Then, hybridomas producing the desired antibody may be screened and singly cloned by conventional limiting dilution methods.

Desired antibodies can be preferably selected and singly cloned by screening known
5 methods based on antigen/antibody reaction. For example, a GPC3-binding monoclonal antibody can bind to GPC3 expressed on the cell surface. Such a monoclonal antibody can be screened by fluorescence activated cell sorting (FACS). FACS is a system that assesses the binding of an antibody to cell surface by analyzing cells contacted with a fluorescent antibody using laser beam, and measuring the fluorescence emitted from individual cells.

10 To screen for hybridomas that produce a monoclonal antibody of the present invention by FACS, GPC3-expressing cells are first prepared. Cells preferably used for screening are mammalian cells in which GPC3 is forcedly expressed. As control, the activity of an antibody to bind to cell-surface GPC3 can be selectively detected using non-transformed mammalian cells as host cells. Specifically, hybridomas producing an anti-GPC3 monoclonal antibody can be
15 isolated by selecting hybridomas that produce an antibody which binds to cells forced to express GPC3, but not to host cells.

Alternatively, the activity of an antibody to bind to immobilized GPC3-expressing cells can be assessed based on the principle of ELISA. For example, GPC3-expressing cells are immobilized to the wells of an ELISA plate. Culture supernatants of hybridomas are contacted
20 with the immobilized cells in the wells, and antibodies that bind to the immobilized cells are detected. When the monoclonal antibodies are derived from mouse, antibodies bound to the cells can be detected using an anti-mouse immunoglobulin antibody. Hybridomas producing a desired antibody having the antigen-binding ability are selected by the above screening, and they can be cloned by a limiting dilution method or the like.

25 Monoclonal antibody-producing hybridomas thus prepared can be passaged in a conventional culture medium, and stored in liquid nitrogen for a long period.

The above hybridomas are cultured by a conventional method, and desired monoclonal antibodies can be prepared from the culture supernatants. Alternatively, the hybridomas are administered to and grown in compatible mammals, and monoclonal antibodies are prepared
30 from the ascites. The former method is suitable for preparing antibodies with high purity.

Antibodies encoded by antibody genes that are cloned from antibody-producing cells such as the above hybridomas can also be preferably used. A cloned antibody gene is inserted into an appropriate vector, and this is introduced into a host to express the antibody encoded by the gene. Methods for isolating antibody genes, inserting the genes into vectors, and
35 transforming host cells have already been established, for example, by Vandamme *et al.* (Eur. J. Biochem. (1990) 192(3), 767-775). Methods for producing recombinant antibodies are also

known as described below.

For example, a cDNA encoding the variable region (V region) of an anti-GPC3 antibody is prepared from hybridoma cells expressing the anti-GPC3 antibody. For this purpose, total RNA is first extracted from hybridomas. Methods used for extracting mRNAs from cells include, for example:

- the guanidine ultracentrifugation method (Biochemistry (1979) 18(24), 5294-5299), and
- the AGPC method (Anal. Biochem. (1987) 162(1), 156-159)

Extracted mRNAs can be purified using the mRNA Purification Kit (GE Healthcare Bioscience) or such. Alternatively, kits for extracting total mRNA directly from cells, such as the QuickPrep mRNA Purification Kit (GE Healthcare Bioscience), are also commercially available. mRNAs can be prepared from hybridomas using such kits. cDNAs encoding the antibody V region can be synthesized from the prepared mRNAs using a reverse transcriptase. cDNAs can be synthesized using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Co.) or such. Furthermore, the SMART RACE cDNA amplification kit (Clontech) and the PCR-based 5'-RACE method (Proc. Natl. Acad. Sci. USA (1988) 85(23), 8998-9002; Nucleic Acids Res. (1989) 17(8), 2919-2932) can be appropriately used to synthesize and amplify cDNAs. In such a cDNA synthesis process, appropriate restriction enzyme sites described below may be introduced into both ends of a cDNA.

The cDNA fragment of interest is purified from the resulting PCR product, and then this is ligated to a vector DNA. A recombinant vector is thus constructed, and introduced into *E. coli* or such. After colony selection, the desired recombinant vector can be prepared from the colony-forming *E. coli*. Then, whether the recombinant vector has the cDNA nucleotide sequence of interest is tested by a known method such as the dideoxy nucleotide chain termination method.

The 5'-RACE method which uses primers to amplify the variable region gene is conveniently used for isolating the gene encoding the variable region. First, a 5'-RACE cDNA library is constructed by cDNA synthesis using RNAs extracted from hybridoma cells as a template. A commercially available kit such as the SMART RACE cDNA amplification kit is appropriately used to synthesize the 5'-RACE cDNA library.

The antibody gene is amplified by PCR using the prepared 5'-RACE cDNA library as a template. Primers for amplifying the mouse antibody gene can be designed based on known antibody gene sequences. The nucleotide sequences of the primers vary depending on the immunoglobulin subclass. Therefore, it is preferable that the subclass is determined in advance using a commercially available kit such as the Iso Strip mouse monoclonal antibody isotyping kit (Roche Diagnostics).

Specifically, for example, primers that allow amplification of genes encoding $\gamma 1$, $\gamma 2a$,

$\gamma 2b$, and $\gamma 3$ heavy chains and κ and λ light chains can be used to isolate mouse IgG-encoding genes. In general, a primer that anneals to a constant region site close to the variable region is used as a 3'-side primer to amplify an IgG variable region gene. Meanwhile, a primer attached to a 5' RACE cDNA library construction kit is used as a 5'-side primer.

5 PCR products thus amplified are used to reconstruct immunoglobulins composed of a combination of heavy and light chains. A desired antibody can be selected using the GPC3-binding activity of a reconstructed immunoglobulin as an indicator. For example, when the objective is to isolate an antibody against GPC3, it is more preferred that the binding of the antibody to GPC3 is specific. A GPC3-binding antibody can be screened, for example, by the
10 following steps:

- (1) contacting a GPC3-expressing cell with an antibody comprising the V region encoded by a cDNA isolated from a hybridoma;
- (2) detecting the binding of the antibody to the GPC3-expressing cell; and
- (3) selecting an antibody that binds to the GPC3-expressing cell.

15 Methods for detecting the binding of an antibody to GPC3-expressing cells are known. Specifically, the binding of an antibody to GPC3-expressing cells can be detected by the above-described techniques such as FACS. Immobilized samples of GPC3-expressing cells are appropriately used to assess the binding activity of an antibody.

Preferred antibody screening methods that use the binding activity as an indicator also
20 include panning methods using phage vectors. Screening methods using phage vectors are advantageous when the antibody genes are isolated from heavy-chain and light-chain subclass libraries from a polyclonal antibody-expressing cell population. Genes encoding the heavy-chain and light-chain variable regions can be linked by an appropriate linker sequence to form a single-chain Fv (scFv). Phages presenting scFv on their surface can be produced by
25 inserting a gene encoding scFv into a phage vector. The phages are contacted with an antigen of interest. Then, a DNA encoding scFv having the binding activity of interest can be isolated by collecting phages bound to the antigen. This process can be repeated as necessary to enrich scFv having a desired binding activity.

After isolation of the cDNA encoding the V region of the anti-GPC3 antibody of interest,
30 the cDNA is digested with restriction enzymes that recognize the restriction sites introduced into both ends of the cDNA. Preferred restriction enzymes recognize and cleave a nucleotide sequence that occurs in the nucleotide sequence of the antibody gene at a low frequency. Furthermore, a restriction site for an enzyme that produces a cohesive end is preferably introduced into a vector to insert a single-copy digested fragment in the correct orientation.
35 The cDNA encoding the V region of the anti-GPC3 antibody is digested as described above, and this is inserted into an appropriate expression vector to construct an antibody expression vector.

In this case, if a gene encoding the antibody constant region (C region) and a gene encoding the above V region are fused in-frame, a chimeric antibody is obtained. Herein, "chimeric antibody" means that the origin of the constant region is different from that of the variable region. Thus, in addition to mouse/human heterochimeric antibodies, human/human allochimeric antibodies
5 are included in the chimeric antibodies of the present invention. A chimeric antibody expression vector can be constructed by inserting the above V region gene into an expression vector that already has the constant region. Specifically, for example, a recognition sequence for a restriction enzyme that excises the above V region gene can be appropriately placed on the 5' side of an expression vector carrying a DNA encoding a desired antibody constant region (C
10 region). A chimeric antibody expression vector is constructed by fusing in frame the two genes digested with the same combination of restriction enzymes.

To produce an anti-GPC3 monoclonal antibody, antibody genes are inserted into an expression vector so that the genes are expressed under the control of an expression regulatory region. The expression regulatory region for antibody expression includes, for example,
15 enhancers and promoters. Furthermore, an appropriate signal sequence may be attached to the amino terminus so that the expressed antibody is secreted to the outside of cells. In the Reference Examples described below, a peptide having the amino acid sequence MGWSCIILFLVATATGVHS (SEQ ID NO: 3) is used as a signal sequence. Meanwhile, other appropriate signal sequences may be attached. The expressed polypeptide is cleaved at the
20 carboxyl terminus of the above sequence, and the resulting polypeptide is secreted to the outside of cells as a mature polypeptide. Then, appropriate host cells are transformed with the expression vector, and recombinant cells expressing the anti-GPC3 antibody-encoding DNA are obtained.

DNAs encoding the antibody heavy chain (H chain) and light chain (L chain) are
25 separately inserted into different expression vectors to express the antibody gene. An antibody molecule having the H and L chains can be expressed by co-transfecting the same host cell with vectors into which the H-chain and L-chain genes are respectively inserted. Alternatively, host cells can be transformed with a single expression vector into which DNAs encoding the H and L chains are inserted (see WO 94/11523).

30 There are various known host cell/expression vector combinations for antibody preparation by introducing isolated antibody genes into appropriate hosts. All of these expression systems are applicable to isolation of domains including antibody variable regions of the present invention. Appropriate eukaryotic cells used as host cells include animal cells, plant cells, and fungal cells. Specifically, the animal cells include, for example, the following cells.
35 (1) mammalian cells: CHO, COS, myeloma, baby hamster kidney (BHK), HeLa, Vero, or such;
(2) amphibian cells: Xenopus oocytes, or such; and

(3) insect cells: sf9, sf21, Tn5, or such.

In addition, as a plant cell, an antibody gene expression system using cells derived from the *Nicotiana* genus such as *Nicotiana tabacum* is known. Callus cultured cells can be appropriately used to transform plant cells.

5 Furthermore, the following cells can be used as fungal cells:

yeasts: the *Saccharomyces* genus such as *Saccharomyces cerevisiae*, and the *Pichia* genus such as *Pichia pastoris*; and

filamentous fungi: the *Aspergillus* genus such as *Aspergillus niger*.

10 Furthermore, antibody gene expression systems that utilize prokaryotic cells are also known. For example, when using bacterial cells, *E. coli* cells, *Bacillus subtilis* cells, and such can suitably be utilized in the present invention. Expression vectors carrying the antibody genes of interest are introduced into these cells by transfection. The transfected cells are cultured *in vitro*, and the desired antibody can be prepared from the culture of transformed cells.

15 In addition to the above-described host cells, transgenic animals can also be used to produce a recombinant antibody. That is, the antibody can be obtained from an animal into which the gene encoding the antibody of interest is introduced. For example, the antibody gene can be constructed as a fusion gene by inserting in frame into a gene that encodes a protein produced specifically in milk. Goat β -casein or such can be used, for example, as the protein secreted in milk. DNA fragments containing the fused gene inserted with the antibody gene is
20 injected into a goat embryo, and then this embryo is introduced into a female goat. Desired antibodies can be obtained as a protein fused with the milk protein from milk produced by the transgenic goat born from the embryo-recipient goat (or progeny thereof). In addition, to increase the volume of milk containing the desired antibody produced by the transgenic goat, hormones can be administered to the transgenic goat as necessary (Ebert, K. M. *et al.*,
25 Bio/Technology (1994) 12 (7), 699-702).

When an antigen-binding molecule described herein is administered to human, a domain derived from a genetically recombinant antibody that has been artificially modified to reduce the heterologous antigenicity against human and such, can be appropriately used as the domain of the antigen-binding molecule including an antibody variable region. Such genetically
30 recombinant antibodies include, for example, humanized antibodies. These modified antibodies are appropriately produced by known methods.

An antibody variable region used to produce a domain of an antigen-binding molecule including an antibody variable region described herein is generally formed by three complementarity-determining regions (CDRs) that are separated by four framework regions (FRs). CDR is a region that substantially determines the binding specificity of an antibody.
35 The amino acid sequences of CDRs are highly diverse. On the other hand, the FR-forming

amino acid sequences often have high identity even among antibodies with different binding specificities. Therefore, generally, the binding specificity of a certain antibody can be introduced to another antibody by CDR grafting.

A humanized antibody is also called a reshaped human antibody. Specifically, humanized antibodies prepared by grafting the CDR of a non-human animal antibody such as a mouse antibody to a human antibody and such are known. Common genetic engineering techniques for obtaining humanized antibodies are also known. Specifically, for example, overlap extension PCR is known as a method for grafting a mouse antibody CDR to a human FR. In overlap extension PCR, a nucleotide sequence encoding a mouse antibody CDR to be grafted is added to primers for synthesizing a human antibody FR. Primers are prepared for each of the four FRs. It is generally considered that when grafting a mouse CDR to a human FR, selecting a human FR that has high identity to a mouse FR is advantageous for maintaining the CDR function. That is, it is generally preferable to use a human FR comprising an amino acid sequence which has high identity to the amino acid sequence of the FR adjacent to the mouse CDR to be grafted.

Nucleotide sequences to be ligated are designed so that they will be connected to each other in frame. Human FRs are individually synthesized using the respective primers. As a result, products in which the mouse CDR-encoding DNA is attached to the individual FR-encoding DNAs are obtained. Nucleotide sequences encoding the mouse CDR of each product are designed so that they overlap with each other. Then, complementary strand synthesis reaction is conducted to anneal the overlapping CDR regions of the products synthesized using a human antibody gene as template. Human FRs are ligated *via* the mouse CDR sequences by this reaction.

The full length V region gene, in which three CDRs and four FRs are ultimately ligated, is amplified using primers that anneal to its 5'- or 3'-end, which are added with suitable restriction enzyme recognition sequences. An expression vector for humanized antibody can be produced by inserting the DNA obtained as described above and a DNA that encodes a human antibody C region into an expression vector so that they will ligate in frame. After the recombinant vector is transfected into a host to establish recombinant cells, the recombinant cells are cultured, and the DNA encoding the humanized antibody is expressed to produce the humanized antibody in the cell culture (see, European Patent Publication No. EP 239400 and International Patent Publication No. WO 1996/002576).

By qualitatively or quantitatively measuring and evaluating the antigen-binding activity of the humanized antibody produced as described above, one can suitably select human antibody FRs that allow CDRs to form a favorable antigen-binding site when ligated through the CDRs. Amino acid residues in FRs may be substituted as necessary, so that the CDRs of a reshaped

human antibody form an appropriate antigen-binding site. For example, amino acid sequence mutations can be introduced into FRs by applying the PCR method used for grafting a mouse CDR into a human FR. More specifically, partial nucleotide sequence mutations can be introduced into primers that anneal to the FR. Nucleotide sequence mutations are introduced
 5 into the FRs synthesized by using such primers. Mutant FR sequences having the desired characteristics can be selected by measuring and evaluating the activity of the amino acid-substituted mutant antibody to bind to the antigen by the above-mentioned method (Sato, K. *et al.*, Cancer Res. (1993) 53: 851-856).

Alternatively, desired human antibodies can be obtained by immunizing transgenic
 10 animals having the entire repertoire of human antibody genes (see International Patent Publication Nos. WO 1993/012227; WO 1992/003918; WO 1994/002602; WO 1994/025585; WO 1996/034096; WO 1996/033735) by DNA immunization.

Furthermore, techniques for preparing human antibodies by panning using human antibody libraries are also known. For example, the V region of a human antibody is expressed
 15 as a single-chain antibody (scFv) on phage surface by the phage display method. Phages expressing an scFv that binds to the antigen can be selected. The DNA sequence encoding the human antibody V region that binds to the antigen can be determined by analyzing the genes of selected phages. The DNA sequence of the scFv that binds to the antigen is determined. An expression vector can be prepared by fusing the V region sequence in frame with the C region
 20 sequence of a desired human antibody, and inserting this into an appropriate expression vector. The expression vector is introduced into cells appropriate for expression such as those described above. The human antibody can be produced by expressing the human antibody-encoding gene in the cells. These methods are already known (see International Patent Publication Nos. WO 1992/001047; WO 1992/020791; WO 1993/006213; WO 1993/011236; WO 1993/019172; WO
 25 1995/001438; WO 1995/015388).

A domain comprising an antibody variable region having glypican 3 (GPC3)-binding activity

Herein, the phrase “a domain comprising an antibody variable region having glypican 3 (GPC3)-binding activity” refers to an antibody portion that comprises a region that specifically
 30 binds to the above-mentioned GPC3 protein, or to all or a portion of a partial peptide of the GPC3 protein, and is also complementary thereto. Domains comprising an antibody variable region may be provided from variable domains of one or a plurality of antibodies. Preferably, domains comprising an antibody variable region comprise antibody light-chain and heavy-chain variable regions (VL and VH). Suitable examples of such domains comprising antibody
 35 variable regions include “single chain Fv (scFv)”, “single chain antibody”, “Fv”, “single chain Fv 2 (scFv2)”, “Fab”, “F(ab')₂”, etc.

A domain comprising an antibody variable region having T-cell receptor complex-binding activity

Herein, the phrase “a domain comprising an antibody variable region having T-cell receptor complex-binding activity” refers to a T-cell receptor complex-binding antibody portion that comprises a region that specifically binds to all or a portion of a T-cell receptor complex and is also complementary thereto. The T-cell receptor complex may be a T-cell receptor itself, or an adaptor molecule constituting a T-cell receptor complex along with a T-cell receptor. CD3 is suitable as an adaptor molecule.

A domain comprising an antibody variable region that has T-cell receptor-binding activity

Herein, the phrase “a domain comprising an antibody variable region having T-cell receptor-binding activity” refers to a T-cell receptor-binding antibody portion produced by including a region that specifically binds to all or a portion of a T-cell receptor and is also complementary thereto.

The portion of a T cell receptor to which the domain of the present invention binds may be a variable region or a constant region, but an epitope present in the constant region is preferred. Examples of the constant region sequence include the T cell receptor α chain of RefSeq Accession No. CAA26636.1 (SEQ ID NO: 4), the T cell receptor β chain of RefSeq Accession No. C25777 (SEQ ID NO: 5), the T cell receptor $\gamma 1$ chain of RefSeq Accession No. A26659 (SEQ ID NO: 6), the T cell receptor $\gamma 2$ chain of RefSeq Accession No. AAB63312.1 (SEQ ID NO: 7), and the T cell receptor δ chain of RefSeq Accession No. AAA61033.1 (SEQ ID NO: 8).

A domain comprising an antibody variable region that has CD3-binding activity

Herein, the phrase “a domain comprising an antibody variable region that has CD3-binding activity” refers to a CD3-binding antibody portion produced by including a region that specifically binds to all or a portion of CD3 and is also complementary thereto. Preferably, the domain comprises the light-chain and heavy-chain variable regions (VL and VH) of an anti-CD3 antibody. Suitable examples of such a domain include “single chain Fv (scFv)”, “single chain antibody”, “Fv”, “single chain Fv 2 (scFv2)”, “Fab”, “F(ab')₂”, etc.

The domain comprising an antibody variable region that has CD3-binding activity of the present invention may be any epitope-binding domain as long as the epitope exists in the γ -chain, δ -chain, or ϵ -chain sequence that constitutes human CD3. In the present invention, preferably, a domain comprising an anti-CD3 antibody light-chain variable region (VL) and an anti-CD3 antibody heavy-chain variable region (VH) that bind to an epitope present in the extracellular

region of the ϵ chain of the human CD3 complex is suitably used. Besides the anti-CD3 antibody light chain variable region (VL) and anti-CD3 antibody heavy chain variable region (VH) described in the Reference Examples, various known CD3-binding domains containing a CD3-binding antibody light chain variable region (VL) and a CD3-binding antibody heavy chain variable region (VH), and those of the OKT3 antibody (Proc. Natl. Acad. Sci. USA (1980) 77, 4914-4917) are suitably used as such domains. One may appropriately use an antibody variable region-containing domain derived from the anti-CD3 antibody having desired properties, which is obtained by immunizing a desired animal by the above-mentioned method using the γ -chain, δ -chain, or ϵ -chain constituting the human CD3. Human antibodies and properly humanized antibodies as described above may be appropriately used as the anti-CD3 antibody to give rise to the domain containing the antibody variable region having CD3-binding activity. Regarding the structure of the γ -chain, δ -chain, or ϵ -chain constituting CD3, their polynucleotide sequences are shown in SEQ ID NOs: 9 (NM_000073.2), 10 (NM_000732.4), and 11 (NM_000733.3), and their polypeptide sequences are shown in SEQ ID NOs: 12 (NP_000064.1), 13 (NP_000723.1), and 14 (NP_000724.1) (the RefSeq accession number is shown in parentheses).

Antibody variable region-containing domains in antigen binding molecules of the present invention may bind to the same epitope. Herein, the same epitope may be present in a protein comprising the amino acid sequence of SEQ ID NO: 2 or 14. Alternatively, antibody variable region-containing domains in antigen binding molecules of the present invention may bind to different epitopes, respectively. Herein, the different epitopes may be present in a protein comprising the amino acid sequence of SEQ ID NO: 2 or 14.

Specific

The term “specific” means that one of molecules involved in specific binding does not show any significant binding to molecules other than a single or a number of binding partner molecules. Furthermore, the term is also used when a domain containing an antibody variable region is specific to a particular epitope among multiple epitopes in an antigen. When an epitope bound by a domain containing an antibody variable region is included in a number of different antigens, antigen-binding molecules comprising the antibody variable region-containing domain can bind to various antigens that have the epitope.

Epitope

“Epitope” means an antigenic determinant in an antigen, and refers to an antigen site to which a domain of an antigen-binding molecule including an antibody variable region disclosed herein binds. Thus, for example, the epitope can be defined according to its structure. Alternatively, the epitope may be defined according to the antigen-binding activity of an

antigen-binding molecule that recognizes the epitope. When the antigen is a peptide or polypeptide, the epitope can be specified by the amino acid residues forming the epitope. Alternatively, when the epitope is a sugar chain, the epitope can be specified by its specific sugar chain structure.

5 A linear epitope is an epitope that contains an epitope whose primary amino acid sequence is recognized. Such a linear epitope typically contains at least three and most commonly at least five, for example, about 8 to 10 or 6 to 20 amino acids in its specific sequence.

10 In contrast to the linear epitope, “conformational epitope” is an epitope in which the primary amino acid sequence containing the epitope is not the only determinant of the recognized epitope (for example, the primary amino acid sequence of a conformational epitope is not necessarily recognized by an epitope-defining antibody). Conformational epitopes may contain a greater number of amino acids compared to linear epitopes. A conformational epitope-recognizing antibody recognizes the three-dimensional structure of a peptide or protein.
15 For example, when a protein molecule folds and forms a three-dimensional structure, amino acids and/or polypeptide main chains that form a conformational epitope become aligned, and the epitope is made recognizable by the antibody. Methods for determining epitope conformations include, for example, X ray crystallography, two-dimensional nuclear magnetic resonance, site-specific spin labeling, and electron paramagnetic resonance, but are not limited
20 thereto. See, for example, Epitope Mapping Protocols in Methods in Molecular Biology (1996), Vol. 66, Morris (ed.).

A method for confirming binding to an epitope by a test antigen-binding molecule comprising a domain that contains an antibody variable region having GPC3-binding activity is exemplified below, and a method for confirming binding to an epitope by a test antigen-binding
25 molecule comprising a domain that contains an antibody variable region having T-cell receptor complex-binding activity may also be performed suitably according to the examples below.

For example, recognition of a linear epitope present in the GPC3 molecule by a test antigen-binding molecule comprising a domain that contains an antibody variable region having GPC3-binding activity can be confirmed below. A linear peptide comprising the amino acid
30 sequence constituting the extracellular domain of GPC3 is synthesized for the above-mentioned objective. The peptide may be synthesized chemically. Alternatively, it can be obtained by genetic engineering methods using a region in the cDNA of GPC3 that encodes an amino acid sequence corresponding to the extracellular domain. Next, the binding activity between a linear peptide comprising the amino acid sequence constituting the extracellular domain and the test
35 antigen-binding molecule comprising a domain that contains an antibody variable region having GPC3-binding activity is evaluated. For example, ELISA which uses an immobilized linear

peptide as the antigen may enable evaluation of the binding activity of the antigen-binding molecule towards the peptide. Alternatively, binding activity towards the linear peptide may be elucidated based on the level of inhibition caused by the linear peptide in the binding of the antigen-binding molecule to GPC3-expressing cells. These tests may elucidate the binding activity of the antigen-binding molecules toward the linear peptide.

Furthermore, recognition of the three-dimensional structure of the epitope by a test antigen-binding molecule comprising a domain that contains an antibody variable region having GPC3-binding activity can be confirmed below. GPC3-expressing cells are prepared for the above-mentioned objective. For example, when the test antigen-binding molecule comprising a domain that contains an antibody variable region having GPC3-binding activity contacts GPC3-expressing cells, it binds strongly to the cells, but on the other hand, there are cases when the antigen-binding molecule does not substantially bind to the immobilized linear peptide comprising the amino acid sequence constituting the extracellular domain of GPC3. In these cases, “does not substantially bind” refers to a binding activity of 80% or less, generally 50% or less, preferably 30% or less, and particularly preferably 15% or less relative to the binding activity towards human GPC3-expressing cells.

Methods for assaying the binding activity of a test antigen-binding molecule containing a GPC3 antigen-binding domain towards GPC3-expressing cells include, for example, the methods described in *Antibodies: A Laboratory Manual* (Ed Harlow, David Lane, Cold Spring Harbor Laboratory (1988) 359-420). Specifically, the assessment can be performed based on the principle of ELISA or fluorescence activated cell sorting (FACS) using GPC3-expressing cells as antigen.

In the ELISA format, the binding activity of a test antigen-binding molecule containing a GPC3 antigen-binding domain towards GPC3-expressing cells can be assessed quantitatively by comparing the levels of signal generated by enzymatic reaction. Specifically, a test antigen-binding molecule is added to an ELISA plate onto which GPC3-expressing cells are immobilized. Then, the test antigen-binding molecule bound to the cells is detected using an enzyme-labeled antibody that recognizes the test antigen-binding molecule. Alternatively, when FACS is used, a dilution series of a test antigen-binding molecule is prepared, and the antibody binding titer for GPC3-expressing cells can be determined to compare the binding activity of the test antigen-binding molecule towards GPC3-expressing cells.

The binding of a test antigen-binding molecule towards an antigen expressed on the surface of cells suspended in buffer or the like can be detected using a flow cytometer. Known flow cytometers include, for example, the following devices:

FACSCanto™ II
FACSAria™

FACSArray™

FACSVantage™ SE

FACSCalibur™ (all are trade names of BD Biosciences)

EPICS ALTRA HyPerSort

5 Cytomics FC 500

EPICS XL-MCL ADC EPICS XL ADC

Cell Lab Quanta/Cell Lab Quanta SC (all are trade names of Beckman Coulter)

Preferable methods for assaying the binding activity of a test antigen-binding molecule containing a GPC3 antigen-binding domain towards an antigen include, for example, the following method. First, GPC3-expressing cells are reacted with a test antigen-binding molecule, and then this is stained with an FITC-labeled secondary antibody that recognizes the polypeptide complex. The test antigen-binding molecule is appropriately diluted with a suitable buffer to prepare the complex at a desired concentration. For example, the complex can be used at a concentration within the range of 10 µg/ml to 10 ng/ml. Then, the fluorescence intensity and cell count are determined using FACSCalibur (BD). The fluorescence intensity obtained by analysis using the CELL QUEST Software (BD), i.e., the Geometric Mean value, reflects the quantity of antibody bound to cells. That is, the binding activity of a test antigen-binding molecule, which is represented by the quantity of the test antigen-binding molecule bound, can be determined by measuring the Geometric Mean value.

20 Whether a test antigen-binding molecule containing a GPC3 antigen-binding domain shares a common epitope with another antigen-binding molecule can be assessed based on the competition between the two complexes for the same epitope. The competition between antigen-binding molecules can be detected by cross-blocking assay or the like. For example, the competitive ELISA assay is a preferred cross-blocking assay.

25 Specifically, in cross-blocking assay, the GPC3 protein immobilized to the wells of a microtiter plate is pre-incubated in the presence or absence of a candidate competitor antigen-binding molecule, and then a test antigen-binding molecule is added thereto. The quantity of test antigen-binding molecule bound to the GPC3 protein in the wells is indirectly correlated with the binding ability of a candidate competitor antigen-binding molecule that competes for the binding to the same epitope. That is, the greater the affinity of the competitor antigen-binding molecule for the same epitope, the lower the binding activity of the test antigen-binding molecule towards the GPC3 protein-coated wells.

30 The quantity of the test antigen-binding molecule bound to the wells via the GPC3 protein can be readily determined by labeling the antigen-binding molecule in advance. For example, a biotin-labeled antigen-binding molecule is measured using an avidin/oxidase conjugate and appropriate substrate. In particular, cross-blocking assay that uses enzyme labels

such as peroxidase is called “competitive ELISA assay”. The antigen-binding molecule can also be labeled with other labeling substances that enable detection or measurement. Specifically, radiolabels, fluorescent labels, and such are known.

5 When the candidate competitor antigen-binding molecule can block the binding by a test antigen-binding molecule containing a GPC3 antigen-binding domain by at least 20%, preferably at least 20 to 50%, and more preferably at least 50% compared to the binding activity in a control experiment conducted in the absence of the competitor antigen-binding molecule, the test antigen-binding molecule is determined to substantially bind to the same epitope bound by the competitor antigen-binding molecule, or compete for the binding to the same epitope.

10 When the structure of an epitope bound by a test antigen-binding molecule containing a GPC3 antigen-binding domain has already been identified, whether the test and control antigen-binding molecules share a common epitope can be assessed by comparing the binding activities of the two antigen-binding molecules towards a peptide prepared by introducing amino acid mutations into the peptide forming the epitope.

15 To measure the above binding activities, for example, the binding activities of test and control antigen-binding molecules towards a linear peptide into which a mutation is introduced are compared in the above ELISA format. Besides the ELISA methods, the binding activity towards the mutant peptide bound to a column can be determined by flowing test and control antigen-binding molecules in the column, and then quantifying the antigen-binding molecule
20 eluted in the elution solution. Methods for adsorbing a mutant peptide to a column, for example, in the form of a GST fusion peptide, are known.

Alternatively, when the identified epitope is a conformational epitope, whether test and control antigen-binding molecules share a common epitope can be assessed by the following method. First, GPC3-expressing cells and cells expressing GPC3 with a mutation introduced
25 into the epitope are prepared. The test and control antigen-binding molecules are added to a cell suspension prepared by suspending these cells in an appropriate buffer such as PBS. Then, the cell suspensions are appropriately washed with a buffer, and an FITC-labeled antibody that recognizes the test and control antigen-binding molecules is added thereto. The fluorescence intensity and number of cells stained with the labeled antibody are determined using
30 FACSCalibur (BD). The test and control polypeptide complexes are appropriately diluted using a suitable buffer, and used at desired concentrations. For example, they may be used at a concentration within the range of 10 µg/ml to 10 ng/ml. The fluorescence intensity determined by analysis using the CELL QUEST Software (BD), i.e., the Geometric Mean value, reflects the quantity of labeled antibody bound to cells. That is, the binding activities of the test and control
35 antigen-binding molecules, which are represented by the quantity of labeled antibody bound, can be determined by measuring the Geometric Mean value.

In the above method, whether an antigen-binding molecule does “not substantially bind to cells expressing mutant GPC3” can be assessed, for example, by the following method. First, the test and control antigen-binding molecules bound to cells expressing mutant GPC3 are stained with a labeled antibody. Then, the fluorescence intensity of the cells is determined.

5 When FACSCalibur is used for fluorescence detection by flow cytometry, the determined fluorescence intensity can be analyzed using the CELL QUEST Software. From the Geometric Mean values in the presence and absence of the antigen-binding molecule, the comparison value (Δ Geo-Mean) can be calculated according to the following formula to determine the ratio of increase in fluorescence intensity as a result of the binding by the antigen-binding molecule.

10

$$\Delta\text{Geo-Mean} = \text{Geo-Mean (in the presence of the antigen-binding molecule)} / \text{Geo-Mean (in the absence of the antigen-binding molecule)}$$

The Geometric Mean comparison value (Δ Geo-Mean value for the mutant GPC3 molecule) determined by the above analysis, which reflects the quantity of a test antigen-binding molecule bound to cells expressing mutant GPC3, is compared to the Δ Geo-Mean comparison value that reflects the quantity of the test antigen-binding molecule bound to GPC3-expressing cells. In this case, the concentrations of the test antigen-binding molecule used to determine the Δ Geo-Mean comparison values for GPC3-expressing cells and cells expressing mutant GPC3 are particularly preferably adjusted to be equal or substantially equal. An antigen-binding molecule that has been confirmed to recognize an epitope in GPC3 is used as a control antigen-binding molecule.

If the Δ Geo-Mean comparison value of a test antigen-binding molecule for cells expressing mutant GPC3 is smaller than the Δ Geo-Mean comparison value of the test antigen-binding molecule for GPC3-expressing cells by at least 80%, preferably 50%, more preferably 30%, and particularly preferably 15%, then the test polypeptide complex “does not substantially bind to cells expressing mutant GPC3”. The formula for determining the Geo-Mean (Geometric Mean) value is described in the CELL QUEST Software User’s Guide (BD biosciences). When the comparison shows that the comparison values are substantially equivalent, the epitope for the test and control antigen-binding molecules can be determined to be the same.

30

Variable fragment (Fv)

Herein, the term “variable fragment (Fv)” refers to the minimum unit of an antibody-derived antigen-binding domain that is composed of a pair of the antibody light chain variable region (VL) and antibody heavy chain variable region (VH). In 1988, Skerra and

35

Pluckthun found that homogeneous and active antibodies can be prepared from the *E. coli* periplasm fraction by inserting an antibody gene downstream of a bacterial signal sequence and inducing expression of the gene in *E. coli* (Science (1988) 240(4855), 1038-1041). In the Fv prepared from the periplasm fraction, VH associates with VL in a manner so as to bind to an antigen.

Herein, Fv preferably includes, for example, a pair of Fv which is an antigen-binding molecule or such comprising:

- (1) a bivalent antigen-binding domain which is a bivalent scFv, wherein one monovalent scFv of the bivalent scFv is linked to one polypeptide forming an Fc domain by a heavy-chain Fv fragment forming a CD3-binding domain, and the other monovalent scFv is linked to the other polypeptide forming an Fc domain by a light-chain Fv fragment forming a CD3-binding domain;
 - (2) a domain comprising an Fc domain that has no Fcγ receptor-binding activity, and which is derived from amino acids forming the Fc domain of IgG1, IgG2a, IgG3, or IgG4; and
 - (3) at least a monovalent CD3-binding domain,
- wherein the light-chain and heavy-chain Fv fragments associate to form a CD3-binding domain such that it can bind to the CD3 antigen.

scFv, single-chain antibody, and sc(Fv)₂

Herein, the terms “scFv”, “single-chain antibody”, and “sc(Fv)₂” all refer to an antibody fragment of a single polypeptide chain that contains variable regions derived from the heavy and light chains, but not the constant region. In general, a single-chain antibody also contains a polypeptide linker between the VH and VL domains, which enables formation of a desired structure that is thought to allow antigen binding. The single-chain antibody is discussed in detail by Pluckthun in “The Pharmacology of Monoclonal Antibodies, Vol. 113, Rosenberg and Moore, eds., Springer-Verlag, New York, 269-315 (1994)”. See also International Patent Publication WO 1988/001649; US Patent Nos. 4,946,778 and 5,260,203. In a particular embodiment, the single-chain antibody can be bispecific and/or humanized.

scFv is an antigen-binding domain in which VH and VL forming Fv are linked together by a peptide linker (Proc. Natl. Acad. Sci. U.S.A. (1988) 85(16), 5879-5883). VH and VL can be retained in close proximity by the peptide linker.

sc(Fv)₂ is a single-chain antibody in which four variable regions of two VL and two VH are linked by linkers such as peptide linkers to form a single chain (J Immunol. Methods (1999) 231(1-2), 177-189). The two VH and two VL may be derived from different monoclonal antibodies. Such sc(Fv)₂ preferably includes, for example, a bispecific sc(Fv)₂ that recognizes two epitopes present in a single antigen as disclosed in the Journal of Immunology (1994) 152(11), 5368-5374. sc(Fv)₂ can be produced by methods known to those skilled in the art.

For example, sc(Fv)2 can be produced by linking scFv by a linker such as a peptide linker.

Herein, the form of an antigen-binding domain forming an sc(Fv)2 include an antibody in which the two VH units and two VL units are arranged in the order of VH, VL, VH, and VL ([VH]-linker-[VL]-linker-[VH]-linker-[VL]) beginning from the N terminus of a single-chain polypeptide. The order of the two VH units and two VL units is not limited to the above form, and they may be arranged in any order. Example order of the form is listed below.

[VL]-linker-[VH]-linker-[VH]-linker-[VL]

[VH]-linker-[VL]-linker-[VL]-linker-[VH]

[VH]-linker-[VH]-linker-[VL]-linker-[VL]

10 [VL]-linker-[VL]-linker-[VH]-linker-[VH]

[VL]-linker-[VH]-linker-[VL]-linker-[VH]

The molecular form of sc(Fv)2 is also described in detail in WO 2006/132352.

According to these descriptions, those skilled in the art can appropriately prepare desired sc(Fv)2 to produce the antigen-binding molecules disclosed herein.

15 Furthermore, the antigen-binding molecules of the present invention may be conjugated with a carrier polymer such as PEG or an organic compound such as an anticancer agent. Alternatively, a sugar chain addition sequence is preferably inserted into the polypeptide complexes such that the sugar chain produces a desired effect.

The linkers to be used for linking the variable regions of an antibody comprise arbitrary peptide linkers that can be introduced by genetic engineering, and synthetic linkers disclosed in, 20 for example, Protein Engineering, 9(3), 299-305, 1996. However, peptide linkers are preferred in the present invention. The length of the peptide linkers is not particularly limited, and can be suitably selected by those skilled in the art according to the purpose. The length is preferably five amino acids or more (without particular limitation, the upper limit is generally 30 amino acids or less, preferably 20 amino acids or less), and particularly preferably 15 amino acids. 25 When sc(Fv)2 contains three peptide linkers, their length may be all the same or different.

For example, such peptide linkers include:

Ser

Gly·Ser

30 Gly·Gly·Ser

Ser·Gly·Gly

Gly·Gly·Gly·Ser (SEQ ID NO: 15)

Ser·Gly·Gly·Gly (SEQ ID NO: 16)

Gly·Gly·Gly·Gly·Ser (SEQ ID NO: 17)

35 Ser·Gly·Gly·Gly·Gly (SEQ ID NO: 18)

Gly·Gly·Gly·Gly·Gly·Ser (SEQ ID NO: 19)

Ser·Gly·Gly·Gly·Gly·Gly (SEQ ID NO: 20)

Gly·Gly·Gly·Gly·Gly·Gly·Ser (SEQ ID NO: 21)

Ser·Gly·Gly·Gly·Gly·Gly·Gly (SEQ ID NO: 22)

(Gly·Gly·Gly·Gly·Ser (SEQ ID NO: 17))_n

5 (Ser·Gly·Gly·Gly·Gly (SEQ ID NO: 18))_n

where n is an integer of 1 or larger. The length or sequences of peptide linkers can be selected accordingly by those skilled in the art depending on the purpose.

Synthetic linkers (chemical crosslinking agents) are routinely used to crosslink peptides, and for example:

10 N-hydroxy succinimide (NHS),

disuccinimidyl suberate (DSS),

bis(sulfosuccinimidyl) suberate (BS³),

dithiobis(succinimidyl propionate) (DSP),

dithiobis(sulfosuccinimidyl propionate) (DTSSP),

15 ethylene glycol bis(succinimidyl succinate) (EGS),

ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS),

disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST),

bis[2-(succinimidoxycarbonyloxy)ethyl] sulfone (BSOCOES),

and bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl] sulfone (sulfo-BSOCOES). These

20 crosslinking agents are commercially available.

In general, three linkers are required to link four antibody variable regions together. The linkers to be used may be of the same type or different types.

Fab, F(ab')₂, and Fab'

25 "Fab" consists of a single light chain, and a CH1 domain and variable region from a single heavy chain. The heavy chain of Fab molecule cannot form disulfide bonds with another heavy chain molecule.

"F(ab')₂" or "Fab'" is produced by treating an immunoglobulin (monoclonal antibody) with a protease such as pepsin and papain, and refers to an antibody fragment generated by
30 digesting an immunoglobulin (monoclonal antibody) at near the disulfide bonds present between the hinge regions in each of the two H chains. For example, papain cleaves IgG upstream of the disulfide bonds present between the hinge regions in each of the two H chains to generate two homologous antibody fragments, in which an L chain comprising VL (L-chain variable region) and CL (L-chain constant region) is linked to an H-chain fragment comprising VH
35 (H-chain variable region) and CH₁ (γ1 region in an H-chain constant region) *via* a disulfide bond at their C-terminal regions. Each of these two homologous antibody fragments is called

Fab'.

“F(ab')₂” consists of two light chains and two heavy chains comprising the constant region of a CH1 domain and a portion of CH2 domains so that disulfide bonds are formed between the two heavy chains. The F(ab')₂ forming an antigen-binding molecule disclosed
5 herein can be preferably produced as follows. A whole monoclonal antibody or such comprising a desired antigen-binding domain is partially digested with a protease such as pepsin; and Fc fragments are removed by adsorption onto a Protein A column. The protease is not particularly limited, as long as it can cleave the whole antibody in a selective manner to produce F(ab')₂ under an appropriate setup enzyme reaction condition such as pH. Such proteases
10 include, for example, pepsin and ficin.

Fc domain

An Fc domain that forms an antigen-binding molecule disclosed herein can be preferably produced in the following manner. An antibody such as a monoclonal antibody is
15 partially digested with a protease such as pepsin. Then, the resulting fragment is adsorbed onto a Protein A or Protein G column, and eluted with an appropriate elution buffer. The protease is not particularly limited, as long as it can cleave antibodies such as monoclonal antibodies under an appropriate setup enzyme reaction condition such as pH. Such proteases include, for example, pepsin and ficin.

20 The antigen-binding molecules described herein comprise an Fc domain with reduced Fcγ receptor-binding activity, which includes amino acids forming the Fc domain of IgG1, IgG2, IgG3, or IgG4.

Antibody isotype is determined according to the structure of the constant region. Constant regions of the isotypes IgG1, IgG2, IgG3, and IgG4 are called Cγ1, Cγ2, Cγ3, and Cγ4,
25 respectively. The amino acid sequences of Fc domain polypeptides forming human Cγ1, Cγ2, Cγ3, and Cγ4 are exemplified in SEQ ID NO: 23, 24, 25, and 26, respectively. The relationship between amino acid residues forming each amino acid sequence and Kabat's EU numbering (herein also referred to as EU INDEX) are shown in Fig. 23.

The Fc domain refers to the region besides F(ab')₂ which comprises two light chains
30 and two heavy chains comprising a portion of the constant region that comprises a CH1 domain and a region between the CH1 and CH2 domains so that disulfide bonds are formed between the two heavy chains. The Fc domain forming an antigen-binding molecule disclosed herein can be preferably produced as follows. A monoclonal IgG1, IgG2, IgG3, or IgG4 antibody or the like is partially digested with a protease such as pepsin, followed by elution of the fraction adsorbed
35 onto a Protein A column. The protease is not particularly limited, as long as it can cleave the whole antibody in a selective manner to produce F(ab')₂ in an appropriate setup enzyme reaction

condition such as pH. Such proteases include, for example, pepsin and ficin.

Fcγ receptor

Fcγ receptor refers to a receptor capable of binding to the Fc domain of monoclonal
 5 IgG1, IgG2, IgG3, or IgG4 antibodies, and includes all members belonging to the family of
 proteins substantially encoded by an Fcγ receptor gene. In human, the family includes FcγRI
 (CD64) including isoforms FcγRIa, FcγRIb and FcγRIc; FcγRII (CD32) including isoforms
 FcγRIIa (including allotype H131 and R131), FcγRIIb (including FcγRIIb-1 and FcγRIIb-2), and
 FcγRIIc; and FcγRIII (CD16) including isoform FcγRIIIa (including allotype V158 and F158)
 10 and FcγRIIIb (including allotype FcγRIIIb-NA1 and FcγRIIIb-NA2); as well as all unidentified
 human FcγRs, FcγR isoforms, and allotypes thereof. However, Fcγ receptor is not limited to
 these examples. Without being limited thereto, FcγR includes those derived from humans, mice,
 rats, rabbits, and monkeys. FcγR may be derived from any organisms. Mouse FcγR includes,
 without being limited to, FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIII-2
 15 (CD16-2), as well as all unidentified mouse FcγRs, FcγR isoforms, and allotypes thereof. Such
 preferred Fcγ receptors include, for example, human FcγRI (CD64), FcγRIIA (CD32), FcγRIIB
 (CD32), FcγRIIIA (CD16), and/or FcγRIIB (CD16). The polynucleotide sequence and amino
 acid sequence of FcγRI are shown in SEQ ID NOs: 27 (NM_000566.3) and 28 (NP_000557.1),
 respectively; the polynucleotide sequence and amino acid sequence of FcγRIIA are shown in
 20 SEQ ID NOs: 29 (BC020823.1) and 30 (AAH20823.1), respectively; the polynucleotide
 sequence and amino acid sequence of FcγRIIB are shown in SEQ ID NOs: 31 (BC146678.1) and
 32 (AAI46679.1), respectively; the polynucleotide sequence and amino acid sequence of
 FcγRIIIA are shown in SEQ ID NOs: 33 (BC033678.1) and 34 (AAH33678.1), respectively; and
 the polynucleotide sequence and amino acid sequence of FcγRIIB are shown in SEQ ID NOs:
 25 35 (BC128562.1) and 36 (AAI28563.1), respectively (RefSeq accession number is shown in
 each parentheses). Whether an Fcγ receptor has binding activity to the Fc domain of a
 monoclonal IgG1, IgG2, IgG3, or IgG4 antibody can be assessed by ALPHA screen (Amplified
 Luminescent Proximity Homogeneous Assay), surface plasmon resonance (SPR)-based
 BIACORE method, and others (Proc. Natl. Acad. Sci. USA (2006) 103(11), 4005-4010), in
 30 addition to the above-described FACS and ELISA formats.

Meanwhile, “Fc ligand” or “effector ligand” refers to a molecule and preferably a
 polypeptide that binds to an antibody Fc domain, forming an Fc/Fc ligand complex. The
 molecule may be derived from any organisms. The binding of an Fc ligand to Fc preferably
 induces one or more effector functions. Such Fc ligands include, but are not limited to, Fc
 35 receptors, FcγR, FcαR, FcεR, FcRn, C1q, and C3, mannan-binding lectin, mannose receptor,
Staphylococcus Protein A, *Staphylococcus* Protein G, and viral FcγRs. The Fc ligands also

include Fc receptor homologs (FcRH) (Davis *et al.*, (2002) Immunological Reviews 190, 123-136), which are a family of Fc receptors homologous to FcγR. The Fc ligands also include unidentified molecules that bind to Fc.

5 Fcγ receptor-binding activity

The impaired binding activity of Fc domain to any of the Fcγ receptors FcγI, FcγIIA, FcγIIB, FcγIIIA, and/or FcγIIIB can be assessed by using the above-described FACS and ELISA formats as well as ALPHA screen (Amplified Luminescent Proximity Homogeneous Assay) and surface plasmon resonance (SPR)-based BIACORE method (Proc. Natl. Acad. Sci. USA (2006)
10 103(11), 4005-4010).

ALPHA screen is performed by the ALPHA technology based on the principle described below using two types of beads: donor and acceptor beads. A luminescent signal is detected only when molecules linked to the donor beads interact biologically with molecules linked to the acceptor beads and when the two beads are located in close proximity. Excited by laser beam,
15 the photosensitizer in a donor bead converts oxygen around the bead into excited singlet oxygen. When the singlet oxygen diffuses around the donor beads and reaches the acceptor beads located in close proximity, a chemiluminescent reaction within the acceptor beads is induced. This reaction ultimately results in light emission. If molecules linked to the donor beads do not interact with molecules linked to the acceptor beads, the singlet oxygen produced by donor beads
20 do not reach the acceptor beads and chemiluminescent reaction does not occur.

For example, a biotin-labeled antigen-binding molecule is immobilized to the donor beads and glutathione S-transferase (GST)-tagged Fcγ receptor is immobilized to the acceptor beads. In the absence of an antigen-binding molecule comprising a competitive mutant Fc domain, Fcγ receptor interacts with an antigen-binding molecule comprising a wild-type Fc
25 domain, inducing a signal of 520 to 620 nm as a result. The antigen-binding molecule having a non-tagged mutant Fc domain competes with the antigen-binding molecule comprising a wild-type Fc domain for the interaction with Fcγ receptor. The relative binding affinity can be determined by quantifying the reduction of fluorescence as a result of competition. Methods for biotinylating antigen-binding molecules such as antibodies using Sulfo-NHS-biotin or the
30 like are known. Appropriate methods for adding the GST tag to an Fcγ receptor include methods that involve fusing polypeptides encoding Fcγ and GST in-frame, expressing the fused gene using cells introduced with a vector carrying the gene, and then purifying using a glutathione column. The induced signal can be preferably analyzed, for example, by fitting to a one-site competition model based on nonlinear regression analysis using software such as
35 GRAPHPAD PRISM (GraphPad; San Diego).

One of the substances for observing their interaction is immobilized as a ligand onto the

gold thin layer of a sensor chip. When light is shed on the rear surface of the sensor chip so that total reflection occurs at the interface between the gold thin layer and glass, the intensity of reflected light is partially reduced at a certain site (SPR signal). The other substance for observing their interaction is injected as an analyte onto the surface of the sensor chip. The mass of immobilized ligand molecule increases when the analyte binds to the ligand. This alters the refraction index of solvent on the surface of the sensor chip. The change in refraction index causes a positional shift of SPR signal (conversely, the dissociation shifts the signal back to the original position). In the Biacore system, the amount of shift described above (*i.e.*, the change of mass on the sensor chip surface) is plotted on the vertical axis, and thus the change of mass over time is shown as measured data (sensorgram). Kinetic parameters (association rate constant (k_a) and dissociation rate constant (k_d)) are determined from the curve of sensorgram, and affinity (K_D) is determined from the ratio between these two constants. Inhibition assay is preferably used in the BIACORE methods. Examples of such inhibition assay are described in Proc. Natl. Acad. Sci. USA (2006) 103(11), 4005-4010.

Herein, “Fcγ receptor-binding activity is reduced” means, for example, that based on the above-described analysis method the competitive activity of a test antigen-binding molecule is 50% or less, preferably 45% or less, 40% or less, 35% or less, 30% or less, 20% or less, or 15% or less, and particularly preferably 10% or less, 9% or less, 8% or less, 7% or less, 6% or less, 5% or less, 4% or less, 3% or less, 2% or less, or 1% or less than the competitive activity of a control antigen-binding molecule.

Antigen-binding molecules comprising the Fc domain of a monoclonal IgG1, IgG2, IgG3, or IgG4 antibody can be appropriately used as control antigen-binding molecules. The Fc domain structures are shown in SEQ ID NOs: 37 (A is added to the N terminus of RefSeq accession number AAC82527.1), 38 (A is added to the N terminus of RefSeq accession number AAB59393.1), 25 (A is added to the N terminus of RefSeq accession number CAA27268.1), and 39 (A is added to the N terminus of RefSeq accession number AAB59394.1). Furthermore, when an antigen-binding molecule comprising an Fc domain mutant of an antibody of a particular isotype is used as a test substance, the effect of the mutation of the mutant on the Fcγ receptor-binding activity is assessed using as a control an antigen-binding molecule comprising an Fc domain of the same isotype. As described above, antigen-binding molecules comprising an Fc domain mutant whose Fcγ receptor-binding activity has been judged to be reduced are appropriately prepared.

Such known mutants include, for example, mutants having a deletion of amino acids 231A-238S (EU numbering) (WO 2009/011941), as well as mutants C226S, C229S, P238S, (C220S) (J. Rheumatol (2007) 34, 11); C226S and C229S (Hum. Antibod. Hybridomas (1990) 1(1), 47-54); C226S, C229S, E233P, L234V, and L235A (Blood (2007) 109, 1185-1192).

Specifically, the preferred antigen-binding molecules include those comprising an Fc domain with a substitution of the amino acid at position 220, 226, 229, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 264, 265, 266, 267, 269, 270, 295, 296, 297, 298, 299, 300, 325, 327, 328, 329, 330, 331, or 332 (EU numbering) in the amino acids forming the Fc domain of an antibody of a particular isotype. The isotype of antibody from which the Fc domain originates is not particularly limited, and it is possible to use an appropriate Fc domain derived from a monoclonal IgG1, IgG2, IgG3, or IgG4 antibody. It is preferable to use Fc domains derived from IgG1 antibodies.

The preferred antigen-binding molecules include, for example, those comprising an Fc domain which has any one of the substitutions shown below, whose positions are specified according to EU numbering (each number represents the position of an amino acid residue in the EU numbering; and the one-letter amino acid symbol before the number represents the amino acid residue before substitution, while the one-letter amino acid symbol after the number represents the amino acid residue before the substitution) in the amino acids forming the Fc domain of IgG1 antibody:

- (a) L234F, L235E, P331S;
- (b) C226S, C229S, P238S;
- (c) C226S, C229S;
- (d) C226S, C229S, E233P, L234V, L235A;
- (e) L234A, L235A or L235R, N297A;
- (f) L235A or L235R, S239K, N297A

as well as those having an Fc domain which has a deletion of the amino acid sequence at positions 231 to 238.

Furthermore, the preferred antigen-binding molecules also include those comprising an Fc domain that has any one of the substitutions shown below, whose positions are specified according to EU numbering in the amino acids forming the Fc domain of an IgG2 antibody:

- (g) H268Q, V309L, A330S, and P331S;
- (h) V234A;
- (i) G237A;
- (j) V234A and G237A;
- (k) A235E and G237A;
- (l) V234A, A235E, and G237A. Each number represents the position of an amino acid residue in EU numbering; and the one-letter amino acid symbol before the number represents the amino acid residue before substitution, while the one-letter amino acid symbol after the number represents the amino acid residue before the substitution.

Furthermore, the preferred antigen-binding molecules also include those comprising an

Fc domain that has any one of the substitutions shown below, whose positions are specified according to EU numbering in the amino acids forming the Fc domain of an IgG3 antibody:

(m) F241A;

(n) D265A;

- 5 (o) V264A. Each number represents the position of an amino acid residue in EU numbering; and the one-letter amino acid symbol before the number represents the amino acid residue before substitution, while the one-letter amino acid symbol after the number represents the amino acid residue before the substitution.

Furthermore, the preferred antigen-binding molecules also include those comprising an Fc domain that has any one of the substitutions shown below, whose positions are specified according to EU numbering in the amino acids forming the Fc domain of an IgG4 antibody:

(p) L235A, G237A, and E318A;

(q) L235E;

- 15 (r) F234A and L235A. Each number represents the position of an amino acid residue in EU numbering; and the one-letter amino acid symbol before the number represents the amino acid residue before substitution, while the one-letter amino acid symbol after the number represents the amino acid residue before the substitution.

The other preferred antigen-binding molecules include, for example, those comprising an Fc domain in which any amino acid at position 233, 234, 235, 236, 237, 327, 330, or 331 (EU numbering) in the amino acids forming the Fc domain of an IgG1 antibody is substituted with an amino acid of the corresponding position in EU numbering in the corresponding IgG2 or IgG4.

The preferred antigen-binding molecules also include, for example, those comprising an Fc domain in which any one or more of the amino acids at positions 234, 235, and 297 (EU numbering) in the amino acids forming the Fc domain of an IgG1 antibody is substituted with other amino acids. The type of amino acid after substitution is not particularly limited; however, the antigen-binding molecules comprising an Fc domain in which any one or more of the amino acids at positions 234, 235, and 297 are substituted with alanine are particularly preferred.

The preferred antigen-binding molecules also include, for example, those comprising an Fc domain in which an amino acid at position 265 (EU numbering) in the amino acids forming the Fc domain of an IgG1 antibody is substituted with another amino acid. The type of amino acid after substitution is not particularly limited; however, antigen-binding molecules comprising an Fc domain in which an amino acid at position 265 is substituted with alanine are particularly preferred.

35 Multispecific antigen-binding molecules

Examples of a preferred embodiment of the “multispecific antigen-binding molecule” of

the present invention include multispecific antibodies. When an Fc region with reduced Fc γ receptor-binding activity is used as the multispecific antibody Fc region, an Fc region derived from the multispecific antibody may be used appropriately. Bispecific antibodies are particularly preferred as the multispecific antibodies of the present invention. In this case, a
5 bispecific antibody is an antibody having two different specificities. IgG-type bispecific antibodies can be secreted from a hybrid hybridoma (quadroma) produced by fusing two types of hybridomas that produce IgG antibodies (Milstein *et al.*, Nature (1983) 305, 537-540).

Furthermore, IgG-type bispecific antibodies are secreted by introducing the genes of L chains and H chains constituting the two types of IgGs of interest, i.e. a total of four genes, into
10 cells, and co-expressing them. However, the number of combinations of H and L chains of IgG that can be produced by these methods is theoretically ten combinations. Accordingly, it is difficult to purify an IgG comprising the desired combination of H and L chains from ten types of IgGs. Furthermore, theoretically the amount of secretion of the IgG having the desired combination will decrease remarkably, and therefore large-scale culturing will be necessary, and
15 production costs will increase further.

Therefore, techniques for promoting the association among H chains and between L and H chains having the desired combinations can be applied to the multispecific antigen-binding molecules of the present invention.

For example, techniques for suppressing undesired H-chain association by introducing
20 electrostatic repulsion at the interface of the second constant region or the third constant region of the antibody H chain (CH₂ or CH₃) can be applied to multispecific antibody association (WO2006/106905).

In the technique of suppressing unintended H-chain association by introducing electrostatic repulsion at the interface of CH₂ or CH₃, examples of amino acid residues in
25 contact at the interface of the other constant region of the H chain include regions corresponding to the residues at EU numbering positions 356, 439, 357, 370, 399, and 409 in the CH₃ region.

More specifically, examples include an antibody comprising two types of H-chain CH₃ regions, in which one to three pairs of amino acid residues in the first H-chain CH₃ region, selected from the pairs of amino acid residues indicated in (1) to (3) below, carry the same type
30 of charge: (1) amino acid residues comprised in the H chain CH₃ region at EU numbering positions 356 and 439; (2) amino acid residues comprised in the H-chain CH₃ region at EU numbering positions 357 and 370; and (3) amino acid residues comprised in the H-chain CH₃ region at EU numbering positions 399 and 409.

Furthermore, the antibody may be an antibody in which pairs of the amino acid residues
35 in the second H-chain CH₃ region which is different from the first H-chain CH₃ region mentioned above, are selected from the aforementioned pairs of amino acid residues of (1) to (3),

wherein the one to three pairs of amino acid residues that correspond to the aforementioned pairs of amino acid residues of (1) to (3) carrying the same type of charges in the first H-chain CH3 region mentioned above carry opposite charges from the corresponding amino acid residues in the first H-chain CH3 region mentioned above.

5 Each of the amino acid residues indicated in (1) to (3) above come close to each other during association. Those skilled in the art can find out positions that correspond to the above-mentioned amino acid residues of (1) to (3) in a desired H-chain CH3 region or H-chain constant region by homology modeling and such using commercially available software, and amino acid residues of these positions can be appropriately subjected to modification.

10 In the antibodies mentioned above, “charged amino acid residues” are preferably selected, for example, from amino acid residues included in either one of the following groups:
 (a) glutamic acid (E) and aspartic acid (D); and
 (b) lysine (K), arginine (R), and histidine (H).

 In the above-mentioned antibodies, the phrase “carrying the same charge” means, for
15 example, that all of the two or more amino acid residues are selected from the amino acid residues included in either one of groups (a) and (b) mentioned above. The phrase “carrying opposite charges” means, for example, that when at least one of the amino acid residues among two or more amino acid residues is selected from the amino acid residues included in either one of groups (a) and (b) mentioned above, the remaining amino acid residues are selected from the
20 amino acid residues included in the other group.

 In a preferred embodiment, the antibodies mentioned above may have their first H-chain CH3 region and second H-chain CH3 region crosslinked by disulfide bonds.

 In the present invention, amino acid residues subjected to modification are not limited to the above-mentioned amino acid residues of the antibody variable regions or the antibody
25 constant regions. Those skilled in the art can identify the amino acid residues that form an interface in mutant polypeptides or heteromultimers by homology modeling and such using commercially available software; and amino acid residues of these positions can then be subjected to modification so as to regulate the association.

 Other known techniques can also be used for the association of multispecific antibodies
30 of the present invention. Fc region-containing polypeptides comprising different amino acids can be efficiently associated with each other by substituting an amino acid side chain present in one of the H-chain Fc regions of the antibody with a larger side chain (knob), and substituting an amino acid side chain present in the corresponding Fc region of the other H chain with a smaller side chain (hole) to allow placement of the knob within the hole (WO1996/027011; Ridgway JB
35 *et al.*, Protein Engineering (1996) 9, 617-621; Merchant A. M. *et al.* Nature Biotechnology (1998) 16, 677-681; and US20130336973).

In addition, other known techniques can also be used for formation of multispecific antibodies of the present invention. Association of polypeptides having different sequences can be induced efficiently by complementary association of CH3 using a strand-exchange engineered domain CH3 produced by changing part of one of the H-chain CH3s of an antibody to a
5 corresponding IgA-derived sequence and introducing a corresponding IgA-derived sequence into the complementary portion of the other H-chain CH3 (Protein Engineering Design & Selection, 23; 195-202, 2010). This known technique can also be used to efficiently form multispecific antibodies of interest.

In addition, technologies for antibody production using association of antibody CH1 and
10 CL and association of VH and VL as described in WO 2011/028952, WO2014/018572, and Nat Biotechnol. 2014 Feb; 32(2):191-8; technologies for producing bispecific antibodies using separately prepared monoclonal antibodies in combination (Fab Arm Exchange) as described in WO2008/119353 and WO2011/131746; technologies for regulating association between antibody heavy-chain CH3s as described in WO2012/058768 and WO2013/063702;
15 technologies for producing bispecific antibodies composed of two types of light chains and one type of heavy chain as described in WO2012/023053; technologies for producing bispecific antibodies using two bacterial cell strains that individually express one of the chains of an antibody comprising a single H chain and a single L chain as described by Christoph *et al.* (Nature Biotechnology Vol. 31, p 753-758 (2013)); and such may be used for the formation of
20 multispecific antibodies.

An embodiment of multispecific antibody formation includes methods for obtaining bispecific antibodies by mixing two types of monoclonal antibodies in the presence of a reducing agent to cleave the disulfide bonds in the core hinge region, followed by re-association for heterodimerization (FAE) as described above. Meanwhile, introduction of electrostatic
25 interactions at the interacting interface of the CH3 region (WO2006/106905) can induce even more efficient heterodimerization during the re-association (WO2015/046467). In FAE using naturally-occurring IgG, re-association takes place randomly; and thus theoretically, bispecific antibodies can only be obtained at 50% efficiency; however, in this method, bispecific antibodies can be produced in high yield.

30 Alternatively, even when a multispecific antibody of interest cannot be formed efficiently, a multispecific antibody of the present invention can be obtained by separating and purifying the multispecific antibody of interest from the produced antibodies. For example, a method for enabling purification of two types of homomeric forms and the heteromeric antibody of interest by ion-exchange chromatography by imparting a difference in isoelectric points by
35 introducing amino acid substitutions into the variable regions of the two types of H chains has been reported (WO2007114325). To date, as a method for purifying heteromeric antibodies,

methods using Protein A to purify a heterodimeric antibody comprising a mouse IgG2a H chain that binds to Protein A and a rat IgG2b H chain that does not bind to Protein A have been reported (WO98050431 and WO95033844). Furthermore, a heterodimeric antibody can be purified efficiently on its own by using H chains comprising substitution of amino acid residues at EU numbering positions 435 and 436, which is the IgG-Protein A binding site, with Tyr, His, or such which are amino acids that yield a different Protein A affinity, or using H chains with a different protein A affinity obtained according to the method of Reference Example 9, to change the interaction of each of the H chains with Protein A, and then using a Protein A column.

Alternatively, a common L chain that can provide binding ability to a plurality of different H chains can be obtained and used as the common L chain of a multispecific antibody. Efficient expression of a multispecific IgG can be achieved by introducing the genes of such a common L chain and a plurality of different H chains into cells to express the IgG (Nature Biotechnology (1998) 16, 677-681). A method for selecting a common L chain that shows a strong binding ability to any of the different H chains can also be used when selecting the common H chain (WO 2004/065611).

Furthermore, an Fc region whose Fc region C-terminal heterogeneity has been improved can be appropriately used as an Fc region of the present invention. More specifically, the present invention provides Fc regions produced by deleting glycine at position 446 and lysine at position 447 as specified by EU numbering from the amino acid sequences of two polypeptides constituting an Fc region derived from IgG1, IgG2, IgG3, or IgG4.

A plurality, such as two or more, of these technologies can be used in combination. Furthermore, these technologies can be appropriately and separately applied to the two H chains to be associated. Furthermore, these techniques can be used in combination with the above-mentioned Fc region which has reduced binding activity to an Fcγ receptor. Furthermore, an antigen-binding molecule of the present invention may be a molecule produced separately so that it has the same amino acid sequence, based on the antigen-binding molecule subjected to the above-described modifications.

A non-limiting embodiment of the present invention provides anticancer agents comprising as an active ingredient a bispecific antibody of any one of (a) to (c) below that comprises an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3-binding activity:

(a) a bispecific antibody in which CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having

CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3

5 regions comprised in SEQ ID NO: 223, respectively;

(b) a bispecific antibody in which the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a
10 common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 223; and

(c) a bispecific antibody which has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence
15 of SEQ ID NO: 402; and common L chains having at least 80% identity to the amino acid sequence of SEQ ID NO: 410.

In the bispecific antibody of any one of (a) to (c) above, the amino acid sequence identity of each specified heavy chain and light chain CDR1, CDR2, and CDR3, heavy chain variable region, light chain variable region, whole heavy chain, and whole light chain is
20 preferably at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, or 90%, or higher, and more preferably at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or higher.

Herein, sequence identity is calculated as the percentage of residues identical to those in the original amino acid sequence of heavy chain or light chain variable region determined after
25 the sequences are aligned and gaps are appropriately introduced as necessary to maximize the sequence identity.

In the bispecific antibody of any one of (a) to (c) above, as long as the antibody has glypican 3- or CD3-binding activity, one or more amino acids may be substituted, deleted, added, and/or inserted in the amino acid sequences of heavy chain and light chain CDR1, CDR2, and
30 CDR3, and heavy chain variable region, light chain variable region, whole heavy chain, and whole light chain. Methods well known to those skilled in the art for preparing such amino acid sequence in which one or more amino acids are substituted, deleted, added, and/or inserted include a method of introducing mutations into proteins. For example, those skilled in the art can prepare mutants that are functionally equivalent to the heavy chain or light chain variable
35 region of the bispecific antibody which has the antibody H chain of SEQ ID NO: 385 having glypican 3-binding activity, the antibody H chain of SEQ ID NO: 402 having CD3-binding

activity, and the antibody common L chains of SEQ ID NO: 410 by appropriately introducing mutations into the amino acid sequence of an antibody having glypican 3- or CD3-binding activity using site-directed mutagenesis (Hashimoto-Gotoh, T, Mizuno, T, Ogasahara, Y, and Nakagawa, M. (1995) An oligodeoxyribonucleotide-directed dual amber method for site-directed mutagenesis. *Gene* 152, 271-275; Zoller, MJ, and Smith, M. (1983) Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol.* 100, 468-500; Kramer, W, Drutsa, V, Jansen, HW, Kramer, B, Pflugfelder, M, and Fritz, HJ (1984) The gapped duplex DNA approach to oligonucleotide-directed mutation construction. *Nucleic Acids Res.* 12, 9441-9456; Kramer W, and Fritz HJ (1987) Oligonucleotide-directed construction of mutations via gapped duplex DNA *Methods. Enzymol.* 154, 350-367; Kunkel, TA (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc Natl Acad Sci U S A.* 82, 488-492) or such. In the present invention, the phrase "functionally equivalent" means that the binding affinities for an antigen are equivalent, or alternatively, it means that the cytotoxic activities against glypican 3-expressing cells or tissues containing these cells are equivalent when it is used as a multispecific antigen-binding molecule. The binding affinity and cytotoxic activity can be measured based on the description herein. The details are described herein below.

The number of amino acids to be altered is not limited, and is, for example, 40 or less, 30 or less, 20 or less, preferably 18 or less, 16 or less, 15 or less, 12 or less, 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less.

When an amino acid residue is altered, the amino acid is preferably mutated into a different amino acid(s) that conserves the properties of the amino acid side chain. Examples of amino acid side chain properties are: hydrophobic amino acids (A, I, L, M, F, P, W, Y, and V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, and T), amino acids having aliphatic side chains (G, A, V, L, I, and P), amino acids having hydroxyl group-containing side chains (S, T, and Y), amino acids having sulfur-containing side chains (C and M), amino acids having carboxylic acid- and amide-containing side chains (D, N, E, and Q), amino acids having basic side chains (R, K, and H), and amino acids having aromatic side chains (H, F, Y, and W) (amino acids are represented by one-letter codes in parentheses). Amino acid substitutions within each of these groups are referred to as conservative substitutions. It is already known that a polypeptide having a modified amino acid sequence in which one or more amino acid residues in a given amino acid sequence are deleted, added, and/or substituted with other amino acids can retain the biological activity (Mark, D. F. *et al.*, *Proc. Natl. Acad. Sci. USA* (1984)81:5662-6; Zoller, M. J. and Smith, M., *Nucleic Acids Res.* (1982)10:6487-500; Wang, A. *et al.*, *Science* (1984) 224:1431-3; Dalbadie-McFarland, G. *et al.*, *Proc. Natl. Acad. Sci. USA* (1982)79:6409-13).

Alternatively, an amino acid sequence having glypican 3- or CD3-binding activity that has a substitution, deletion, addition, and/or insertion of one or more amino acids in the amino acid sequence of the CDR region, heavy chain variable region, light chain variable region, whole heavy chain region, or whole light chain region can be obtained from nucleic acids that hybridize under stringent conditions with nucleic acids comprising the nucleotide sequence encoding the amino acid sequence of the CDR region, heavy chain variable region, light chain variable region, whole heavy chain region, or whole light chain region. Stringent hybridization conditions for isolating a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of the CDR region, heavy chain variable region, light chain variable region, whole heavy chain region, or whole light chain region include, for example, the conditions of 6 M urea, 0.4% SDS, 0.5x SSC, and 37°C, or hybridization conditions with a stringency equivalent thereto. Isolation of nucleic acids with a much higher homology can be expected with more stringent conditions, for example, the conditions of 6 M urea, 0.4% SDS, 0.1x SSC, and 42°C. The washing conditions following the hybridization are, for example, washing with 0.5x SSC (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 0.1% SDS at 60°C, more preferably washing with 0.2x SSC and 0.1% SDS at 60°C, even more preferably washing with 0.2x SSC and 0.1% SDS at 62°C, yet even more preferably washing with 0.2x SSC and 0.1% SDS at 65°C, and still more preferably washing with 0.1x SSC and 0.1% SDS at 65°C. The washing time and the number of washes may be appropriately adjusted, for example, a 20-minute wash may be performed three times. The sequences of the isolated nucleic acids can be determined by known methods described below. The overall nucleotide sequence homology of the isolated nucleic acid is at least 50% or higher, preferably 70% or higher, 75% or higher, 80% or higher, 85% or higher, and more preferably 90% or higher (for example, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher) sequence identity.

Instead of the above-described methods using hybridization techniques, gene amplification methods, for example, polymerase chain reaction (PCR), using primers synthesized based on the information of a nucleotide sequence encoding the amino acid sequence of the CDR region, heavy chain variable region, light chain variable region, whole heavy chain region, or whole light chain region can also be employed to isolate a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of the region.

The identity of one nucleotide sequence or amino acid sequence to another can be determined using algorithm BLAST by Karlin and Altschul (Proc. Natl. Acad. Sci. USA (1993) 90:5873-7). Programs called BLASTN and BLASTX were developed based on this algorithm (Altschul *et al.*, J. Mol. Biol. (1990) 215:403-10). Parameters for the analysis of nucleotide

sequences by BLASTN based on BLAST include, for example, score= 100 and wordlength= 12. On the other hand, parameters for the analysis of amino acid sequences by BLASTX based on BLAST include, for example, score= 50 and wordlength= 3. Default parameters for each program are used when using the BLAST and Gapped BLAST programs. Specific techniques
 5 for such analyses are known (see the website of the National Center for Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST); <http://www.ncbi.nlm.nih.gov>).

A non-limiting embodiment of the present invention provides anticancer agents comprising as an active ingredient a bispecific antibody that has the two H chains and common L
 10 chains of the antibody of sample number 38 (Table 17) described in Examples and Reference Examples. A non-limiting embodiment of the present invention also provides anticancer agents comprising as an active ingredient a bispecific antibody that has the variable regions of the two H chains and common L chains of the antibody of sample number 38 and has glypican 3- and CD3-binding activities. A non-limiting embodiment of the present invention also provides
 15 anticancer agents comprising as an active ingredient a bispecific antibody that has the respective amino acid sequences of CDR1, CDR2, and CDR3 regions comprised in the two H chains and common L chains of the antibody of sample number 38 and has glypican 3- and CD3-binding activities.

A non-limiting embodiment of the present invention provides anticancer agents
 20 comprising as an active ingredient a bispecific antibody that has the antibody H chain of SEQ ID NO: 385 having glypican 3-binding activity, the antibody H chain of SEQ ID NO: 402 having CD3-binding activity, and the antibody common L chains of SEQ ID NO: 410.

A non-limiting embodiment of the present invention provides anticancer agents comprising as an active ingredient a bispecific antibody in which CDR1, CDR2, and CDR3
 25 comprised in the antibody variable region having glypican 3-binding activity are the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in the antibody variable
 30 region of the common L chain are the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively.

The amino acid sequences of CDR1, CDR2, and CDR3 comprised in the antibody variable region can be identified by methods known to those skilled in the art, and such methods are not particularly limited. The CDR1, CDR2, and CDR3 regions may be those based on
 35 numbering known to those skilled in the art, for example, Kabat, Chothia, or Contact numbering, or may be those based on other criteria. In a non-limiting embodiment of the present invention,

the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206 include the regions of amino acid sequences of positions 31 to 35 (SEQ ID NO: 456), positions 50 to 66 (SEQ ID NO: 457), and positions 99 to 104 (SEQ ID NO: 458), respectively (Kabat numbering); the regions of amino acid sequences of positions 26 to 32 (SEQ ID NO: 459), positions 52 to 57 (SEQ ID NO: 460), and positions 97 to 104 (SEQ ID NO: 461), respectively (Chothia numbering); and the regions of amino acid sequences of positions 30 to 35 (SEQ ID NO: 462), positions 47 to 59 (SEQ ID NO: 463), and positions 97 to 103 (SEQ ID NO: 464), respectively (Contact numbering); however, the CDR regions may be those based on other criteria.

In a non-limiting embodiment of the present invention, the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168 include the regions of amino acid sequences of positions 31 to 35 (SEQ ID NO: 465), positions 50 to 68 (SEQ ID NO: 466), and positions 101 to 111 (SEQ ID NO: 467), respectively (Kabat numbering); the regions of amino acid sequences of positions 26 to 32 (SEQ ID NO: 468), positions 52 to 59 (SEQ ID NO: 469), and positions 99 to 111 (SEQ ID NO: 470), respectively (Chothia numbering); and the regions of amino acid sequences of positions 30 to 35 (SEQ ID NO: 471), positions 47 to 61 (SEQ ID NO: 472), and positions 99 to 110 (SEQ ID NO: 473), respectively (Contact numbering); however, the CDR regions may be those based on other criteria.

In a non-limiting embodiment of the present invention, the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223 include the regions of amino acid sequences of positions 24 to 39 (SEQ ID NO: 474), positions 55 to 61 (SEQ ID NO: 475), and positions 94 to 102 (SEQ ID NO: 476), respectively (Kabat numbering and Chothia numbering); and the regions of amino acid sequences of positions 35 to 41 (SEQ ID NO: 477), positions 51 to 56 (SEQ ID NO: 478), and positions 94 to 101 (SEQ ID NO: 479), respectively (Contact numbering); however, the CDR regions may be those based on other criteria (see Figs. 24-1, 24-2, and 25).

A non-limiting embodiment of the present invention provides anticancer agents comprising as an active ingredient a bispecific antibody in which the antibody variable region having glypican 3-binding activity is the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is the amino acid sequence of SEQ ID NO: 168; and the antibody variable region of the common L chain is the amino acid sequence of SEQ ID NO: 223.

An appropriate multispecific antigen-binding molecule comprised in the anticancer agent or the pharmaceutical composition of the present invention comprises

- (1) a domain comprising an antibody variable region having glypican 3-binding activity;
- (2) a domain comprising an antibody variable region having T-cell receptor complex-binding activity; and

(3) a domain comprising an Fc region with reduced Fc γ receptor-binding activity mentioned above, without limitation to its structure.

In the present invention, each of the above-mentioned domains can be linked directly by peptide bonds. For example, when using F(ab')₂ as the domain comprising an antibody
5 variable region of (1) and (2), and these Fc regions as the domain comprising an Fc region with reduced Fc γ receptor-binding activity of (3), the polypeptides formed by linking the antibody variable region-containing domains of (1) and (2) and the Fc region-containing domain of (3) by peptide bonds will form an antibody structure. Such antibodies can be produced by purification from the above-mentioned hybridoma culture medium, and also by purifying antibodies from the
10 culture medium of desired host cells that stably carry polynucleotides encoding the polypeptides constituting the antibody.

Examples of a preferred antibody H-chain variable region comprised in the anticancer agent or the pharmaceutical composition of the present invention contained in the antibody variable region having glypican 3-binding activity comprises the antibody H-chain variable
15 regions of Table 1, or antibody H-chain variable regions having CDR sequences whose CDR1, CDR2, and CDR3 amino acid sequences are the same as the CDR1, CDR2, and CDR3 amino acid sequences contained in the H-chain variable regions of Table 1, or antibody H-chain variable regions which are functionally equivalent to the above-mentioned variable regions.

Table 1

Sequence Name	SEQ ID NO:	Sequence Name	SEQ ID NO:
H0000	40	GCH042	193
GCH003	170	GCH043	194
GCH005	171	GCH045	195
GCH006	172	GCH053	196
GCH007	173	GCH054	197
GCH008	174	GCH055	198
GCH010	175	GCH056	199
GCH012	176	GCH057	200
GCH013	177	GCH059	201
GCH014	178	GCH060	202
GCH015	179	GCH061	203
GCH016	180	GCH062	204
GCH019	181	GCH064	205
GCH022	182	GCH065	206
GCH023	183	GCH066	207
GCH025	184	GCH067	208
GCH026	185	GCH068	209
GCH027	186	GCH073	210
GCH029	187	GCH094	211
GCH032	188	GCH098	212
GCH034	189	GCH099	213
GCH035	190	GCH100	214
GCH039	191	H0610	215
GCH040	192		

- Examples of a preferred antibody variable region having T-cell receptor
- 5 complex-binding activity which is comprised in the anticancer agent or the pharmaceutical composition of the present invention include antibody variable regions having T-cell receptor-binding activity. Of the T-cell receptors, CD3 is preferred, and CD3 ϵ is particularly preferred. Examples of an antibody H-chain variable region contained in such antibody
- 10 variable regions include the antibody H-chain variable regions of Table 2, antibody H-chain variable regions having CDR sequences whose CDR1, CDR2, and CDR3 amino acid sequences are the same as the CDR1, CDR2, and CDR3 amino acid sequences contained in the antibody H-chain variable regions of Table 2, and antibody H-chain variable regions that are functionally equivalent to the above-mentioned variable regions.

Table 2

Sequence Name	SEQ ID NO:	Sequence Name	SEQ ID NO:	Sequence Name	SEQ ID NO:
hCE115HA	52	TR01H036	99	TR01H074	135
CE115HA177	64	TR01H037	100	TR01H075	136
CE115HA178	65	TR01H038	101	TR01H076	137
CE115HA179	66	TR01H039	102	TR01H077	138
CE115HA180	67	TR01H040	103	TR01H079	139
hCE115HAa	68	TR01H041	104	TR01H080	140
TR01H006	69	TR01H042	105	TR01H081	141
TR01H007	70	TR01H043	106	TR01H082	142
TR01H008	71	TR01H044	107	TR01H083	143
TR01H009	72	TR01H045	108	TR01H084	144
TR01H010	73	TR01H046	109	TR01H090	145
TR01H011	74	TR01H047	110	TR01H091	146
TR01H012	75	TR01H048	111	TR01H092	147
TR01H013	76	TR01H049	112	TR01H093	148
TR01H014	77	TR01H050	113	TR01H094	149
TR01H015	78	TR01H051	114	TR01H095	150
TR01H016	79	TR01H052	115	TR01H096	151
TR01H017	80	TR01H053	116	TR01H097	152
TR01H018	81	TR01H054	117	TR01H098	153
TR01H019	82	TR01H055	118	TR01H099	154
TR01H020	83	TR01H056	119	TR01H100	155
TR01H021	84	TR01H057	120	TR01H101	156
TR01H022	85	TR01H058	121	TR01H102	157
TR01H023	86	TR01H061	122	TR01H103	158
TR01H024	87	TR01H062	123	TR01H104	159
TR01H025	88	TR01H063	124	TR01H105	160
TR01H026	89	TR01H064	125	TR01H106	161
TR01H027	90	TR01H065	126	TR01H107	162
TR01H028	91	TR01H066	127	TR01H108	163
TR01H029	92	TR01H067	128	TR01H109	164
TR01H030	93	TR01H068	129	TR01H110	165
TR01H031	94	TR01H069	130	TR01H111	166
TR01H032	95	TR01H070	131	TR01H112	167
TR01H033	96	TR01H071	132	TR01H113	168
TR01H034	97	TR01H072	133	TR01H114	169
TR01H035	98	TR01H073	134	TR01H001	420
				TR01H002	421
				TR01H003	422
				TR01H004	423
				rCE115H	424
				CE115HA121	425
				CE115HA122	426
				CE115HA124	427
				CE115HA192	428
				CE115HA236	429
				CE115HA251	430
				CE115HA252	431

The relationship between the CDR regions of the amino acid residues constituting the antibody H chain amino acid sequence and Kabat numbering is as shown in Fig. 24 (Figs. 24-1 and 24-2).

5 For the antibody L-chain variable regions contained in the antibody variable region having glypican 3-binding activity and the antibody variable region having T-cell receptor complex-binding activity which are comprised in the anticancer agent or the pharmaceutical composition of the present invention, it is preferable to obtain a common L chain that may provide a binding activity to the H chain having glypican 3-binding activity and a binding
10 activity to the H chain having T-cell receptor complex, and to use this as the common L-chain variable region of the multispecific antigen-binding molecule.

 Examples of the common L-chain variable region to be used in the present invention include the L-chain variable regions of Table 3, antibody L-chain variable regions having CDR sequences whose CDR1, CDR2, and CDR3 amino acid sequences are the same as the CDR1,
15 CDR2, and CDR3 amino acid sequences contained in the antibody L-chain variable regions of Table 3, and antibody L-chain variable regions that are functionally equivalent to the above-mentioned variable regions.

Table 3

Sequence Name	SEQ ID NO.	Sequence Name	SEQ ID NO.	Sequence Name	SEQ ID NO.
L0000	53	L0125	264	L0214	312
L0002	217	L0126	265	L0215	313
L0003	218	L0127	266	L0216	314
L0006	219	L0129	267	L0217	315
L0007	220	L0132	268	L0218	316
L0008	221	L0134	269	L0219	317
L0009	222	L0136	270	L0220	318
L0011	223	L0137	271	L0222	319
L0012	224	L0138	272	L0223	320
L0013	225	L0139	273	L0224	321
L0014	226	L0140	274	L0226	322
L0015	227	L0141	275	L0227	323
L0016	228	L0143	276	L0228	324
L0032	229	L0144	277	L0229	325
L0038	230	L0145	278	L0230	326
L0039	231	L0147	279	L0231	327
L0041	232	L0148	280	L0232	328
L0042	233	L0149	281	L0233	329
L0043	234	L0151	282	L0234	330
L0044	235	L0152	283	L0235	331
L0045	236	L0154	284	L0236	332
L0046	237	L0155	285	L0237	333
L0047	238	L0157	286	L0238	334
L0062	239	L0160	287	L0239	335
L0063	240	L0161	288	L0240	336
L0064	241	L0163	289	L0241	337
L0065	242	L0167	290	L0242	338
L0066	243	L0168	291	L0243	339
L0069	244	L0173	292	L0246	340
L0075	245	L0175	293	L0247	341
L0079	246	L0180	294	L0248	342
L0082	247	L0181	295	L0249	343
L0085	248	L0186	296	L0250	344
L0089	249	L0187	297	L0258	345
L0090	250	L0200	298	L0259	346
L0091	251	L0201	299	L0260	347
L0093	252	L0202	300	L0261	348
L0104	253	L0203	301	L0262	349
L0106	254	L0204	302	L0263	350
L0107	255	L0205	303	L0264	351
L0109	256	L0206	304	L0265	352
L0113	257	L0207	305	L0266	353
L0115	258	L0208	306	L0267	354
L0117	259	L0209	307	L0268	355
L0120	260	L0210	308	L0269	356
L0122	261	L0211	309	L0270	357
L0123	262	L0212	310	L0271	358
L0124	263	L0213	311	L0272	359

The relationship between the CDR regions of the amino acid residues constituting the antibody L-chain amino acid sequence and Kabat numbering is as shown in Fig. 25.

In the present invention, the phrase “functionally equivalent” means that the binding
 5 affinities for an antigen are equivalent, or alternatively, it means that the cytotoxic activities against glypican 3-expressing cells or tissues containing these cells are equivalent when it is used as a multispecific antigen-binding molecule. The binding affinity and cytotoxic activity can be measured based on the description herein. The cells used for measurement of cytotoxic activity may be the desired GPC3-expressing cells or a desired tissue containing these cells, and for
 10 example, PC-10 or NCI-H446 which are GPC3-expressing human cancer cell lines can be used. Regarding the antibody constant regions, the phrase may mean that the decreases in Fcγ receptor-binding activity are equivalent.

For example, an antibody H-chain variable region functionally equivalent to the antibody H chain variable region described herein (i.e., the original H chain variable region)
 15 means that this region has the same binding affinity when it is combined with the antibody L-chain variable region described herein which forms a pair with the original H chain, or alternatively that the region has the same cytotoxic activity towards glypican 3-expressing cells or a tissue containing these cells when used for a multispecific antigen-binding molecule. Furthermore, an antibody L-chain variable region functionally equivalent to the antibody L-chain
 20 variable region described herein (i.e., the original L-chain variable region) means that this region has the same binding affinity when it is combined with the antibody H-chain variable region described herein which forms a pair with the original L chain, or alternatively that the region has the same cytotoxic activity towards glypican 3-expressing cells or a tissue containing these cells when used for a multispecific antigen-binding molecule.

25 The term “equivalent” does not necessarily have to mean the same degree of activity, and the activity may be enhanced. Specifically, for antigen-binding affinity, examples include the case where the value (KD value / parent KD value) obtained by comparison to the binding affinity of the antibody variable region serving as the control (parent KD value) is 1.5 or less. The value of KD value / parent KD value is preferably 1.3 or less, more preferably 1.2 or less,
 30 1.1 or less, 1.0 or less, 0.9 or less, 0.8 or less, 0.7 or less, 0.6 or less, or 0.5 or less. While there is no lower limit, examples include 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , or 10^{-6} . More specifically, in the present invention, the value of KD value / parent KD value is preferably 10^{-6} to 1.5×10^{-0} , more preferably 10^{-6} to 10^{-1} , even more preferably 10^{-6} to 10^{-2} , and yet even more preferably 10^{-6} to 10^{-3} . For cytotoxic activity, examples include the case where the value (cell proliferation
 35 inhibition rate / parent cell proliferation inhibition rate) obtained by comparison to the cell proliferation inhibition rate of the multispecific antigen-binding molecule serving as the control

(parent cell proliferation inhibition rate) is 0.7 or more. The concentration of the added multispecific antigen-binding molecule can be determined appropriately, but is preferably, for example, 0.01 nM, 0.05 nM, 0.1 nM, 0.5 nM, or 1 nM; and preferably, measurements are taken at 0.05 nM or 0.1 nM. The value for cell proliferation inhibition rate / parent cell proliferation inhibition rate is preferably 0.8 or higher, more preferably 0.9 or higher, 1.0 or higher, 1.2 or higher, 1.5 or higher, 2 or higher, 3 or higher, 5 or higher, 10 or higher, or 20 or higher. While there is no upper limit, the value may be 10, 10^2 , 10^3 , 10^4 , 10^5 , or 10^6 .

Furthermore, for cytotoxic activity, examples include the case where the value (concentration for 50% inhibition of cell proliferation / parent concentration for 50% inhibition of cell proliferation) obtained by comparison to the concentration of the original multispecific antigen-binding molecule for 50% inhibition of cell proliferation (parent concentration for 50% inhibition of cell proliferation) is 1.5 or less. Concentration for 50% growth inhibition refers to the concentration of the multispecific antigen-binding molecule necessary for reducing the cell proliferation rate to one half compared to when the multispecific antigen-binding molecule is not added. The value of “concentration for 50% inhibition of cell proliferation / parent concentration for 50% inhibition of cell proliferation” is preferably 1.3 or less, more preferably 1.2 or less, 1.1 or less, 1.0 or less, 0.9 or less, 0.8 or less, 0.7 or less, 0.6 or less, or 0.5 or less. While there is no lower limit, the value may be, for example, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , or 10^{-6} . Specifically, the value is preferably 10^{-6} to 1.5×10^{-0} , more preferably 10^{-6} to 10^{-1} , even more preferably 10^{-6} to 10^{-2} , and yet even more preferably 10^{-6} to 10^{-3} .

Regarding the domain comprising an antibody variable region having GPC3-binding activity, the KD value towards GPC3 (for example, human GPC3) may be, for example, 5×10^{-9} M or less, preferably 4×10^{-9} M or less, such as 3×10^{-9} M or less, 2×10^{-9} M or less, 1×10^{-9} M or less, 8×10^{-10} M or less, 5×10^{-10} M or less, 4×10^{-10} M or less, 3×10^{-10} M or less, 2×10^{-10} M or less, 1×10^{-10} M or less, 8×10^{-11} M or less, 5×10^{-11} M or less, 4×10^{-11} M or less, 3×10^{-11} M or less, 2×10^{-11} M or less, 1×10^{-11} M or less, 8×10^{-12} M or less, 5×10^{-12} M or less, 4×10^{-12} M or less, 3×10^{-12} M or less, 2×10^{-12} M or less, 1×10^{-12} M or less, 8×10^{-13} M or less, 5×10^{-13} M or less, 4×10^{-13} M or less, 3×10^{-13} M or less, 2×10^{-13} M or less, or 1×10^{-13} M or less.

Regarding the domain comprising an antibody variable region having T-cell receptor complex-binding activity, the KD value towards a human T-cell receptor complex such as a human T cell receptor, or more specifically for example human CD3 ϵ may be, for example, 2×10^{-7} M or less, preferably 1.5×10^{-7} M or less, such as 1.4×10^{-7} M or less, 1.3×10^{-7} M or less, 1.2×10^{-7} M or less, 1×10^{-7} M or less, 3×10^{-8} M or less, 2×10^{-8} M or less, 1×10^{-8} M or less, 8×10^{-9} M or less, 5×10^{-9} M or less, 4×10^{-9} M or less, 3×10^{-9} M or less, 2×10^{-9} M or less, 1×10^{-9} M or less, 8×10^{-10} M or less, 5×10^{-10} M or less, 4×10^{-10} M or less, 3×10^{-10} M or less, $2 \times$

10^{-10} M or less, 1×10^{-10} M or less, 8×10^{-11} M or less, 5×10^{-11} M or less, 4×10^{-11} M or less, 3×10^{-11} M or less, 2×10^{-11} M or less, 1×10^{-11} M or less, 8×10^{-12} M or less, 5×10^{-12} M or less, 4×10^{-12} M or less, 3×10^{-12} M or less, 2×10^{-12} M or less, or 1×10^{-12} M or less.

The multispecific antigen-binding molecules of the present invention preferably have
 5 KD values toward human GPC3 and human T-cell receptor complex (for example, human CD3 ϵ chain) that are 5×10^{-9} M or less and 2×10^{-7} M or less, respectively, and more preferably 1×10^{-9} M or less and 5×10^{-8} M or less, respectively.

In the present invention, antibody variable regions that are “functionally equivalent” are not particularly limited as long as they are antibody H-chain and/or antibody L-chain variable
 10 regions that satisfy the above-described conditions. Examples of such antibody variable regions include regions produced by introducing substitution, deletion, addition, and/or insertion of one or more amino acids (for example, 1, 2, 3, 4, 5, or 10 amino acids) into the amino acid sequences of the variable regions of Tables 1 to 3 mentioned above. A method well known to those skilled in the art for introducing one or more amino-acid substitutions, deletions, additions,
 15 and/or insertions into an amino acid sequence is a method of introducing mutations into proteins. For example, those skilled in the art can prepare variable regions that are functionally equivalent to the antibody variable regions having the above-mentioned functions by appropriately introducing mutations into amino acid sequences using methods such as site-directed mutagenesis (Hashimoto-Gotoh, T., Mizuno, T., Ogasahara, Y., and Nakagawa, M. (1995) An
 20 oligodeoxyribonucleotide-directed dual amber method for site-directed mutagenesis. *Gene* 152, 271-275; Zoller, M.J., and Smith, M. (1983) Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol.* 100, 468-500; Kramer, W., Drutsa, V., Jansen, H.W., Kramer, B., Pflugfelder, M., and Fritz, H.J. (1984) The gapped duplex DNA approach to oligonucleotide-directed mutation construction. *Nucleic Acids Res.* 12, 9441-9456;
 25 Kramer, W., and Fritz, H.J. (1987) Oligonucleotide-directed construction of mutations *via* gapped duplex DNA *Methods. Enzymol.* 154, 350-367; and Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc Natl Acad. Sci. U S A.* 82, 488-492).

When an amino acid residue is altered, the amino acid is preferably mutated into a
 30 different amino acid(s) that conserves the properties of the amino acid side-chain as mentioned above. Examples of amino-acid side chain properties are: hydrophobic amino acids (A, I, L, M, F, P, W, Y, and V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, and T), amino acids containing aliphatic side chains (G, A, V, L, I, and P), amino acids containing hydroxyl group-containing side chains (S, T, and Y), amino acids containing sulfur atom-containing side
 35 chains (C and M), amino acids containing carboxylic acid- and amide-containing side chains (D, N, E, and Q), amino acids containing basic side chains (R, K, and H), and amino acids

containing aromatic side chains (H, F, Y, and W) (amino acids are represented by one-letter codes in parentheses). Amino acid substitutions within each of these groups are called conservative substitutions. It is already known that a polypeptide containing a modified amino acid sequence in which one or more amino acid residues in a given amino acid sequence are

5 deleted, added, and/or substituted with other amino acids can retain the original biological activity (Mark, D. F. *et al.*, Proc. Natl. Acad. Sci. USA; (1984) 81: 5662-6; Zoller, M. J. and Smith, M., Nucleic Acids Res. (1982) 10: 6487-500; Wang, A. *et al.*, Science (1984) 224: 1431-3; Dalbadie-McFarland, G. *et al.*, Proc. Natl. Acad. Sci. USA (1982) 79: 6409-13).

Variable regions of the present invention containing such amino acid modifications have an

10 amino acid sequence identity of at least 70%, more preferably at least 75%, even more preferably at least 80%, still more preferably at least 85%, yet more preferably at least 90%, and most preferably at least 95%, with the amino acid sequence of the CDR sequences, FR sequences, or whole variable regions of the variable region prior to modification. Herein, sequence identity is defined as the percentage of residues identical to those in the original amino acid sequence of the

15 H-chain variable region or L-chain variable region determined after the sequences are aligned, and gaps are appropriately introduced to maximize the sequence identity as necessary. The identity of amino acid sequences can be determined by the method described below.

Furthermore, a “functionally equivalent antibody variable region” can be obtained, for example, from nucleic acids that hybridize under stringent conditions with nucleic acids

20 comprising a nucleotide sequence encoding the amino acid sequence of a variable region in Tables 1 to 3 mentioned above. Stringent hybridization conditions for isolating a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of a variable region include, for example, the conditions of 6 M urea, 0.4% SDS, 0.5x SSC, and 37°C, or hybridization conditions with a stringency equivalent

25 thereto as mentioned above. Isolation of nucleic acids with a much higher homology can be expected with more stringent conditions, for example, the conditions of 6 M urea, 0.4% SDS, 0.1x SSC, and 42°C. The washing conditions following the hybridization are, for example, washing using 0.5x SSC (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate at pH7.0) and 0.1% SDS at 60°C, more preferably washing using 0.2x SSC and 0.1% SDS at 60°C, even more

30 preferably washing using 0.2x SSC and 0.1% SDS at 62°C, yet even more preferably washing using 0.2x SSC and 0.1% SDS at 65°C, and still more preferably washing using 0.1x SSC and 0.1% SDS at 65°C as mentioned above. The sequences of the isolated nucleic acids can be determined by the known methods described below. The overall nucleotide sequence homology of the isolated nucleic acid is at least 50% or higher, preferably 70% or higher, and

35 more preferably 90% or higher (for example, 95%, 96%, 97%, 98%, 99%, or higher) sequence identity.

Nucleic acids that hybridize under stringent conditions to a nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of a variable region can also be isolated by using, instead of the above-described methods using hybridization techniques, gene amplification methods such as polymerase chain reaction (PCR) that uses primers synthesized
 5 based on information of the nucleotide sequence encoding the variable-region amino acid sequence.

The identity of one nucleotide sequence or amino acid sequence to another can be determined using the algorithm BLAST, by Karlin and Altschul (Proc. Natl. Acad. Sci. USA (1993) 90: 5873-7) as mentioned above. Programs called BLASTN and BLASTX were
 10 developed based on this algorithm (Altschul *et al.*, J. Mol. Biol. (1990) 215: 403-10). To analyze nucleotide sequences according to BLASTN based on BLAST, the parameters are set, for example, as score = 100 and wordlength = 12. On the other hand, parameters used for the analysis of amino acid sequences by BLASTX based on BLAST include, for example, score = 50 and wordlength = 3. Default parameters for each program are used when using the BLAST
 15 and Gapped BLAST programs. Specific techniques for such analyses are known in the art (see the website of the National Center for Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST); <http://www.ncbi.nlm.nih.gov>).

The combination of the antibody variable region having glypican 3-binding activity and the antibody variable region having T-cell receptor complex binding activity as comprised in the
 20 multispecific antigen-binding molecule which is comprised in the anticancer agent or the pharmaceutical composition of the present invention is not particularly limited as long as it has the above-described activities. However, in the present invention, the cytotoxic activity of the multispecific antigen-binding molecule is preferably equivalent to or greater than that of the bispecific antibody GPC3_ERY22_rCE115 described in Reference Example 3. Here, the term
 25 “equivalent” does not necessarily have to mean the same degree of activity as described above, and the activity may be enhanced. Being equivalent to GPC3_ERY22_rCE115 is, for example, when the value of (cell proliferation inhibition rate / cell proliferation inhibition rate (GPC3_ERY22_rCE115)) relative to the cell proliferation inhibition rate of GPC3_ERY22_rCE115 (cell proliferation inhibition rate (GPC3_ERY22_rCE115)) is 0.7 or
 30 greater, preferably 0.8 or greater, 0.9 or greater, 1.0 or greater, 1.2 or greater, 1.5 or greater, 2 or greater, 3 or greater, 5 or greater, 10 or greater, or 20 or greater. While there is no upper limit, the value may be, for example, 10, 10², 10³, 10⁴, 10⁵, or 10⁶. The concentration of the multispecific antigen-binding molecule to be added can be determined appropriately, but is preferably, for example, 0.01 nM, 0.05 nM, 0.1 nM, 0.5 nM, or 1 nM; and preferably,
 35 measurements are taken at 0.05 nM or 0.1 nM.

Furthermore, examples include the case where the value (concentration for 50%

inhibition of cell proliferation / concentration for 50% inhibition of cell proliferation (GPC3_ERY22_rCE115)) obtained by comparison to the concentration for 50% inhibition of growth of GPC3_ERY22_rCE115 cells (concentration for 50% inhibition of cell proliferation (GPC3_ERY22_rCE115)) is 1.5 or less. The value for “concentration for 50% inhibition of cell proliferation / concentration for 50% inhibition of cell proliferation (GPC3_ERY22_rCE115)” is preferably 1.3 or less, more preferably 1.2 or less, 1.1 or less, 1.0 or less, 0.9 or less, 0.8 or less, 0.7 or less, 0.6 or less, or 0.5 or less. While there is no lower limit, the value may be for example, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , or 10^{-6} . Specifically, the value is preferably 10^{-6} to 1.5×10^{-0} , more preferably 10^{-6} to 10^{-1} , even more preferably 10^{-6} to 10^{-2} , and yet even more preferably 10^{-6} to 10^{-3} .

The preferred specific KD values for human GPC3 and human T cell receptor complex (for example, human CD3 ϵ chain) are also as indicated above. Desired cells showing GPC3 expression or desired tissues containing these cells may be used for the cells, and for example, PC-10 or NCI-H446 which are GPC3-expressing human cancer cell lines can be used.

Examples of such a combination of the antibody variable region having glypican 3-binding activity and the antibody variable region having T-cell receptor complex binding activity, include the combinations of antibody H-chain variable regions shown in Table 4, combinations of antibody H-chain variable regions having CDR sequences whose CDR1, CDR2, and CDR3 amino acid sequences are the same as the CDR1, CDR2, and CDR3 amino acid sequences carried by the antibody H-chain variable regions of Table 4, and combinations of antibody H-chain variable regions functionally equivalent to these variable regions. Here, “functionally equivalent” has the same meaning described above.

Table 4

GPC3 side / T cell receptor complex side	SEQ ID NO:
H0000/hCE115HA	40/52
H0000/CE115HA251	40/500
H0000/CE115HA236	40/429
H0000/TR01H002	40/421
H0000/CE115HA122	40/426
H0610/rCE115H	215/424
H0610/TR01H040	215/103
H0610/TR01H061	215/122
H0610/TR01H068	215/129
H0610/TR01H071	215/132
GCH054/TR01H067	197/128
GCH094/TR01H082	211/142
GCH094/TR01H084	211/144
GCH065/TR01H084	206/144
GCH065/TR01H082	206/142
GCH094/TR01H109	211/164
GCH065/TR01H109	206/164
GCH094/TR01H113	211/168
GCH065/TR01H113	206/168

- A preferred common L chain for such combinations of an antibody variable region having glypican 3-binding activity and an antibody variable region having T-cell receptor complex binding activity includes, for example, L0000, L0011, L0201, L0203, L0204, L0206, L0208, L0209, L0211, L0212, L0222, and a common L chain having CDR sequences (CDR1, CDR2, and CDR3 amino acid sequences) identical to the CDR1, CDR2, and CDR3 amino acid sequences as in the above common L chain. Specific combinations include, for example, the combinations of antibody H-chain variable regions and a common L chain shown in Table 5, combinations of antibody variable regions having CDR sequences (CDR1, CDR2, and CDR3 amino acid sequences) identical to the amino acid sequences of CDR1, CDR2, and CDR3 carried by the antibody variable regions and a common L chain of Table 5, and combinations of antibody H-chain variable regions and a common L chain functionally equivalent to these variable regions. Here, "functionally equivalent" has the same meaning as described above.

Table 5

GPC3 side / T cell receptor complex side / common L chain	SEQ ID NO:
H0610/rCE115H/L0000	215/424/53
H0610/TR01H040/L0000	215/103/53
H0610/TR01H040/L0201	215/103/299
H0610/TR01H040/L0203	215/103/301
H0610/TR01H040/L0204	215/103/302
H0610/TR01H040/L0206	215/103/304
H0610/TR01H040/L0208	215/103/306
H0610/TR01H040/L0209	215/103/307
H0610/TR01H040/L0211	215/103/309
H0610/TR01H061/L0000	215/122/53
H0610/TR01H068/L0000	215/129/53
H0610/TR01H071/L0000	215/132/53
GCH054/TR01H067/L0201	197/128/299
GCH054/TR01H067/L0212	197/128/310
GCH054/TR01H067/L0222	197/128/319
GCH054/TR01H067/L0000	197/128/53
GCH094/TR01H082/L0201	211/142/299
GCH094/TR01H082/L0011	211/142/223
GCH094/TR01H084/L0011	211/144/223
GCH065/TR01H084/L0011	206/144/223
GCH065/TR01H082/L0011	206/142/223
GCH094/TR01H109/L0011	211/164/223
GCH065/TR01H109/L0011	206/164/223
GCH094/TR01H113/L0011	211/168/223
GCH065/TR01H113/L0011	206/168/223

The Fc region comprised in the multispecific antigen-binding molecule which is
 5 comprised in the anticancer agent or the pharmaceutical composition of the present invention is
 not particularly limited as long as it is an Fc region having reduced Fc γ receptor-binding activity,
 but examples of a preferred Fc region of the present invention include a combination of the
 Fc-region portion of E22Hh and the Fc-region portion of E22Hk, a combination of the Fc-region
 portion of E2702GsKsc and the Fc-region portion of E2704sEpse, and a combination of the
 10 Fc-region portion of E2702sKsc and the Fc-region portion of E2704sEpse.

Examples of a preferred multispecific antigen-binding molecule comprised in an
 anticancer agent or a pharmaceutical composition of the present invention include bispecific

antibodies comprising an antibody variable region having glypican 3-binding activity and an antibody variable region having CD3 ϵ -binding activity. More preferably, the cytotoxic activity is the same or greater than that of the GPC3_ERY22_rCE115 bispecific antibody. Examples of such bispecific antibodies include bispecific antibodies comprising H and L chains described in Table 17, and bispecific antibodies that bind to an epitope overlapping with an epitope bound by the above antibodies, and which contain an Fc region with reduced Fc γ receptor-binding activity.

Whether an antibody recognizes an epitope that overlaps with an epitope recognized by another antibody can be confirmed by the competition between the two antibodies against the epitope. Competition between the antibodies can be evaluated by competitive binding assays using means such as enzyme-linked immunosorbent assay (ELISA), fluorescence energy transfer method (FRET), and fluorometric microvolume assay technology (FMAT (Registered trademark)). The amount of an antibody bound to an antigen indirectly correlates with the binding ability of a candidate competitor antibody (a test antibody) that competitively binds to the overlapping epitope. In other words, as the amount or affinity of a test antibody against the overlapping epitope increases, the amount of the antibody bound to the antigen decreases, and the amount of the antigen-bound test antibody increases. Specifically, the appropriately labeled antibody and antibody to be evaluated are simultaneously added to the antigen, and the antibody bound as a result are detected using the label. The amount of the antigen-bound antibody can be easily determined by labeling the antibody beforehand. This label is not particularly limited, and the labeling method is selected according to the assay technique used. Specifically, the labeling method includes fluorescent labeling, radiolabeling, enzymatic labeling, and such.

For example, the fluorescently labeled antibody and the unlabeled antibody or test antibody are simultaneously added to beads immobilized with GPC3 or CD3 ϵ , and the labeled antibody is detected by fluorometric microvolume assay technology.

Herein, the “antibody that binds to the overlapping epitope” refers to a test antibody that can reduce the amount of the bound labeled antibody by at least 50% at a concentration that is usually 100 times higher, preferably 80 times higher, more preferably 50 times higher, even more preferably 30 times higher, and still more preferably 10 times higher than the concentration at which the non-labeled antibody reduces 50% of the amount of the labeled antibody bound (IC₅₀).

Multispecific antigen-binding molecules, which have the antigen-binding sites of antibodies that bind to epitopes overlapping with epitopes bound by the above-mentioned antibodies, can yield excellent cytotoxic activity.

The multispecific antigen-binding molecules of the present invention are produced by the same technique as the method for producing recombinant antibodies mentioned above.

Combination therapies and pharmaceutical compositions

In a non-limiting embodiment of the present invention, the combination therapy of the present invention provides methods for damaging cells, for suppressing cell proliferation, for activating immunity towards cancer cells or cancer cell-comprising tumor tissues, for treating cancer, or for preventing cancer, each of the methods comprising administering effective
5 amounts of the above-described bispecific antibody and another anticancer agent. In several embodiments, the combination therapy of the present invention is highly effective for damaging cells, suppressing cell proliferation, activating immunity towards cancer cells or cancer cell-comprising tumor tissues, treating cancer, or preventing cancer, as compared to monotherapy using the above-described bispecific antibody or the other anticancer agent. In another
10 embodiment, the combination therapy of the present invention has synergistic effects or additive effects on damaging cells, suppressing cell proliferation, activating immunity towards cancer cells or cancer cell-comprising tumor tissues, treating cancer, or preventing cancer.

In several embodiments, the term “effective amount” in the present invention refers to a dose of the above-described bispecific antibody and/or another anticancer agent that is effective
15 for treating or preventing a disease in an individual. The disease is not particularly limited but is preferably cancer.

In several embodiments, “treatment/treating/therapeutic” in the present invention means that the combination therapy of the present invention decreases the number of cancer cells in individuals, suppresses cancer cell proliferation, decreases tumor size, suppresses infiltration of
20 cancer cells into peripheral organs, suppresses cancer cell metastasis, or ameliorates various symptoms caused by cancer. Furthermore, in several embodiments, “prevention/preventing/prophylactic” in the present invention refers to inhibiting increase in the number of cancer cells due to repopulation of cancer cells that have been decreased, inhibiting repopulation of cancer cells whose proliferation has been suppressed, and inhibiting the
25 decreased tumor size to become large again.

In several embodiments, the combination therapy of the present invention provides methods for enhancing therapeutic or prophylactic effects of the other anticancer agent by using the above-described bispecific antibody, in cancer treatment or prevention with the other anticancer agent. In another embodiment, the combination therapy of the present invention
30 provides methods for enhancing therapeutic or prophylactic effects of the above-described bispecific antibody by using the other anticancer agent, in cancer treatment or prevention with the bispecific antibody. Herein, enhancement of therapeutic or prophylactic effects refers to, for example, increase in efficacy rate of the treatment, decrease in the amount of the anticancer agent that is administered for the treatment, and/or shortening of the period of the treatment with
35 an anticancer agent, but is not limited thereto. In another embodiment, the combination therapy of the present invention provides methods for extending progression-free survival in individuals,

the method comprising administering an effective amount of the above-described bispecific antibody and another anticancer agent.

In several embodiments, the combination therapy of the present invention comprises administering the above-described bispecific antibody and another anticancer agent. The bispecific antibody and the other anticancer agent can be administered by any appropriate methods known in the art. For example, the bispecific antibody and the other anticancer agent can be administered in parallel (i.e., simultaneously) or successively (i.e., at different time points). In several embodiments, when the bispecific antibody and the other anticancer agent are administered successively (i.e., at different time points), the interval between administration of the bispecific antibody and the other anticancer agent is not particularly limited and the interval can be determined by taking account for factors such as the administration route and dosage form. The interval is, for example, 0 to 168 hours, preferably 0 to 72 hours, more preferably 0 to 24 hours, and even more preferably 0 to 12 hours, but is not limited thereto.

In several embodiments, the above-described bispecific antibody and the other anticancer agent are administered simultaneously. In several embodiments, the bispecific antibody is administered at intervals (i.e., intermittently). In several embodiments, the bispecific antibody is administered before administration of the other anticancer agent. In several embodiments, the bispecific antibody is administered after administration of the other anticancer agent.

In several embodiments, the other anticancer agent is administered at intervals (i.e., intermittently). In several embodiments, the other anticancer agent is administered before administration of the bispecific antibody. In several embodiments, the other anticancer agent is administered after administration of the bispecific antibody.

In several embodiments, the bispecific antibodies described herein and anticancer agents which are known or described herein can be used in the above-described combination therapies using bispecific antibodies and other anticancer agents.

In several embodiments, an additional therapy can be performed in addition to the combination therapies using the above-described bispecific antibody and another anticancer agent. In several embodiments, a therapy to add to the combination therapy of the present invention may comprise additional administration of the bispecific antibody and/or the other anticancer agent.

A non-limiting embodiment of the present invention provides agents for inducing cytotoxicity, agents for suppressing cell proliferation (agents for inhibiting cell proliferation), agents for activating immune response towards cancer cells or cancer cell-comprising tumor tissues, agents for treating cancer, and agents for preventing cancer (herein below, referred to as pharmaceutical compositions and such), each comprising the above-described bispecific

antibody, another anticancer agent, or a combination of the bispecific antibody and the other anticancer agent. In several embodiments, the pharmaceutical compositions and such of the present invention can be used in the combination therapy of the present invention. In several embodiments, the pharmaceutical compositions and such of the present invention are highly effective for damaging cells, suppressing cell proliferation, activating immunity towards cancer cells or cancer cell-comprising tumor tissues, treating cancer, or preventing cancer due to combined use of the above-described bispecific antibody and the other anticancer agent, as compared to monotherapy using the bispecific antibody or the other anticancer agent. In another embodiment, the pharmaceutical compositions of the present invention have synergistic effects or additive effects on damaging cells, suppressing cell proliferation, activating immunity towards cancer cells or cancer cell-comprising tumor tissues, treating cancer, or preventing cancer due to combined use of the above-described bispecific antibody and the other anticancer agent.

In several embodiments, the pharmaceutical compositions and such according to the present invention “comprising a combination of a bispecific antibody and another anticancer agent” refers to pharmaceutical compositions and such in which the above-described bispecific antibody and the other anticancer agent are combined for use in simultaneous, separate, or sequential administration in treatment or prevention of a disease. For example, the pharmaceutical compositions and such of the present invention can be provided in the form of a combination preparation containing both a bispecific antibody and another anticancer agent. Alternatively, for example, as the pharmaceutical compositions and such of the present invention, a pharmaceutical agent containing a bispecific antibody and a pharmaceutical agent containing another anticancer agent can be separately provided, and these pharmaceutical agents may be used simultaneously or sequentially. The disease is not particularly limited but is preferably cancer.

In several embodiments, the present invention provides pharmaceutical compositions and such for use in combination with another anticancer agent, the compositions comprising the above-described bispecific antibody as an active ingredient.

In several embodiments, the present invention provides pharmaceutical compositions and such for use in combination with the above-described bispecific antibody, the compositions comprising another anticancer agent as an active ingredient.

In several embodiments, the present invention provides pharmaceutical compositions and such for enhancing therapeutic effects of another anticancer agent in cancer treatment with said another anticancer agent, by using the above-described bispecific antibody in combination with said another anticancer agent.

In several embodiments, the present invention provides pharmaceutical compositions

and such for enhancing therapeutic effects of the above-described bispecific antibody in cancer treatment with the bispecific antibody, by using another anticancer agent in combination with the bispecific antibody.

5 In several embodiments, the present invention provides use of the above-described bispecific antibody and/or another anticancer agent for the production of pharmaceutical compositions and such comprising as active ingredients the bispecific antibody and/or the other anticancer agent.

10 In the present invention, “comprising as active ingredients the above-described bispecific antibody and/or another anticancer agent” means “containing the bispecific antibody and/or the other (another) anticancer agent as major active component(s)”, and does not limit the content of the bispecific antibody and/or the other anticancer agent.

In several embodiments, the bispecific antibodies described herein and other anticancer agents which are known or described herein can be used in the above-described pharmaceutical compositions and others.

15 In a non-limiting embodiment of the present invention, the above-described other anticancer agents include, but are not limited to, nitrogen mustard analogues, alkyl sulfonates, ethylene imines, nitrosoureas, epoxides, other alkylating agents, folic acid analogues, purine analogues, pyrimidine analogues, other antimetabolites, vinca alkaloids or analogues, podophyllotoxin derivatives, camptothecin analogues, colchicine derivatives, taxanes, other
20 plant alkaloids or natural substances, actinomycines, anthracyclines or related substances, other cytotoxic antibiotics, platinum compounds, methylhydrazines, kinase inhibitors, angiogenic inhibitors, hormonal agents, inhibitors of DNA modification enzymes, immunostimulators, proteasome inhibitors, enzymes, histone deacetylase inhibitors, inhibitors of DNA modification enzymes, cytokine preparations, retinoids, immune checkpoint inhibitors, indoleamine
25 2,3-Dioxygenase (IDO) inhibitors, co-stimulatory molecule activators, natural killer cell activators, monoclonal antibodies, other molecular-targeted agents, and other anticancer agents. In a non-limiting embodiment, other anticancer agents in the present invention include, but are not limited to, for example, antibodies described in WO2015/174439 and WO2015/156268.

30 In several embodiments, an “immune checkpoint” of the present invention refers to a molecule that is expressed on immunocompetent cells (including T cells) and binds to a ligand to thereby transduce to the immunocompetent cells signals inhibiting immune response. Examples of immune checkpoints and ligands thereof include, but are not limited to, molecules such as PD-1, CTLA-4, TIM3, LAG3, PD-L1, PD-L2, BTNL2, B7-H3, B7-H4, CD48, CD80, 2B4, BTLA, CD160, CD60, CD86, and VISTA. In several embodiments, an “immune
35 checkpoint inhibitor” of the present invention refers to a pharmaceutical agent that inhibits binding between an immune checkpoint and its ligand, and thereby inhibits signal transduction

mediated by the immune checkpoint.

A non-limiting embodiment of the present invention provides pharmaceutical compositions and such in which the other anticancer agent is a chemotherapeutic agent, a T cell-activating agonist agent, an immune checkpoint inhibitor, or an angiogenic inhibitor.

5 In a non-limiting embodiment of the present invention, chemotherapeutic agents include, but are not limited to, antimetabolites, plant alkaloids, and platinum compounds. Preferred examples of an antimetabolite include, but are not limited to, enocitabine, capecitabine, carmofur, gemcitabine, cytarabine, tegafur, tegafur uracil, nelarabine, fluorouracil, fludarabine, pemetrexed, pentostatin, and methotrexate. Particularly preferred antimetabolites include, for example,
10 capecitabine. Preferred examples of a plant alkaloid include, but are not limited to, irinotecan, etoposide, sobuzoxane, docetaxel, nogitecan, paclitaxel, vinorelbine, vincristine, vindesine, and vinblastine. Particularly preferred plant alkaloids include, for example, paclitaxel. Preferred examples of a platinum compound include, but are not limited to, oxaliplatin, carboplatin, cisplatin, and nedaplatin. Particularly preferred platinum compounds include, for example,
15 cisplatin.

In a non-limiting embodiment of the present invention, T cell-activating agonist agents include, but are not limited to, TNF receptor superfamily (TNFRSF) agonist antibodies and co-stimulatory molecule agonist antibodies against. Target molecules of “TNF receptor superfamily agonist antibodies” are not particularly limited as long as they are factors that
20 activate cells expressing the TNF receptor superfamily (for example, T cells and NK cells), but are preferably factors belonging to the “TNF superfamily” or the “TNF receptor superfamily”. Known factors belonging to the “TNF superfamily” or the “TNF receptor superfamily” include ligands having a trimeric structure and receptors having a trimeric structure to which the ligands bind, which contribute to activation of various immune cells (Nat. Rev. Immunol., 2012, 12,
25 339-51). Examples of a factor belonging to the TNF superfamily or TNF receptor superfamily include CD137, CD137L, CD40, CD40L, OX40, OX40L, CD27, CD70, HVEM, LIGHT, RANK, RANKL, CD30, CD153, GITR, GITRL, TNFRSF25, and TL1A. Preferred factors include, for example, CD137. Examples of a CD137 agonist antibody include Urelumab (CAS No. 934823-49-1), PF-05082566, and various known CD137 agonist antibodies.

30 Factors belonging to co-stimulatory molecules include TMIGD2, HHLA2, ICOS, ICOS ligand, CD28, CD80, CD86, and such. Examples of an OX40 agonist antibody include MOXR0916, MEDI6469, MEDI0562, MEDI6383, PF-04518600, GSK-3174998, and various known OX40 agonist antibodies. Examples of a CD40 agonist antibody include RG-7876, ADC-1013, SEA-CD40, APX005M, Dacetuzumab, and various known CD40 agonist antibodies.
35 Examples of a GITR agonist antibody include AMG228, AMK-1248, MK-4166, BMS-986156, TRX518, and various known GITR agonist antibodies. Examples of a CD27 agonist antibody

include Varlilumab (CAS No. 1393344-72-3) and various known CD27 agonist antibodies.

In a non-limiting embodiment of the present invention, preferred examples of an immune checkpoint inhibitor include, but are not limited to, PD1 antibodies, PDL1 antibodies, CTLA-4 antibodies, TIM3 antibodies, and LAG3 antibodies. Examples of a PD-1 antibody
 5 include Pembrolizumab (CAS No. 1374853-91-4), Nivolumab (CAS No. 946414-94-4), MEDI0680, PDR001, BGB-A317, REGN2810, SHR-1210, PF-06801591, and various known PD1 antibodies. Examples of a PD-L1 antibody include Atezolizumab (CAS No. 1380723-44-3), Avelumab (CAS No. 1537032-82-8), Durvalumab (CAS No. 1428935-60-7), MDX-1105, and various known PD-L1 antibodies. Examples of a CTLA-4 antibody include
 10 Ipilimumab (CAS No. 477202-00-9), Tremelimumab (CAS No. 745013-59-6), and various known CTLA-4 antibodies. Examples of a TIM3 antibody include MBG452 and various known TIM3 antibodies. Examples of an LAG3 antibody include BMS-986016, LAG525, and various known LAG3 antibodies.

In a non-limiting embodiment of the present invention, preferred examples of an
 15 angiogenic inhibitor include, but are not limited to, VEGFR2 antibodies. Examples of an angiogenic inhibitor include Bevacizumab, Sorafenib, Everolimus, Temsirolimus, and various known angiogenic inhibitors.

In several embodiments, other anticancer agents of the present invention are not particularly limited and any anticancer agent can be used as long as, when used in combination
 20 with the bispecific antibody of the present invention, therapeutic effect or prophylactic effect of the other anticancer agent is enhanced or therapeutic effect or prophylactic effect of the bispecific antibody is enhanced.

In a non-limiting embodiment of the present invention, the combination therapy of the present invention may comprise the above-described bispecific antibody and at least one other
 25 therapeutic agent, an immunomodulator, a therapeutic cancer vaccine, adoptive T cell therapy, Treg depletion, or such, but the therapy is not limited thereto. Preferred therapeutic cancer vaccines include, but are not limited to, whole tumor cell vaccines, tumor antigen vaccines, vector-based vaccines, oncolytic viral vaccines, and dendritic cell vaccines. Multimodality therapy may be performed using surgical operation, radiation treatment, or such in combination,
 30 in addition to the above-described therapies.

In a non-limiting embodiment of the present invention, the combination therapy of the present invention can be performed using the above-described bispecific antibody in combination with cytokine therapy using a cytokine as an anti-tumor immune
 response-enhancing agent. In such therapy, cytokines include, but are not limited to, IL-2, IL-7,
 35 IL-12, IL-15, IL-17, IL-18, IL-21, IL-23, IL-27, GM-CSF, interferon- α (IFN α), IFN α -2b, IFN β , and IFN γ .

A non-limiting embodiment of the present invention provides agents for inducing cytotoxicity, agents for suppressing cell proliferation, agents for inhibiting cell proliferation, agents for activating immune response, agents for treating cancer, and agents for preventing cancer, each comprising the above-described pharmaceutical composition.

5 In several embodiments, an “individual” to which the above-described bispecific antibody and/or another anticancer agent is administered refers to a human or a non-human animal, for example, a mammal such as cattle, horse, dog, sheep, or cat. The individual is preferably a human. The individual includes patients (including human and non-human mammals). In several embodiments, the individual is a patient who has cancer cells or cancer
10 cell-comprising tumor tissues. Cancer cells or cancer cell-comprising tumor tissues which become targets of the anticancer agent or the combination therapy of the present invention are not particularly limited, as long as they express glypican 3. In the present invention, preferred glypican 3-expressing cells, i.e., glypican 3-positive cells, are cancer cells. More preferred cancer types include, but are not limited to, for example, gastric cancer, head and neck cancer
15 (H&N), esophageal cancer, lung cancer, liver cancer, ovary cancer, breast cancer, colon cancer, kidney cancer, skin cancer, muscle tumor, pancreas cancer, prostate cancer, testis cancer, uterine cancer, cholangiocarcinoma, Merkel cell carcinoma, bladder cancer, thyroid cancer, schwannoma, adrenal cancer (adrenal gland), anus cancer, central nervous system tumor, neuroendocrine tissue tumor, penis cancer, pleura tumor, salivary gland tumor, vulva cancer, thymoma, and childhood
20 cancer (Wilms tumor, neuroblastoma, sarcoma, hepatoblastoma, and germ cell tumor). Still more preferred cancer types include, but are not limited to, gastric cancer, head and neck cancer (H&N), esophageal cancer, lung cancer, liver cancer, ovary cancer, breast cancer, colon cancer, kidney cancer, skin cancer, muscle tumor, pancreas cancer, prostate cancer, testis cancer, and uterine cancer (Tumori. (2012) 98, 478-484; Tumor Biol. (2015) 36, 4671-4679; Am J Clin
25 Pathol (2008) 130, 224-230; Adv Anat Pathol (2014) 21, 450-460; Med Oncol (2012) 29, 663-669; Clinical Cancer Research (2004) 10, 6612-6621; Appl Immunohistochem Mol Morphol (2009) 17, 40-46; Eur J Pediatr Surg (2015) 25, 138-144; J Clin Pathol (2011) 64, 587-591; Am J Surg Pathol (2006) 30, 1570-1575; Oncology (2007) 73, 389-394; Diagnostic Pathology (2010) 64, 1-6; Diagnostic Pathology (2015) 34, 1-6; Am J Clin Pathol (2008) 129, 899-906; Virchows
30 Arch (2015) 466, 67-76).

In several embodiments, patients are those who have received treatment with the above-described bispecific antibody and/or some kind of anticancer agent(s) prior to the combination therapy using the bispecific antibody and another anticancer agent. In several
35 embodiments, patients are those who cannot receive standard therapy or for whom standard therapy is ineffective. In several embodiments, cancer which a patient has is early-stage or end-stage.

As used herein, “cancer” refers not only to epithelial malignancy such as ovary cancer or gastric cancer but also to non-epithelial malignancy including hematopoietic tumors such as chronic lymphocytic leukemia or Hodgkin’s lymphoma. Herein, the terms “cancer”, “carcinoma”, “tumor”, “neoplasm” and such are not differentiated from each other and are mutually interchangeable.

Meanwhile, in several embodiments, cancer types which become targets of anticancer agents or pharmaceutical compositions (combination therapy) of the present invention are preferably those in which the number of glypican-3 antigens on the cell surface per cell is 100 or more, more preferably those in which the number of glypican-3 antigens on the cell surface per cell is 200 or more, 300 or more, 400 or more, 500 or more, 600 or more, 700 or more, 800 or more, 900 or more, 1000 or more, 1200 or more, 1400 or more, 1600 or more, 1800 or more, or 2000 or more, and still more preferably those in which the number of glypican-3 antigens on the cell surface per cell is 3000 or more, 4000 or more, 5000 or more, 6000 or more, 7000 or more, 8000 or more, 9000 or more, 10000 or more, 20000 or more, 30000 or more, 40000 or more, or 50000 or more.

The number of glypican-3 antigens on cell surface per cell can be appropriately determined using methods described herein or known to those skilled in the art, for example, by calculating antibody binding capacity (ABC) of GPC3 on the cell surface with flow cytometry using QIFIKIT (DAKO). The number of glypican-3 antigens on cell surface per cell in a tissue sample isolated from a target candidate can be determined in order to assess whether the candidate can be a target to which an anticancer agent or pharmaceutical composition (combination therapy) of the present invention is administered. When in the sample the number of glypican-3 antigens on cell surface per cell meets the criterion described above, the target from which the sample is derived can be the target to which the anticancer agent or pharmaceutical composition (combination therapy) of the present invention is administered.

In a non-limiting embodiment of the present invention, anticancer agents of the present invention can be used to treat patients who have cancer which is refractory to treatment with an immune checkpoint inhibitor. For example, patients with glypican 3-positive cancer, in whom administration of an immune checkpoint inhibitor has failed to achieve a desired drug efficacy, can be treated with the anticancer agent of the present invention. In other words, glypican 3-positive cancer that has been already treated with therapy using an immune checkpoint inhibitor can be treated with the anticancer agent of the present invention.

In a non-limiting embodiment of the present invention, pharmaceutical compositions (combination therapy) of the present invention can be used to treat patients who have cancer which is refractory to treatment with an immune checkpoint inhibitor. For example, patients with GPC-positive cancer, in whom administration of an immune checkpoint inhibitor has failed

to achieve a desired drug efficacy, can be treated with the pharmaceutical composition (combination therapy) of the present invention. In other words, glypican 3-positive cancer that has been already treated with therapy using an immune checkpoint inhibitor can be treated with the pharmaceutical composition (combination therapy) of the present invention. Preferred
5 examples of another anticancer agent comprised in the pharmaceutical composition include immune checkpoint inhibitors, but are not limited thereto.

In a non-limiting embodiment of the present invention, pharmaceutical compositions (combination therapy) of the present invention can be used to treat patients who have cancer which is refractory to treatment with the anticancer agent of the present invention. For example,
10 patients with GPC-positive cancer, whose cancer has become resistant to the anticancer agent of the present invention after administration of the anticancer agent or in whom administration of the anticancer agent of the present invention has failed to achieve a desired drug efficacy, can be treated with the pharmaceutical composition (combination therapy) of the present invention. In other words, glypican 3-positive cancer that has been already treated with therapy using the
15 anticancer agent of the present invention can be treated with the pharmaceutical composition (combination therapy) of the present invention. Preferred examples of another anticancer agent comprised in the pharmaceutical composition include immune checkpoint inhibitors, but are not limited thereto.

With respect to glypican 3-positive cancer (cancer confirmed to express glypican 3),
20 those skilled in the art can appropriately examine positivity for glypican 3 using methods known to those skilled in the art such as immunohistochemical staining or flow cytometry.

From another viewpoint, the present invention provides anticancer agents comprising as the active ingredient a multispecific antigen-binding molecule that comprises: (1) a domain comprising an antibody variable region having glypican 3-binding activity, (2) a domain
25 comprising an antibody variable region having T-cell receptor complex-binding activity, and (3) a domain comprising an Fc region with reduced binding activity towards an Fcγ receptor.

In the present invention “comprising as the active ingredient a multispecific antigen-binding molecule that comprises (1) a domain comprising an antibody variable region having glypican 3-binding activity, (2) a domain comprising an antibody variable region having
30 T-cell receptor complex-binding activity, and (3) a domain comprising an Fc region with reduced binding activity towards an Fcγ receptor” means comprising the antigen-binding molecule as a major active component, without limitation to the content ratio of the antigen-binding molecule.

If necessary, multispecific antigen-binding molecules of the present invention may be encapsulated in microcapsules (e.g., those made of hydroxymethylcellulose, gelatin, and
35 poly(methylmetacrylate)), or incorporated as components of a colloidal drug delivery system (e.g., liposomes, albumin microspheres, microemulsion, nanoparticles, and nanocapsules) (see,

for example, "Remington's Pharmaceutical Science 16th edition", Oslo Ed. (1980)). Methods for preparing the pharmaceutical agents as controlled-release pharmaceutical agents are also well known, and such methods may be applied to the multispecific antigen-binding molecules of the present invention (J. Biomed. Mater. Res. (1981) 15: 267-277; Chemtech. (1982) 12: 98-105; 5 U.S. Patent No. 3,773,719; European Patent Application Publication Nos. EP 58,481 and EP 133,988; Biopolymers (1983) 22: 547-556).

The pharmaceutical compositions or anticancer agents of the present invention may be administered to patients by oral or parenteral administration, and parenteral administration is preferred. Specific examples of the administration method include administration by injection, 10 transnasal administration, transpulmonary administration, and transdermal administration. Examples of administration by injection include intravenous injection, intramuscular injection, intraperitoneal injection, and subcutaneous injection. A pharmaceutical composition or anticancer agent of the present invention can be administered systemically or locally, for example, through administration by injection. The method of administration can be selected 15 appropriately according to the age and symptoms of the patient. The dose can be selected from the range of 0.0001 mg to 1000 mg per kilogram body weight for a single administration. Alternatively, for example, the dose may be selected from the range of 0.001 mg/body to 100000 mg/body per patient. The dose may be defined, for example, as the amount of the multispecific antigen-binding molecule of the present invention comprised as the active ingredient in the 20 pharmaceutical composition. However, the pharmaceutical compositions or anticancer agents of the present invention are not limited to these doses.

The pharmaceutical compositions or anticancer agents of the present invention can be formulated according to conventional methods (for example, Remington's Pharmaceutical Science, latest edition, Mark Publishing Company, Easton, U.S.A), and may also contain 25 pharmaceutically acceptable carriers and additives. Examples include, but are not limited to surfactants, excipients, coloring agents, perfumes, preservatives, stabilizers, buffers, suspending agents, isotonization agents, binders, disintegrants, lubricants, fluidity promoting agents, and flavoring agents; and other commonly used carriers can be suitably used. Specific examples of the carriers include light anhydrous silicic acid, lactose, crystalline cellulose, mannitol, starch, 30 carmellose calcium, carmellose sodium, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyvinylacetal diethylaminoacetate, polyvinylpyrrolidone, gelatin, medium chain fatty acid triglyceride, polyoxyethylene hardened castor oil 60, saccharose, carboxymethyl cellulose, corn starch, inorganic salt, and such.

A non-limiting embodiment of the present invention also provides methods for 35 damaging glypican 3 antigen-expressing cells or tumor tissues containing the antigen-expressing cells, or methods for suppressing growth of these cells or tumor tissues by contacting the

glypican 3 antigen-expressing cells with a multispecific antigen-binding molecule of the present invention that binds to the antigen and another anticancer agent. The multispecific antigen-binding molecule that binds to the antigen is as described above for an antigen-binding molecule of the present invention that binds to the antigen, which is comprised in the anticancer agents of the present invention. The cells bound by a multispecific antigen-binding molecule of the present invention that binds to the antigen are not particularly limited as long as they are cells expressing the antigen.

In the present invention, “contact” is carried out, for example, by adding a multispecific antigen-binding molecule of the present invention which binds to the antigen and another anticancer agent to the culture medium of GPC3 antigen-expressing cells cultured *in vitro*. In this case, a liquid or a solid obtained by freeze-drying or such may be suitably used as the form of the added antigen-binding molecule and/or another anticancer agent. When added as an aqueous solution, it may be an aqueous solution that simply contains only the multispecific antigen-binding molecule of the present invention, or it may be a solution containing also, for example, the above-mentioned surfactants, excipients, coloring agents, perfumes, preservatives, stabilizers, buffers, suspending agents, isotonization agents, binders, disintegrants, lubricants, fluidity promoting agents, and flavoring agents. The concentration at which the addition is performed is not particularly limited, but a suitable final concentration in the culture solution is preferably in the range of 1 pg/ml to 1 g/ml, more preferably 1 ng/ml to 1 mg/ml, and even more preferably 1 µg/mL to 1 mg/mL.

Furthermore, in another embodiment, “contact” of the present invention is also carried out by administering an antigen-binding molecule of the present invention and another anticancer agent to non-human animals with cells expressing the GPC3 antigen transplanted into their bodies, and to animals carrying cells that intrinsically express the antigen. The method of administration may be oral or parenteral, and parenteral administration is particularly preferred. Specific examples of the administration method include administration by injection, transnasal administration, transpulmonary administration, and transdermal administration. Examples of administration by injection include intravenous injection, intramuscular injection, intraperitoneal injection, and subcutaneous injection. A pharmaceutical composition or anticancer agent of the present invention can be administered systemically or locally, for example, through administration by injection. The method of administration can be selected appropriately according to the age and symptoms of the test animal. When administered as an aqueous solution, an aqueous solution containing simply only a multispecific antigen-binding molecule of the present invention may be used, or a solution containing also the above-mentioned surfactants, excipients, coloring agents, perfumes, preservatives, stabilizers, buffers, suspending agents, isotonization agents, binders, disintegrants, lubricants, fluidity promoting agents, flavoring

agents, and such may be used. The dose can be selected from the range of 0.0001 mg to 1000 mg per kilogram body weight for a single administration. Alternatively, for example, the dose may be selected from the range of 0.001 mg/body to 100000 mg/body per patient. The dose may be defined, for example, as the amount of the multispecific antigen-binding molecule of the present invention comprised as the active ingredient in the pharmaceutical composition.
5 However, the amount of the multispecific antigen-binding molecule of the present invention administered is not limited to these doses.

The following method is suitably used as a method for evaluating or measuring cytotoxicity induced in cells expressing the glypican 3 antigen which is bound by a domain carrying an antibody variable region having glypican 3-binding activity that constitutes the antigen-binding molecule as a result of contacting the cells with a multispecific antigen-binding molecule or another anticancer agent of the present invention. Examples of a method for evaluating or measuring the cytotoxic activity *in vitro* include methods for measuring cytotoxic T cell activity and such. Whether or not a multispecific antigen-binding molecule of the present invention has T cellular cytotoxicity can be measured by known methods (for example, Current protocols in Immunology, Chapter 7. Immunologic studies in humans, Editor, John E. Coligan *et al.*, John Wiley & Sons, Inc., (1993) and the like). For activity measurements, an antigen-binding molecule that binds to an antigen different from glypican 3, which is an antigen not expressed in the cells used for the examination, can be used as a control in the same manner
10 as a multispecific antigen-binding molecule of the present invention, and the activity can be determined to be present when the multispecific antigen-binding molecule of the present invention shows a stronger cytotoxic activity than when the antigen-binding molecule is used as a control.
20

To evaluate or measure cytotoxic activity *in vivo*, for example, cells expressing a glypican 3 antigen are intradermally or subcutaneously transplanted to a non-human test animal, and then a test antigen-binding molecule and/or another anticancer agent is intravenously or intraperitoneally administered daily or with an interval of few days, starting from the day of transplantation or the following day. Cytotoxic activity can be determined by daily measurement of tumor size and by observing difference in the change of tumor size. In a similar manner to the *in vitro* evaluation, the cytotoxic activity of an antigen-binding molecule of the present invention can be determined to be present when administration of a control antigen-binding molecule shows that the tumor size in the group subjected to administration of an antigen-binding molecule of the present invention is significantly smaller than the tumor size in the group subjected to administration of the control antigen-binding molecule.
30

35 As a method for evaluating or measuring the suppressive effect on proliferation of cells expressing a glypican 3 antigen, a method of measuring the uptake of isotope-labeled thymidine

into cells or the MTT method may be suitably used. As a method for evaluating or measuring the cell proliferation-suppressing activity *in vivo*, the same method described above for evaluating or measuring cytotoxic activity *in vivo* may be suitably used.

5 Kits

In several embodiments, the present invention provides a kit comprising:

- (1) the above-described bispecific antibody;
- (2) a container; and
- (3) an instruction or a label indicating that the bispecific antibody and at least one type of anticancer agent are administered in combination to a test subject for treating cancer in an individual. In another embodiment, the present invention provides a kit comprising:
 - (1) another anticancer agent;
 - (2) a container; and
 - (3) an instruction or a label indicating that said another anticancer agent and at least one type of the above-described bispecific antibody are administered in combination to an individual for treating cancer in the individual.

In another embodiment, the present invention provides a kit comprising:

- (1) the above-described bispecific antibody;
- (2) another anticancer agent;
- (3) a container; and
- (4) an instruction or a label indicating that the bispecific antibody and the other anticancer agent are administered in combination to an individual for treating cancer in the individual.

In several embodiments, the kit further comprises a pharmaceutically acceptable carrier. The kit can further comprise a sterile diluent preferably stored in a separate, additional container. The kit can also comprise an instruction relating to a combination therapy for treating or preventing cancer.

In several embodiments, an “instruction” refers to the written instruction usually contained in a commercially available box carrying a pharmaceutical, and can include information on indications, usage, dose, administration, contraindications, and/or warnings regarding the use of the pharmaceutical.

The kits may be those which are used exclusively for the purpose of combined use of the bispecific antibody of the present invention and another anticancer agent. Alternatively, the kits may be those which are used for other purposes as long as they are used for the purpose of combined use of the bispecific antibody of the present invention and another anticancer agent. For example, as long as the instruction or label of the kit of the present invention indicates that the bispecific antibody and other anticancer agent are administered in combination to an

individual, the instruction or label may indicate other embodiments, for example, in which the bispecific antibody or other anticancer agent is used alone.

The present invention also relates to molecules having GPC3-binding activity, which contain a domain comprising an antibody variable region having GPC3-binding activity of the multispecific antigen-binding molecule comprised in the anticancer agent or pharmaceutical composition of the present invention. Furthermore, the present invention relates to a molecule having GPC3-binding activity, which comprises the antibody variable regions of H and L chains respectively comprising the three CDRs of the H and L chains (total of six CDRs) contained in the molecule. The present invention also relates to molecules having T-cell receptor complex-binding activity, which contain a domain comprising an antibody variable region having T-cell receptor complex-binding activity of the multispecific antigen-binding molecule comprised in the anticancer agent or pharmaceutical composition of the present invention. Furthermore, the present invention relates to a molecule having T-cell receptor complex-binding activity that comprises the antibody variable regions of the H and L chains respectively comprising the three CDRs of the H and L chains (total of six CDRs) contained in the molecule. Such molecules may be antibodies or polypeptides comprising antigen-binding fragments of an antibody. The present invention also relates to antibodies that bind to epitopes overlapping or competing with these molecules or polypeptides containing the antigen-binding fragments thereof. Suitable examples of such polypeptides comprising antigen-binding fragments of an antibody include scFv, single chain antibody, Fv, single chain Fv 2 (scFv2), Fab, and F(ab')₂. Furthermore, these molecules do not have to be multispecific (bispecific), and may bind only to either GPC3 or a T cell receptor complex (for example, the CD3ε chain).

These molecules include a molecule comprising a domain that comprises an antibody variable region having GPC-binding activity of the multispecific antigen-binding molecule exemplified in detail in the Reference Examples herein (which comprises the H-chain variable regions having GPC3-binding activity and the common L-chain variable region), a molecule comprising a domain that comprises an antibody variable region having T cell receptor complex-binding activity of the multispecific antigen-binding molecule exemplified in the Reference Examples herein (which comprises the H-chain variable regions having T cell receptor complex-binding activity and the common L-chain variable region), and also a molecule having an activity to bind to the same antigenic protein (GPC3 or T-cell receptor complex), which comprises the three CDRs of each of the H and L chains (total of six CDRs) contained in the above molecule.

These molecules have CDRs that are in common with those of a multispecific antigen-binding molecule of the present invention; and therefore, they are expected to bind to an epitope overlapping with an epitope for the multispecific antigen-binding molecule of the present

invention. Therefore, these molecules can compete with multispecific antigen-binding molecules of the present invention when they coexist with the multispecific antigen-binding molecules of the present invention. Accordingly, these molecules can be used, for example, as regulatory agents for suppressing activities (such as antigen-binding activity, cytotoxic activity, and antitumor activity) of the multispecific antigen-binding molecules of the present invention. Furthermore, such a molecule can be bound to a target protein (GPC3 or T cell receptor complex) in advance, and when a multispecific antigen-binding molecule of the present invention is added, the molecules that dissociate through competition can be detected. This way, the molecule is useful as an agent for detecting binding of a multispecific antigen-binding molecule of the present invention to a target protein. Here, such molecules may be labeled appropriately with fluorescent substances or such. Alternatively, these molecules are useful for screening novel antibodies that bind to epitopes overlapping with the epitopes bound by the multispecific antigen-binding molecules of the present invention. As described above, such a molecule can be bound to a target protein (GPC3 or T cell receptor complex) in advance, and when a test antibody is added, if the bound molecules dissociate, then the test antibody is a candidate for an antibody against an epitope overlapping with the epitope bound by the multispecific antigen-binding molecule of the present invention. This will enable efficient screening of novel multispecific antigen-binding molecules.

The combinations presented as examples herein as combinations of each CDR of the multispecific antigen-binding molecules of the present invention can be directly used as specific combinations of CDRs of the H-chain and L-chain variable regions in these molecules. The antigen affinity of these molecules (KD values) is preferably a value exemplified herein as the KD value of a multispecific antigen-binding molecule of the present invention, but is not limited thereto.

In the present invention, the indefinite article “a” or “an” refers to one, or two or more (i.e., at least one) grammatical object referred to by the indefinite article. For example, “a component” refers to one component or two or more components.

Those skilled in the art will naturally appreciate that any combinations of one or more of the embodiments described herein are also included in the present invention as long as they are not technically inconsistent based on common technical knowledge of those skilled in the art.

All prior art documents cited herein are incorporated by reference into this description.

Examples

Hereinbelow, the present invention will be specifically described with reference to the Examples, but it is not to be construed as being limited thereto.

[Example 1] T cell-dependent cellular cytotoxicity (TDCC activity) of each test antibody when

human peripheral blood mononuclear cells are used as effector cells

TDCC activity of each test antibody was measured by the method described below.

Human peripheral blood mononuclear cells (hereinafter referred to as human PBMCs) were used as effector cells and TDCC activity of each test antibody was measured as follows.

5 (1) Preparation of human PBMC solution

50 ml of peripheral blood was collected from a healthy person in Chugai Pharmaceutical Co. Ltd. using a syringe pre-loaded with 500 μ l of 5000 units/5 ml heparin solution. The peripheral blood diluted two-fold with PBS was divided into four aliquots and added to Leucosep tubes for lymphocyte separation (GE Healthcare) which had been loaded with 15 ml of
10 Ficoll-Paque PLUS and centrifuged in advance. The separation tubes containing the aliquoted peripheral blood were centrifuged at a speed of 1000 g for 10 minutes at room temperature, and then, the mononuclear cell layer fraction was collected. After cells contained in each layer fraction were washed once with RPMI-1640 (SIGMA) containing 10% FBS (hereinafter referred to as 10% FBS/RPMI-1640), the cells were suspended at a cell density of 2×10^6 cells/ml in
15 culture medium for each target cell. The cell suspensions were used as effector cells in subsequent experiments.

(2) LDH release test (TDCC activity)

TDCC activity was assessed by an LDH release method (LDH Cytotoxicity Detection Kit, TAKARA). First, an antibody solution was diluted with each target cell culture medium to
20 concentrations (0.000004, 0.00004, 0.0004, 0.004, 0.04, 0.4, 4, and 40 μ g/ml), which were four times greater than the final concentrations, and 50- μ l aliquots of antibody solutions of respective concentrations were added to each well of a 96-well round-bottomed plate. Then, 50- μ l aliquots of target cells prepared at 2×10^5 cells/ml in culture medium for each target cell were seeded (1×10^4 cells/well) and allowed to stand at room temperature for 15 minutes. 100- μ l
25 aliquots of the human PBMC suspension prepared in culture medium for each target cell (2×10^5 cells/well) as described in (1) were added to each well of the plate. The plate was allowed to stand in a 5% carbon dioxide gas incubator at 37°C for about 24 hours, followed by centrifugation. 100 μ l of the culture supernatant in each well of the plate was transferred to a 96-well flat-bottomed plate. A catalyst solution was dissolved in 1 ml of H₂O and mixed with a
30 dye solution at ratio of 1:45. The mixed solution of catalyst and dye solutions was aliquoted at 100 μ l/well to the 96-well flat-bottomed plate where the culture supernatants had been transferred. The plate was allowed to stand at room temperature for 15 to 30 minutes. The absorbance at 490 to 492 nm was measured with a plate reader. The reference wavelength used was 600 to 620 nm, and the absorbance was subtracted from that at 490 to 492 nm. Values
35 obtained by subtracting the mean value for wells containing culture medium alone (blank) were plugged into the following equation.

Equation :

$$\text{Cytotoxicity (TDCC) (\%)} = ((A-B)-C) \times 100 / (D-C)$$

5 Cytotoxic activity was determined based on this equation.

Here, A represents the absorbance of a mixture of target cells, effector cells, and antibody; B represents the absorbance of effector cells; C represents the absorbance of target cells; and D represents the absorbance of target cells with Triton X-100 added.

10 As a result, TDCC was clearly observed for an anti-human CD3ε chain and anti-human GPC3 bispecific antibody (the antibody of sample No. 38 described in Table 17 of Reference Example 3) (Fig. 1).

[Example 2] Determination of the amount of GPC3 expressed on cell surface in each cell line

15 Antibody binding capacity (ABC) of GPC3 on cell surface was calculated for each cell line by flow cytometry using QIFIKIT (DAKO).

After washing with CellWASH (BD Bioscience) supplemented with 0.5% BSA (hereinafter referred to as FACS/PBS), 5×10^5 cells of each cell line were prepared in 50 μl of a solution containing mouse anti-human GPC antibody or control antibody at a final concentration of 20 μg/ml and allowed to stand on ice for 30 minutes. The cells were washed with
20 FACS/PBS. Then, 50 μl of a solution containing FITC-labeled goat anti-mouse IgG antibody which had been diluted 50-fold with FACS/PBS was added to the cells. The cells were allowed to stand on ice for 30 minutes. After washing with FACS/PBS, the cells were analyzed by flow cytometry. ABC was calculated by the method described in the instruction manual of QIFI KIT.

25 ABC of GPC3 on cell surface was calculated for each cell line and is shown in Table 6.

Table 6

Cell name	Tissue	Histology	ABC
MKN-74	Gastric	Adenocarcinoma	2. 97E3
FU-97	Gastric	Adenocarcinoma	5. 52E5
SNU-1	Gastric	Adenocarcinoma	1. 85E4
STM-03	Gastric	Adenocarcinoma	Not applicable (PDX)
SCC152	Hypopharynx	Squamous carcinoma	3. 77E4
KYSE70	Esophageal	Squamous carcinoma	5. 35E4
PC-10	Lung	Squamous carcinoma	1. 21E5
NCI-H446	Lung	Small cell carcinoma	6. 95E4
huH-1	Liver	Carcinoma	5. 66E4
HuH-7	Liver	Carcinoma	4. 85E4
RMG-1	Ovary	Adenocarcinoma	3. 00E2
TOV-21G	Ovary	Clear cell adenocarcinoma	1. 15E4
MDA-MB-134VI	Breast	Carcinoma, ductal carcinoma	1. 07E4
HCC1419	Breast	Carcinoma, ductal carcinoma	1. 76E4
C2BBel	Colorectal	Adenocarcinoma	5. 77E4
RCC-HB	Kidney	Adenocarcinoma, clear cell	1. 03E4
WM-115	Skin	Melanoma	1. 37E3
VMRC-MELG	Skin	Melanoma	1. 58E3
SJCRH30	Striated muscle	Sarcoma	9. 26E3
BxPC-3	Pancreas	Adenocarcinoma	1. 06E3
Shmac 4	Prostate	Carcinoma	3. 46E3
NTERA-2	Testis	Teratocarcinoma	7. 60E3
C-33 A	Uterine cervix	Carcinoma	2. 10E3

[Example 3] Evaluation of *in vivo* drug efficacy (anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibody) (drug efficacy evaluation studies when antibodies of sample Nos. 30, 31, 32, 33, and 38, or antibodies of sample Nos. 39 and 40 described in Table 17 of Reference example 3 are used as a single agent)

In vivo drug efficacy was evaluated using tumor-bearing models of some of the lines in which cytotoxic activity was observed by the *in vitro* assay described in Example 1 as well as *in vivo*-passaged lines.

Methods called an NOD scid/T cell-injected model, a humanized NOG mouse model, and a human CD3 $\epsilon\delta\gamma$ gene-modified mouse model were used in the *in vivo* drug efficacy

evaluation. Assay using an NOD scid/T cell-injected model was performed as follows. Some of the lines in which cytotoxic activity was observed in the *in vitro* assay and *in vivo*-passaged cell lines were transplanted into NOD scid mice. T cells expanded by culturing human PBMCs *in vitro* were injected into the NOD scid mice with confirmed tumor establishment. The mice
5 were treated by administering bispecific antibody-38 and bispecific antibody-30, -31, -32, or -33. Assay using a humanized NOG mouse model was performed as follows. CD34-positive hematopoietic stem cells derived from umbilical cord blood were transplanted into NOG mice by tail vein injection. Human T cells are constantly supplied in the mice, which are called humanized NOG mice. The PC-10 cell line was transplanted into humanized NOG mice. The
10 humanized NOG mice with confirmed establishment of PC-10 tumor were treated by administering antibody-38. Assay using a human CD3 $\epsilon\delta\gamma$ gene-modified mouse model was performed as follows. Cells of mouse-derived cell line forced to express human GPC3 were transplanted into a human CD3 $\epsilon\delta\gamma$ gene-modified mouse model, which had been prepared according to Reference Example 10. The human CD3 $\epsilon\delta\gamma$ gene-modified mice with confirmed
15 tumor establishment were treated by administering antibody-38.

The drug efficacy evaluation studies for bispecific antibody-38, and bispecific antibody-30, -31, -32, and -33 in NOD scid/T cell-injected model were performed as follows. T cell expansion culture was performed using PBMCs separated from blood collected from a healthy person and T cell activation/ expansion kit/ human. Each of human cancer cell lines
20 (MKN74, FU-97, SNU-1, SCC152, KYSE70, PC-10, HuH-7, TOV-21G, RMG-1, and SK-pca31a (SK-HEP-1/hGPC3)) was mixed with MatrigelTM Basement Membrane Matrix, and transplanted subcutaneously into NOD scid mice (CLEA Japan). An *in vivo*-passaged line (STM-03) was transplanted as about 2-mm tumor tissue cubes subcutaneously into NOD scid mice (CLEA Japan). The day of transplantation was defined as Day 0. On the day before
25 transplantation, anti-asialo GM1 antibody (Wako Pure Chemical Industries) was administered intraperitoneally at 0.2 mg/mouse. When tumor was clearly established after transplantation, the mice were separated into groups based on tumor size and body weight. Then, anti-asialo GM1 antibody was administered intraperitoneally at 0.2 mg/mouse again. On the following day, T cells obtained by the aforementioned expansion culture were transplanted intraperitoneally at
30 1.5×10^7 to 3×10^7 cells/mouse. About two to five hours after T cell transplantation, antibody-38 was administered at 1 mg/kg (MKN74, FU-97, SNU-1, SCC152, KYSE70, PC-10, HuH-7, TOV-21G, RMG-1, and STM-03) through the tail vein. The administration was performed only once. Bispecific antibody-30, -31, -32, and -33 were administered at 5 mg/kg (SK-pca31a) through the tail vein. The administration was performed only once.

35 Bispecific antibody-39 and -40 were administered at 1 mg/kg (PC-10) through the tail vein. The administration was performed only once. Antibody-39 and -40 used in the

experiment have different constant region sequences, but they share the same amino acid sequences of the CD3-binding variable region, GPC3-binding variable region, and common L chain variable region (CD3-binding variable region: SEQ ID NO: 433, GPC3-binding variable region: SEQ ID NO: 434, common L chain variable region: SEQ ID NO: 435). The antibodies
 5 were produced by a method known to those skilled in the art.

As a result, bispecific antibody-38 produced an evident anti-tumor effect as compared to the vehicle-administered group (Fig. 2).

As a result, bispecific antibody-39 and -40 produced an evident anti-tumor effect as compared to the vehicle-administered group (Fig. 3).

10 As a result, bispecific antibody-30, -31, -32, and -33 produced an evident anti-tumor effect as compared to the vehicle-administered group (Fig. 4).

The drug efficacy evaluation study for antibody-38 in the humanized NOG mouse model was performed as follows. 2.5 Gy of X ray was irradiated to NOG mice (In-Vivo Science Inc., ♀). On the following day, 1×10^5 CD34-positive hematopoietic stem cells which
 15 are derived from umbilical cord blood were transplanted to the NOG mice by tail vein injection. After 16 weeks, human PC-10 cancer cells were mixed with MatrigelTM Basement Membrane Matrix, and transplanted subcutaneously into the humanized NOG mice. The day of transplantation was defined as Day 0. When tumor was clearly established, the mice were separated into groups based on tumor size and body weight. Antibody-38 was administered at
 20 0.008, 0.04, 0.2, or 1 mg/kg through the tail vein. Antibody-38 was administered only once.

As a result, an evident, dose-dependent anti-tumor effect was produced in the antibody-38-administered group as compared to the vehicle-administered group (Fig. 5).

The drug efficacy evaluation study for antibody-38 in assay using a human CD3 $\epsilon\delta\gamma$ gene-modified mouse model was performed as follows. LLC1/hGPC3 cancer cells, which are
 25 mouse-derived cancer cell line with over-expression of human GPC3, were transplanted subcutaneously into human CD3 $\epsilon\delta\gamma$ gene-modified mice (Chugai Pharmaceutical Co. Ltd., ♀). The day of transplantation was defined as Day 0. On Day 11, the mice were separated into groups based on tumor size and body weight. Antibody-38 was administered at 5 mg/kg through the tail vein. Antibody-38 was administered twice (Day 11 and Day 14). An
 30 anti-human GPC3 antibody (WO2006/006693, clone name: GC33), anti-mouse CTLA-4 antibody (BioXCell, Catalog# BE0032), anti-mouse PD-1 antibody (BioXCell, Catalog# BE0146), and anti-mouse PD-L1 antibody (BioXCell, Catalog# BE0101), which were pharmaceutical agents used as controls, were administered at 25 mg/kg through the tail vein. The anti-human GPC3 antibody, anti-mouse CTLA-4 antibody, anti-mouse PD-1 antibody, and
 35 anti-mouse PD-L1 antibody were administered twice (Day 11 and Day 14).

As a result, antibody-38 produced an evident anti-tumor effect as compared to the

solvent-administered group, or the anti-human GPC3 antibody, anti-mouse CTLA-4 antibody, anti-mouse PD-1 antibody, and anti-mouse PD-L1 antibody (Fig. 6).

5 [Example 4] Evaluation of *in vivo* drug efficacy (drug efficacy evaluation study for combination of antibody-38 and other agents)

In vivo drug efficacy evaluation study for combination of antibody-38 and other agents was carried out using tumor-bearing models. *In vivo* drug efficacy by the combination was evaluated using the NOD scid/T cell-injected model described in Example 3, the human CD3 ϵ δ γ gene-modified mouse model described in Example 3, or a human CD3 ϵ gene-modified mouse
10 model. Combination study using the NOD scid/T cell-injected model was performed as follows. MKN45 or NCI-H446 cell line was transplanted into NOD scid mice. T cells expanded by culturing human PBMCs *in vitro* were injected into the NOD scid mice with confirmed evident tumor establishment. The mice were treated by administering antibody-38 in combination with capecitabine, cisplatin, or paclitaxel. Combination study using the human CD3 ϵ δ γ
15 gene-modified mouse model was performed as follows. LLC1/hGPC3 cancer cells or Hepa1-6/hGPC3 cells, which are mouse-derived cell lines forced to express human GPC3, were transplanted into human CD3 ϵ δ γ gene-modified mice. The human CD3 ϵ δ γ gene-modified mice with confirmed evident tumor establishment were treated by administering antibody-38 in combination with an anti-mouse TIM-3 antibody (BioXCell, Catalog# BE0115), anti-mouse
20 LAG-3 antibody (BioXCell, Catalog# BE0174), anti-mouse CD137 antibody (BioXCell, Catalog# BE0169), or anti-mouse VEGFR2 antibody (BioXCell, Catalog# BP0060). Combination study using a human CD3 ϵ gene-modified mouse model was performed as follows. Hepa1-6/hGPC3 cancer cells, which are a mouse-derived cancer line forced to express human GPC3, were transplanted into human CD3 ϵ gene-modified mice. The human CD3 ϵ
25 gene-modified mice with confirmed evident tumor establishment were treated by administering antibody-38 in combination with an anti-mouse PD-1 antibody (BioXCell, Catalog# BE0146) or anti-mouse PD-L1 (BioXCell, Catalog# BE0101) antibody.

The drug efficacy evaluation study for antibody-38 and other agents using the NOD scid/T cell-injected model was performed as follows. T cell expansion culture was performed
30 using PBMCs separated from blood collected from a healthy person and T cell activation/ expansion kit/ human. Each of human cancer cell lines (MKN45 and NCI-H446) was mixed with MatrigelTM Basement Membrane Matrix, and transplanted subcutaneously into NOD scid mice (CLEA Japan). The day of transplantation was defined as Day 0. On the day before transplantation, anti-asialo GM1 antibody was administered intraperitoneally at 0.2 mg/mouse.
35 When evident tumor establishment was confirmed after transplantation, the mice were separated into groups based on tumor size and body weight. Then, anti-asialo GM1 antibody was

administered intraperitoneally at 0.2 mg/mouse again. On the following day, T cells obtained by the aforementioned expansion culture were transplanted intraperitoneally at 3×10^7 cells/mouse. About two to five hours after T cell transplantation, antibody-38 was administered. In combination with antibody-38, capecitabine, cisplatin, or paclitaxel was administered according to the dose and dosing regimen shown in Table 7.

Table 7

Cell line	Pharmaceutical agent for use in combination	Pharmaceutical agent for use in combination Dose and administration route	Pharmaceutical agent for use in combination Dosing regimen	Antibody-38 Dose	Antibody-38 Dosing regimen
MKN45	capecitabine	431 mg/kg Oral administration	Repetitive administration for five days from Day 17	5 mg/kg, Tail vein administration	Single administration Day 14
	cisplatin	7.5 mg/kg Tail vein administration	Single administration Day 13	1 mg/kg, Tail vein administration	Single administration Day 14
	paclitaxel	20 mg/kg Tail vein administration	Single administration Day 13	1 mg/kg, Tail vein administration	Single administration Day 14
NCI-H446	cisplatin	7.5 mg/kg Tail vein administration	Single administration Day 14	1 mg/kg, Tail vein administration	Single administration Day 15
	paclitaxel	20 mg/kg Tail vein administration	Days 14 and 21	1 mg/kg, Tail vein administration	Single administration Day 15

As a result, the anti-tumor effect was enhanced in the combination groups to which antibody-38 and the pharmaceutical agent for use in combination were administered as compared to groups where antibody-38 or the pharmaceutical agent for use in combination was used alone (Figs. 7 and 8).

RNA was extracted from tumor samples after administration of paclitaxel, cisplatin, or capecitabine and antibody-38. Comprehensive RNA analysis using nCounter, tumor infiltrating lymphocyte (TIL) analysis, and pathological analysis were performed as follows.

The tumor samples were prepared as follows. The tumor samples when antibody-38 was used in combination with a chemotherapeutic agent in the humanized NOG mouse model were prepared by the procedure described below. 2.5 Gy of X ray was irradiated to NOG mice (In-Vivo Science Inc., ♀). On the following day, 1×10^5 CD34-positive hematopoietic stem cells which are derived from umbilical cord blood were transplanted into the NOG mice by tail vein injection. After 16 to 20 weeks, each of human cancer cell lines (MKN45 and NCI-H446) was mixed with Matrigel™ Basement Membrane Matrix, and transplanted subcutaneously into the humanized NOG mice. The day of transplantation was defined as Day 0.

When tumor was established, the mice were separated into groups based on tumor size and body weight. Antibody-38 and the pharmaceutical agents for use in combination were administered according to the dose and dosing regimen described in Table 21. The mice were euthanized at the timing described in Table 21, and tumor was excised and preserved for use in tumor infiltrating lymphocyte (TIL) analysis, pathological analysis, or RNA analysis.

Table 21

Cell line	Pharmaceutical agent for use in combination	Pharmaceutical agent for use in combination Dose and administration route	Pharmaceutical agent for use in combination Dosing regimen	Antibody-38 Dose	Antibody-38 Dosing regimen	Tumor sampling	Analysis
MKN45	capecitabine	359 mg/kg Oral administration	Repetitive administration for five days from Day 21	5 mg/kg Tail vein administration	Single administration Day 22	14th day from the start of capecitabine administration	RNA
NCI-H446	cisplatin	7.5 mg/kg Tail vein administration	Single administration Day 20	5 mg/kg Tail vein administration	Single administration Day 21	Sixth day after antibody-38 administration	TIL
	paclitaxel	20 mg/kg Tail vein administration	Day 20	5 mg/kg Tail vein administration	Single administration Day 21	Sixth day after antibody-38 administration	TIL, RNA, Pathology

RNA analysis was performed as follows. RNA was extracted (miRNeasy Mini Kit, QIAGEN) from the above-described tumor samples and the RNA concentrations were determined (NanoDrop, Thermo Fisher Scientific). 100 ng of RNA was subjected to comprehensive (human) RNA expression analysis using nCounter PanCancer Pathway Panel and PanCancer Immune Profiling Panel (NanoString). Normalization was performed using the housekeeping gene included in the Panel. The analysis software used was nSolver (NanoString).

As a result, the expressions of immune cell markers, chemokines, cytokines, genes involved in cell death, genes involved in cell cycle regulation were increased and the expressions of genes involved in the progress of cell cycle were suppressed when antibody-38 was used in combination with a chemotherapeutic agent, paclitaxel or capecitabine, as compared to the group to which antibody-38 or the chemotherapeutic agent was administered alone (Fig. 35-1 to 35-6).

TIL analysis was performed as follows. Tumor tissues which were dissected from mice transplanted with NCI-H446 described in Table 21 on the sixth day after antibody-38 administration were dissociated into cells by enzyme treatment using gentle MACS™ Octo Dissociator. The cells were labeled with CD45, CD3, CD4, CD8, and GZMB antibodies and examined for their positive rates in each fraction of TIL using BD LSRFortessa X-20.

As a result, cell populations expressing T cell markers, activated T cell markers, and

proteins involved in cytotoxic activity were increased when antibody-38 was used in combination with a chemotherapeutic agent, paclitaxel or capecitabine, as compared to the group to which antibody-38 or the chemotherapeutic agent was administered alone (Figs. 36-1 and 36-2).

5 With respect to histological analysis of tumor tissues, tumor samples from mice six days after administration of antibody-38 and paclitaxel were fixed in 10% formaldehyde neutral buffer solution. Then, HE-stained samples were prepared according to a conventional method, and histopathological evaluation was performed using a light microscope.

10 As a result, the immune cell infiltration was observed around the tumor periphery when antibody 38 was administered, and the infiltration area was expanded when antibody-38 was used in combination with paclitaxel.

15 The drug efficacy evaluation study for antibody-38 and other agents using the human CD3 $\epsilon\delta\gamma$ gene-modified mouse model was performed as follows. Hepal-6/hGPC3 and LLC1/hGPC3 cancer cell lines, which are a mouse cancer cell line with over-expression of human GPC3, were transplanted subcutaneously into human CD3 $\epsilon\delta\gamma$ gene-modified mice (Chugai Pharmaceutical Co. Ltd.). The day of transplantation was defined as Day 0. When evident tumor establishment was confirmed, the mice were separated into groups based on tumor size and body weight. Treatment was performed by administering antibody-38 in combination with the anti-mouse TIM-3 antibody, anti-mouse LAG-3 antibody, anti-mouse CD137 antibody, 20 or anti-mouse VEGFR2 antibody. The dose of each pharmaceutical agent that was used in combination with antibody-38 is shown in Table 8.

Table 8

Cell line	Model	Pharmaceutical agent for use in combination	Pharmaceutical agent for use in combination Dose and administration route	Pharmaceutical agent for use in combination Dosing regimen	Antibody-38 Dose	Antibody-38 Dosing regimen
Hepa1-6/hGPC3	Human CD3 $\epsilon\delta\gamma$ gene-modified mouse	TIM3 Ab (Clone RMT 3-23)	10 mg/kg Intravenous administration	Day 13 Single administration	0.2 mg/kg. Tail vein administration	Day 13 Single administration
		LAG-3 Ab (Clone C9B7W)	10 mg/kg Tail vein administration	Day 13 Single administration	0.2 mg/kg. Tail vein administration	Day 13 Single administration
		CD137 Ab (Clone LOB12.3)	10 mg/kg Tail vein administration	Day 13 Single administration	0.2 mg/kg. Tail vein administration	Day 13 Single administration
		VEGFR2 Ab (Clone DC101)	10 mg/kg Tail vein administration	Day 13 Single administration	0.2 mg/kg. Tail vein administration	Day 13 Single administration
LLC1/hGPC3	Human CD3 $\epsilon\delta\gamma$ gene-modified mouse	CD137 Ab (Clone LOB12.3)	10 mg/kg Intraperitoneal administration	Days 11 and 15	5 mg/kg. Tail vein administration	Days 11 and 15

As a result, the anti-tumor effect was enhanced in the combination groups to which antibody-38 and a pharmaceutical agent for use in combination were administered as compared to the groups where antibody-38 or the pharmaceutical agent for use in combination was used alone (Figs. 9 and 10).

The drug efficacy evaluation study for antibody-38 with other agents in the human CD3 ϵ gene-modified mouse model was performed as follows. Hepa1-6/hGPC3 cancer cell line, which is a mouse cancer cell line with over-expression of human GPC3, was transplanted subcutaneously into human CD3 ϵ gene-modified mice (Chugai Pharmaceutical Co. Ltd.). The day of transplantation was defined as Day 0. On Day 15, the mice were separated into groups based on tumor size and body weight. Treatment was performed by administering antibody-38 in combination with the anti-mouse PD-1 antibody or anti-mouse PD-L1 antibody. The dose of each pharmaceutical agent that was used in combination with antibody-38 is shown in Table 9.

Table 9

Cell line	Model	Pharmaceutical agent for use in combination	Pharmaceutical agent for use in combination Dose and administration route	Pharmaceutical agent for use in combination Dosing regimen	Antibody-38 Dose	Antibody-38 Dosing regimen
Hepa1-6/hGPC3	Human CD3 ϵ gene-modified mouse	PD-1 Ab (clone RMP1-14)	200 μ g/mouse Tail vein administration	Days 15 and 18	5 mg/kg, Tail vein administration	Days 15 and 18
		PD-L1 Ab (clone 10F.9G2)	200 μ g/mouse Tail vein administration	Days 15 and 18	5 mg/kg, Tail vein administration	Days 15 and 18

As a result, the anti-tumor effect was enhanced in the combination groups to which antibody-38 and a pharmaceutical agent for use in combination were administered as compared to groups where antibody-38 or the pharmaceutical agent for use in combination was used alone (Fig. 11).

Reference Examples

[Reference Example 1] Production of GPC3_ERY22_rCE115 and measurement of cytotoxic activity

(1-1) Production of GPC3_ERY22_rCE115

A molecule in which one of the Fabs has been replaced with a CD3 epsilon-binding domain was produced using IgG against a cancer antigen (GPC3) as the basic structure. In this case, the IgG Fc used as the basic structure was a silent Fc with attenuated affinity for Fc γ R (an Fc γ (Fc gamma) receptor). An anti-GPC3 antibody, H0000 (SEQ ID NO: 40) / GL4 (SEQ ID NO: 41), was used as the GPC3-binding domain. An anti-CD3 antibody, rCE115H/rCE115L (SEQ ID NO: 42 / SEQ ID NO: 43), was used as the CD3-binding domain.

G1d produced by removing Gly and Lys at the C terminus of IgG1 was used as the antibody H-chain constant region, and this was used in combination with H0000/GL4 and rCE115H/rCE115L. When the antibody H-chain constant region was named H1, the sequence corresponding to the H chain of the antibody carrying H0000 in the variable region was shown as H0000-H1. Here, an amino acid alteration was shown, for example, as D356K. The first alphabet (corresponding to D in D356K) is the one-letter code representation for the amino acid residue before modification, the number that follows (corresponding to 356 of D356K) is the position of modification indicated by EU numbering, and the final alphabet (corresponding to K of D356K) is the one-letter code representation for the amino acid residue after modification. G1dh (SEQ ID NO: 44) produced by removing Gly and Lys at the C terminus of IgG1, ERY22_Hk (SEQ ID NO: 45) produced by introducing the L234A/L235A/Y349C/T366W mutations into G1dh, and ERY22_Hh (SEQ ID NO: 46) produced by introducing the

L234A/L235A/D356C/T366S/L368A/Y407V mutations into G1dh were prepared according to the method of Reference Example 5. The L234A and L235A mutations were introduced into the respective H chains to attenuate affinity for FcγR (an Fcγ receptor), and the Y349C/T366W and D356C/T366S/L368A/Y407V mutations were introduced to efficiently form heteromers of each H chain when producing heterodimeric antibodies comprising two types of H chains.

The heterodimeric antibody, GPC3_ERY22_rCE115, produced by substitution with the VH and VL domains of Fab against GPC3 was prepared according to Reference Example 5 (Fig. 12a).

A series of expression vectors inserted with a polynucleotide encoding each of GL4-ERY22_Hk (SEQ ID NO: 47), H0000-ERY22_L (SEQ ID NO: 48), rCE115H-ERY22_Hh (SEQ ID NO: 49), and rCE115L-k0 (SEQ ID NO: 50) were produced by methods well-known to those skilled in the art, such as PCR methods using primers added with an appropriate sequence similar to those in the above-described method.

The following combination of expression vectors were introduced into FreeStyle 293-F cells for transient expression of each target molecule.

Target molecule: GPC3_ERY22_rCE115

Polypeptides encoded by the polynucleotides inserted into the expression vectors: GL4-ERY22_Hk, H0000-ERY22_L, rCE115H-ERY22_Hh, rCE115L-k0

20 (1-2) Purification of GPC3_ERY22_rCE115

The obtained culture supernatant was added to an anti-FLAG M2 column (Sigma), and then the column was washed, followed by elution using 0.1 mg/mL of a FLAG peptide (Sigma). The fractions containing the molecule of interest were added to a HisTrap HP column (GE Healthcare), and then the column was washed, followed by elution using an imidazole concentration gradient. Fractions containing the molecule of interest were concentrated using an ultrafiltration membrane, then the fractions were added to a Superdex 200 column (GE Healthcare), and each of the purified molecules of interest was obtained by collecting only the monomeric fractions from the eluted solution.

30 (1-3) Measurement of the cytotoxic activity of GPC3_ERY22_rCE115 using human peripheral blood mononuclear cells

The *in vitro* cytotoxic activity of GPC3_ERY22_rCE115 was assessed.

(1-3-1) Preparation of a human peripheral blood mononuclear cell (PBMC) solution

Using a syringe preloaded with 100 μL of 1,000 units/mL heparin solution (Novo Heparin for injection, 5000 units, Novo Nordisk), 50 mL of peripheral blood was collected from each healthy volunteer (adult individual). This peripheral blood was diluted two-fold in PBS(-),

divided into four aliquots, and added into a Leucosep tube for lymphocyte separation (Cat. No. 227290, Greiner Bio-One) that had been loaded with 15 mL of Ficoll-Paque PLUS and subjected to centrifugation in advance. This separation tube was centrifuged (at 2150 rpm for ten minutes at room temperature), and then the mononuclear cell fraction was collected. The cells in the mononuclear cell fraction were washed once with the Dulbecco's Modified Eagle's Medium containing 10% FBS (manufactured by SIGMA, hereinafter referred to as 10% FBS/D-MEM), and then prepared to have a cell density of 4×10^6 cells/mL using 10% FBS/D-MEM. The cell suspension prepared this way was used as the human PBMC solution in the experiment below.

(1-3-2) Measurement of cytotoxic activity

Cytotoxic activity was assessed by the rate of cell proliferation inhibition using the xCELLigence Real-Time Cell Analyzer (Roche Diagnostics). The NCI-H446 human cancer cell line or the PC-10 human cancer cell line, which expresses human GPC3, was used as the target cell. NCI-H446 or PC-10 was detached from the dish, then the cells were plated into E-Plate 96 (Roche Diagnostics) in aliquots of 100 μ L/well by adjusting the cells to 1×10^4 cells/well, and measurement of live cells was begun using the xCELLigence Real-Time Cell Analyzer. On the following day, the plate was removed from the xCELLigence Real-Time Cell Analyzer, and 50 μ L of the respective antibodies prepared at each concentration (0.004, 0.04, 0.4, 4, or 40 nM) were added to the plate. After 15 minutes of reaction at room temperature, 50 μ L of the human PBMC solution prepared in (1-2) was added (2×10^5 cells/well), and measurement of live cells was begun by setting the plate into the xCELLigence Real-Time Cell Analyzer again. The reaction was carried out under the conditions of 5% carbon dioxide gas at 37°C, and from the Cell Index value obtained 72 hours after addition of the human PBMC, the cell proliferation inhibition rate (%) was determined using the equation below. The Cell Index value used in the calculation was a normalized value where the Cell Index value immediately before antibody addition was defined as 1.

$$\text{Cell proliferation inhibition rate (\%)} = (A-B) \times 100/(A-1)$$

A represents the mean value of the Cell Index values in wells without antibody addition (containing only the target cells and human PBMCs), and B represents the mean value of the Cell Index values in each well. The examinations were performed in triplicate.

When peripheral blood mononuclear cells (PBMCs) prepared from human blood were used as the effector cell to measure the cytotoxicity of GPC3_ERY22_rCE115, a very strong activity was observed (Fig. 13).

[Reference Example 2] Humanization of the H chain of the anti-CD3 antibody, rCE115, and

sharing of a common L chain

(2-1) Design of hCE115HA, the humanized rCE115 H-chain variable region

The H-chain variable region of the rCE115 anti-CD3 antibody (SEQ ID NO: 42) was humanized. CDR and FR were determined as defined by Kabat (Kabat numbering).

5 First, a human FR sequence was selected by comparing the human antibody variable region sequences in a database to the rCE115 rat variable region sequence. The IMGT Database (<http://www.imgt.org/>) and NCBI GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) were used for the database. A humanized H-chain variable region sequence was designed by linking the H-chain CDR sequence of the rCE115 variable region with the selected human FR
10 sequence. This yielded a humanized H-chain variable region sequence, hCE115HL (SEQ ID NO: 51).

The amino acid residue at position 93 indicated by Kabat numbering is Ala in the selected human H-chain FR3 sequence, but is Arg in the rCE115 variable region sequence. Using the database of rat and human germline sequences (IMGT Database
15 (<http://www.imgt.org/>)), only few sequences were found to contain Arg at this site. It is reported that the amino acid residue at position 94 indicated by Kabat numbering contributes to stabilization of the antibody structure by upper core formation (Ewert *et al.* Methods. 2004 Oct;34(2):184-99). Based on such information, a humanized H-chain variable region sequence, in which the amino acid residues at Kabat positions 93 and 94 in the H-chain FR3 were
20 substituted with those residues present in the rCE115 variable region sequence, was newly designed. This was the humanized H-chain variable region sequence, hCE115HA (SEQ ID NO: 52).

(2-2) Design of the common L chain, L0000, for the rCE115 anti-CD3 antibody and the
25 anti-GPC3 antibody

The FR/CDR shuffling of the L-chain variable region rCE115L (SEQ ID NO: 43) of the rCE115 anti-CD3 antibody and the L-chain variable region GL4 (SEQ ID NO: 41) of the anti-GPC3 antibody was performed.

The FR sequence of GL4 was selected as the L-chain FR sequence. L-chain CDR2
30 was the same for rCE115L and GL4. The L-chain CDR1 was selected from the CDR sequences of GL4, and the L-chain CDR3 was selected from the CDR sequences of rCE115L, respectively. Furthermore, the L-chain CDR3 produced by substituting the amino acid residue Asp at Kabat position 94 of the selected L-chain CDR3 with the Val residue present in GL4 was newly designed.

35 A humanized L chain variable region sequence was designed by linking FR and CDR selected above. This yielded a humanized L-chain variable region sequence, L0000 (SEQ ID

NO: 53).

(2-3) Evaluation of the affinity for human GPC3

The activity to bind human GPC3 when using GL4 (SEQ ID NO: 41) and L0000 (SEQ ID NO: 53) as the L-chain variable regions was evaluated. This was performed using the molecular form of a single-arm antibody having a single Fab at the Fc region of a human IgG1 heterodimerized by the knobs-into-hole technique. H0000 (SEQ ID NO: 40) was used for the anti-GPC3 antibody H-chain variable region.

The affinity and binding rate constants of an anti-GPC3 antibody for an antigen were measured by the multi-cycle kinetics method of a surface plasmon resonance assay using Biacore™-T200 (GE Healthcare Japan). HBS-EP+ (GE Healthcare Japan) was used for the running buffer, and an amine coupling kit (GE Healthcare Japan) was used to covalently bind Protein A/G to the CM5 chip (carboxymethyl dextran-coated chip). Each anti-GPC3 antibody was prepared so that approximately 100 RU will be captured by Protein A/G. Human GPC3 used as the analyte was prepared at 8, 16, 32, 64, and 128 nM using HBS-EP+. Measurements were carried out by first allowing Protein A/G to capture the antibody solution, and then injecting the human GPC3 solution at a flow rate of 30 μ L/min for three minutes to allow reaction to take place. Then, the solution was switched to HBS-EP+ and the dissociation phase was measured for 15 minutes. After completion of the dissociation phase measurement, the sensor chip was regenerated by washing with 10 mM Gly-HCl at pH 1.5. Measurement at the concentration of 0 was similarly carried out by allowing Protein A/G to capture the antibody solution, performing a three-minute HBS-EP+ injection to allow reaction to take place, and then switching to HBS-EP+ to measure the dissociation phase for 15 minutes. After completion of the dissociation phase measurement, the sensor chip was regenerated by washing with 10 mM Gly-HCl at pH 1.5. A data analysis software exclusively for Biacore, Biacore T200 Evaluation Software Version 1.0, was used to perform kinetic analyses to calculate the binding rate constant (k_a), dissociation rate constant (k_d), and the rate constant ratio from the obtained sensorgrams. The results are shown in Table 10.

Table 10

Variable region		Affinity for human GPC3		
H-chain variable region	L-chain variable region	KD (M)	k_a (1/Ms)	k_d (1/s)
H0000	GL4	4.2×10^{-9}	4.3×10^5	1.8×10^{-3}
H0000	L0000	3.6×10^{-8}	3.0×10^5	1.1×10^{-2}

(2-4) Evaluation of the affinity for human CD3

The activity to bind human CD3 when using hCE115HA (SEQ ID NO: 52) as the H chain variable region and L0000 (SEQ ID NO: 53) as the L-chain variable region was evaluated. This was performed using the molecular form of a single-arm antibody having a single Fab at the Fc region of a human IgG1 heterodimerized by the knobs-into-hole technique.

5 The affinity and binding rate constants of an anti-CD3 antibody for an antigen were measured by the single-cycle kinetics method of a surface plasmon resonance assay using Biacore™-T200 (GE Healthcare Japan). HBS-EP+ (GE Healthcare Japan) was used for the running buffer, and an amine coupling kit (GE Healthcare Japan) was used to covalently bind human CD3 to the CM4 chip (carboxymethyl dextran-coated chip). The anti-CD3 antibody
10 used as the analyte was prepared at 5 and 20 µg/mL using HBS-EP+. Measurements were carried out by first injecting each of the 5- and 20-µg/mL anti-CD3 antibody solutions for three minutes continuously at a flow rate of 20 µL/min to allow reaction to take place. Then, the solution was switched to HBS-EP+ and the dissociation phase was measured for 3 minutes. After completion of the dissociation phase measurement, the sensor chip was regenerated by
15 washing with 10 mM Gly-HCl at pH 1.5. Measurement at the concentration of 0 was carried out by performing each of the three-minute HBS-EP+ injections twice successively to allow reaction to take place, and then switching to HBS-EP+ to measure the dissociation phase for 3 minutes. After completion of the dissociation phase measurement, the sensor chip was regenerated by washing with 10 mM Gly-HCl at pH 1.5. A data analysis software exclusively
20 for Biacore, Biacore T200 Evaluation Software Version 1.0, was used to perform kinetic analyses to calculate the binding rate constant (k_a), dissociation rate constant (k_d), and the rate constant ratio from the obtained sensorgrams. The results are shown in Table 11.

Table 11

Variable region		Affinity for human CD3		
H-chain variable region	L-chain variable region	KD (M)	k_a (1/Ms)	k_d (1/s)
rCE115H	rCE115L	1.0×10^{-7}	5.9×10^4	6.0×10^{-3}
hCE115HA	L0000	1.2×10^{-7}	1.9×10^5	2.3×10^{-2}

(2-5) Preparation of GPC3_ERY27_hCE115

The IgG4 against a cancer antigen (GPC3) was used as the basic structure to produce the ERY27 molecule (Fig. 12b), in which the H-chain variable region of one of the Fabs has been
30 replaced with a CD3 epsilon-binding domain, and the L chain is common to both Fabs. In this case, the IgG4 Fc used as the basic structure was a silent Fc with attenuated affinity for FcγR (an Fcγ receptor). H0000 (SEQ ID NO: 40) was used as the H-chain variable region of the GPC3-binding domain, and hCE115HA (SEQ ID NO: 52) was used as the H-chain variable

region of the CD3-binding domain. L0000 (SEQ ID NO: 53) was used as the L-chain variable region. The D356K and K439E mutations introduced into the respective H chains were introduced for efficient heteromer formation of each H chain when producing heterodimeric antibodies comprising two types of H chains (WO2006/106905). H435R is a modification that interrupts binding to Protein A, and was introduced for efficient separation of the heteromer and homomer (WO/2011/078332).

A series of expression vectors inserted with a polynucleotide encoding each of H0000-ERY27_HK (SEQ ID NO: 54), hCE115HA-ERY27_HE (SEQ ID NO: 55), and L0000-k0 (SEQ ID NO: 56) were produced by well-known methods.

The following combination of expression vectors were introduced into FreeStyle 293-F cells for transient expression of each target molecule.

Target molecule: GPC3_ERY27_hCE115

Polypeptides encoded by the polynucleotides inserted into the expression vectors: H0000-ERY27_HK, hCE115HA-ERY27_HE, and L0000-k0

(2-6) Purification of GPC3_ERY27_hCE115

Each molecule of interest was purified by the method described in Reference Example 1-2.

(2-7) Measurement of cytotoxic activity using human peripheral blood mononuclear cells

(2-7-1) Preparation of a human peripheral blood mononuclear cell (PBMC) solution

The solution was prepared by the method described in Reference Example 1-3-1.

(2-7-2) Measurement of cytotoxic activity

Cytotoxic activity was measured by the method described in Reference Example 1-3-2.

When PBMCs prepared from human blood were used as the effector cell to measure the cytotoxicity of GPC3_ERY27_hCE115, reduction of the activity was observed as a result of humanization of the H chain of rCE115 and sharing of a common L chain (Fig. 13).

[Reference Example 3] Production and evaluation of humanized bispecific antibody variants for improvement of various properties

The T-cell-dependent cytotoxic activity of the humanized anti-human CD3 ϵ (CD3 epsilon) chain and anti-human GPC3 bispecific antibody obtained in Reference Example 2, GPC3_ERY27_hCE115 (SEQ ID NOs: 54, 55, and 56), was lower than the T-cell-dependent cytotoxic activity of GPC3_ERY22_rCE115 (SEQ ID NOs: 47, 48, 49, and 50). This may be due to attenuation of affinity for GPC3 and the CD3 ϵ chain as a result of humanization and sharing of a common L chain. Regarding GPC3 and CD3 ϵ -chain antigens which have

independent sequences, there has been no report so far on humanized bispecific antibodies whose T-cell dependent cytotoxic activity has been enhanced and whose affinity for both antigens has been improved by using a common antibody L chain. Therefore, it has been considered difficult to obtain humanized antibodies with dual specificity that show a drug efficacy equivalent to or greater than that of GPC3_ERY22_rCE115.

Under such circumstances, the Applicants produced modified humanized bispecific antibodies with modified affinity for human GPC3 and human CD3 ϵ chain by methods known to those skilled in the art, which involves comprehensively substituting amino acid residues encoded by the antibody gene to produce antibody variants against both the human GPC3 and human CD3 ϵ -chain antigens, and by performing various evaluations by screening. Furthermore, similar methods were used to produce modified humanized bispecific antibodies with modified physicochemical properties. Furthermore, by combining substitutions of amino acid residues effective for modifying affinity and physicochemical properties, optimized bispecific antibodies having a TDCC activity equivalent to or greater than the T-cell dependent cellular cytotoxicity of GPC3_ERY22_rCE115 prior to humanization were produced.

Introduction of point mutations, expression and purification of antibodies, antigen affinity measurements, and determination of T-cell dependent cellular cytotoxicity in the optimization of humanized bispecific antibodies were performed by methods similar to those in Reference Examples 1 and 2. CDR and FR were determined according to the Kabat definition (Kabat numbering).

Depending on the objective, the following were used as the antibody H-chain constant regions (the numbers indicate EU numbering): E22Hh (SEQ ID NO: 57) produced by introducing L234A/L235A/N297A/D356C/T366S/L368A/Y407V/G446 deletion/K447 deletion mutations into human IgG1; E22Hk (SEQ ID NO: 58) produced by introducing L234A/L235A/N297A/Y349C/T366W/G446 deletion/K447 deletion mutations and a Ser-Ser insertion mutation immediately before position 118 into human IgG1; G1dh produced by introducing D356C/T366S/L368A/Y407V/G446 deletion/K447 deletion mutations into human IgG1; none-Hi-Kn010G3 produced by introducing 118-215 deletion and C220S/Y349C/T366W/H435R mutations into human IgG1; E2702GsKsc (SEQ ID NO: 60) produced by introducing L235R/S239K/N297A/E356K/R409K/H435R/L445P/G446 deletion/K447 deletion mutations into human IgG4; E2704sEpSc (SEQ ID NO: 61) produced by introducing K196Q/L235R/S239K/N297A/R409K/K439E/L445P/G446 deletion/K447 deletion mutations into human IgG4; and E2702sKsc (SEQ ID NO: 62) produced by introducing L235R/S239K/N297A/E356K/R409K/L445P/G446 deletion/K447 deletion mutations into human IgG4. Furthermore, human κ (kappa) chain k0 (SEQ ID NO: 63) and E22L (SEQ ID NO: 432) produced by introducing R108A/T109S mutations into human κ chain were used as

the antibody L-chain constant regions.

The mutation that substitutes Cys for Asp at EU numbering position 356, the mutation that substitutes Ser for Thr at EU numbering position 366, the mutation that substitutes Ala for Leu at EU numbering position 368, the mutation that substitutes Val for Tyr at EU numbering position 407, the mutation that substitutes Cys for Tyr at EU numbering position 349, the mutation that substitutes Trp for Thr at EU numbering position 366, and the mutation that inserts Ser-Ser immediately before position 118 are mutations for efficient formation of heterodimeric molecules for each H chain when producing heteromeric antibodies. Similarly, the mutation that substitutes Lys for Glu at EU numbering position 356 and the mutation that substitutes Glu for Lys at EU numbering position 439 are also mutations for efficient formation of heterodimeric molecules for each H chain when producing heteromeric antibodies. They are expected to improve the efficiency of bispecific antibody production.

The mutation that substitutes Ala for Leu at EU numbering position 234, the mutation that substitutes Ala or Arg for Leu at EU numbering position 235, the mutation that substitutes Lys for Ser at EU numbering position 239, and the mutation that substitutes Ala for Asn at EU numbering position 297 are mutations for attenuating affinity for an Fcγ receptor and a complement (C1q). They are expected to suppress the binding of Fab to CD3 and Fc-mediated crosslinking of an Fcγ receptor or a complement, and avoid cytokine release syndrome that accompanies enhancement of non-specific effector functions.

The H chain introduced with deletion mutations at EU numbering positions 118 to 215 can be combined with a full-length H chain sequence to produce an antibody that has only one Fab (monovalent antibody), and it is useful for affinity evaluation.

The mutation that substitutes Lys for Arg at EU numbering position 409 and the mutation that substitutes Arg for His at EU numbering position 435 are mutations for modifying the antibody properties to be close to the properties of human IgG1 and human IgG3, respectively.

(3-1) Modifying the affinity of a humanized anti-CD3 antibody by point mutations

First, point mutations were introduced into FR1, FR2, FR3, CDR1, CDR2, and CDR3 of the humanized anti-human CD3ε chain antibody sequence produced in Reference Example 2, hCE115HA-ERY27_HE (SEQ ID NO: 55), to prepare modified antibodies. Next, the affinity of these modified antibodies for the soluble human CD3ε chain was determined. Combining sites that have an affinity-enhancing effect yielded modified antibodies having the affinities shown in Table 12.

Table 12

Antibody name	KD (Human CD3)
ICE115HA-E22Hh//Hi-Kn010G3/L0000-k0	1.43E-07
TR01H083-E22Hh/none-Hi-Kn010G3/L0212-k0	5.86E-11
TR01H040-E22Hh/none-Hi-Kn010G3/L0240-k0	2.17E-09
TR01H002-E22Hh/GLS3108-k0/GL4-E22Hh/H0610-E22L	2.04E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0212-k0	2.17E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0235-k0	2.81E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0238-k0	2.91E-09
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L016-k0	2.52E-09
TR01H083-E22Hh/none-Hi-Kn010G3/L0262-k0	2.45E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0207-k0	2.60E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0241-k0	3.48E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0242-k0	3.58E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0206-k0	2.90E-09
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L019-k0	3.20E-09
TR01H080-E22Hh/none-Hi-Kn010G3/L0000-k0	3.25E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0211-k0	3.22E-09
TR01H002-E22Hh//Hi-Kn010G3/GLC3108-k0	4.81E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0209-k0	4.25E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0208-k0	4.18E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0224-k0	5.06E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0236-k0	5.64E-09
TR01H083-E22Hh/none-Hi-Kn010G3/L0201-k0	4.42E-09
TR01H084-E2702GsKsc/none-Hi-E2704sE/L0011-k0	4.14E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0210-k0	5.06E-09
TR01H114-E2702GsKsc/none-Hi-E2704sE/L0011-k0	4.22E-09
CE115HA236-E22Hh/GLS3108-k0/GL4-E22Hh/H0610-E22L	6.08E-09
TR01H077-E22Hh/none-Hi-Kn010G3/L0200-k0	6.12E-09
TR01H071-E22Hh/none-Hi-Kn010G3/L0200-k0	6.13E-09
TR01H111-E2702GsKsc/none-Hi-E2704sE/L0011-k0	4.91E-09
TR01H081-E22Hh/none-Hi-Kn010G3/L0262-k0	5.76E-09
TR01H001-E22Hh//Hi-Kn010G3/GLC3108-k0	8.22E-09
CE115HA179-G1dh//Hi-Kn010G3/L0000-k0	8.35E-09
TR01H112-E2702GsKsc/none-Hi-E2704sE/L0011-k0	5.12E-09
TR01H113-E2702GsKsc/none-Hi-E2704sE/L0011-k0	5.14E-09
TR01H082-E22Hh/none-Hi-Kn010G3/L0212-k0	4.75E-09
CE115HA236-E22Hh//Hi-Kn010G3/GLC3108-k0	9.10E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0231-k0	7.75E-09
TR01H037-E22Hh/none-Hi-Kn010G3/L0000-k0	6.93E-09
CE115HA252-E22Hh//Hi-Kn010G3/L0000-k0	9.48E-09
TR01H083-E22Hh/none-Hi-Kn010G3/L0011-k0	6.70E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0223-k0	8.15E-09
TR01H083-E22Hh/none-Hi-Kn010G3/L0000-k0	6.83E-09
TR01H071-E22Hh/none-Hi-Kn010G3/L0000-k0	8.85E-09
TR01H067-E22Hh/none-Hi-Kn010G3/L0212-k0	5.88E-09
CE115HA178-G1dh//Hi-Kn010G3/L0000-k0	1.09E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0237-k0	1.02E-08
TR01H083-E22Hh/none-Hi-Kn010G3/L0222-k0	9.42E-09
TR01H084-E22Hh/none-Hi-Kn010G3/L0262-k0	8.51E-09
TR01H071-E22Hh/none-Hi-Kn010G3/L0215-k0	9.51E-09
TR01H040-E22Hh/none-Hi-Kn010G3/L0218-k0	8.20E-09
TR01H081-E22Hh/none-Hi-Kn010G3/L0201-k0	9.46E-09
TR01H071-E22Hh/none-Hi-Kn010G3/L0222-k0	1.04E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0220-k0	9.15E-09
TR01H067-E22Hh/none-Hi-Kn010G3/TR01L016-k0	1.09E-08
TR01H002-E22Hh/GLS3108-k0/GL4-E22Hh/H0000-E22L	4.78E-09
TR01H067-E22Hh/none-Hi-Kn010G3/TR01L019-k0	1.21E-08
TR01H038-E22Hh/none-Hi-Kn010G3/L0000-k0	1.24E-08
TR01H061-E22Hh/none-Hi-Kn010G3/L0200-k0	1.27E-08
TR01H082-E2702GsKsc/none-Hi-E2704sE/L0011-k0	1.01E-08
CE115HA180-G1dh//Hi-Kn010G3/L0000-k0	1.68E-08
CE115HA251-E22Hh/L0000-k0/GL4-E22Hh/H0610-F22L	1.37E-08
TR01H100-E2702GsKsc/none-Hi-E2704sE/L0011-k0	1.11E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0228-k0	1.60E-08
TR01H081-E22Hh/none-Hi-Kn010G3/L0011-k0	1.35E-08
TR01H061-E22Hh/none-Hi-Kn010G3/L0215-k0	1.54E-08
TR01H110-E2702GsKsc/none-Hi-E2704sE/L0011-k0	1.26E-08
TR01H043-E22Hh/none-Hi-Kn010G3/L0000-k0	1.52E-08
TR01H081-E22Hh/none-Hi-Kn010G3/L0000-k0	1.56E-08
CE115HA251-E22Hh//Hi-Kn010G3/L0000-k0	2.23E-08
TR01H091-E2702GsKsc/none-Hi-E2704sE/L0011-k0	1.39E-08
CE115HA236-E22Hh/GLS3108-k0/GL4-E22Hh/H0000-E22L	6.95E-09
TR01H084-E22Hh/none-Hi-Kn010G3/L0201-k0	1.65E-08
TR01H072-E22Hh/none-Hi-Kn010G3/L0000-k0	2.03E-08
TR01H099-E2702GsKsc/none-Hi-E2704sE/L0011-k0	1.46E-08
TR01H061-E22Hh/none-Hi-Kn010G3/L0222-k0	1.88E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0239-k0	2.31E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0262-k0	1.81E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0234-k0	2.40E-08
TR01H012-E22Hh/none-Hi-Kn010G3/L0000-k0	7.94E-08
TR01H061-E22Hh/none-Hi-Kn010G3/L0000-k0	1.71E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0243-k0	2.46E-08
TR01H109-E2702GsKsc/none-Hi-E2704sE/L0011-k0	1.64E-08
TR01H047-E22Hh/none-Hi-Kn010G3/L0000-k0	2.04E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0267-k0	2.29E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0266-k0	2.29E-08
TR01H084-E22Hh/none-Hi-Kn010G3/L0011-k0	1.98E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0250-k0	2.15E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0204-k0	2.21E-08
TR01H084-E22Hh/none-Hi-Kn010G3/L0000-k0	2.13E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0213-k0	2.01E-08

Antibody name	KD (Human CD3)
hCE115HA-E22Hh//Hi-Kn010G3/L0000-k0	1.43E-07
TR01H040-E22Hh/none-Hi-Kn010G3/L0214-k0	2.02E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0217-k0	2.07E-08
TR01H071-E22Hh/none-Hi-Kn010G3/L0226-k0	2.51E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0200-k0	2.87E-08
TR01H074-E22Hh/none-Hi-Kn010G3/L0000-k0	2.81E-08
TR01H039-E22Hh/none-Hi-Kn010G3/L0000-k0	2.61E-08
CE115HA177-G1dh//Hi-Kn010G3/L0000-k0	3.55E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0201-k0	2.81E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0263-k0	3.08E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0000-k0	3.60E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0216-k0	2.53E-08
TR01H051-E22Hh/none-Hi-Kn010G3/L0000-k0	2.91E-08
TR01H003-E22Hh//Hi-Kn010G3/L0000-k0	4.03E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0264-k0	3.44E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0232-k0	3.66E-08
TR01H041-E22Hh/none-Hi-Kn010G3/L0000-k0	3.16E-08
CE115HA122-E22Hh//Hi-Kn010G3/L0000-k0	4.28E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0233-k0	4.01E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0215-k0	3.37E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0203-k0	3.24E-08
TR01H015-E2702GsKsc/GCH019-E2704sEpsc/L0000-k0	2.86E-08
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L008-k0	2.83E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0205-k0	3.42E-08
TR01H015-E22Hh/L0000-k0/GL4-E22Hh/H0610-E22L	3.57E-08
TR01H064-E22Hh/none-Hi-Kn010G3/L0000-k0	3.07E-08
TR01H044-E22Hh/none-Hi-Kn010G3/L0000-k0	3.52E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0262-k0	3.98E-08
TR01H062-E22Hh/none-Hi-Kn010G3/L0000-k0	3.13E-08
CE115HA251-E22Hh/L0000-k0/GL4-E22Hh/H0000-E22L	1.48E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0011-k0	3.48E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0222-k0	4.65E-08
CE115HA192-E22Hh//Hi-Kn010G3/L0000-k0	5.05E-08
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L010-k0	3.28E-08
TR01H025-E22Hh/none-Hi-Kn010G3/L0000-k0	3.86E-08
TR01H082-E22Hh/none-Hi-Kn010G3/TR01L023-k0	4.25E-08
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L015-k0	3.95E-08
TR01H055-E22Hh/none-Hi-Kn010G3/L0000-k0	3.88E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0260-k0	4.53E-08
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L009-k0	3.56E-08
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L011-k0	3.57E-08
TR01H017-E22Hh/none-Hi-Kn010G3/L0000-k0	3.50E-08
CE115HA122-E22Hh/L0000-k0/GL4-E22Hh/H0000-E22L	1.69E-08
TR01H076-E22Hh/none-Hi-Kn010G3/L0000-k0	4.78E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0258-k0	4.70E-08
TR01H046-E22Hh/none-Hi-Kn010G3/L0000-k0	4.23E-08
hCE115H-G1dh//Hi-Kn010G3/L0000-k0	5.76E-08
TR01H082-E22Hh/none-Hi-Kn010G3/TR01L024-k0	4.76E-08
TR01H016-E22Hh/none-Hi-Kn010G3/L0000-k0	3.69E-08
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L018-k0	4.51E-08
TR01H084-E22Hh/none-Hi-Kn010G3/L0271-k0	2.76E-08
TR01H084-E22Hh/none-Hi-Kn010G3/L0270-k0	2.76E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0000vk1-k0	4.69E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0219-k0	3.94E-08
TR01H014-E22Hh/none-Hi-Kn010G3/L0000-k0	3.87E-08
TR01H061-E22Hh/none-Hi-Kn010G3/L0226-k0	4.71E-08
TR01H048-E22Hh/none-Hi-Kn010G3/L0000-k0	4.52E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0259-k0	5.07E-08
TR01H028-E22Hh/none-Hi-Kn010G3/L0000-k0	4.80E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0201-k0	4.49E-08
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L013-k0	4.15E-08
TR01H033-E22Hh/none-Hi-Kn010G3/L0000-k0	4.88E-08
hCE115HA-G1dh//Hi-Kn010G3/L0000-k0	6.50E-08
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L012-k0	4.22E-08
TR01H065-E22Hh/none-Hi-Kn010G3/L0000-k0	4.31E-08
TR01H079-E22Hh/none-Hi-Kn010G3/L0000-k0	5.05E-08
TR01H042-E22Hh/none-Hi-Kn010G3/L0000-k0	4.48E-08
TR01H063-E22Hh/none-Hi-Kn010G3/L0000-k0	4.35E-08
TR01H084-E22Hh/none-Hi-Kn010G3/L0272-k0	3.10E-08
CE115HA121-E22Hh//Hi-Kn010G3/L0000-k0	6.76E-08
TR01H026-E22Hh/none-Hi-Kn010G3/L0000-k0	5.12E-08
TR01H067-E22Hh/none-Hi-Kn010G3/L0262-k0	4.92E-08
TR01H073-E22Hh/none-Hi-Kn010G3/L0000-k0	5.97E-08
TR01H045-E22Hh/none-Hi-Kn010G3/L0000-k0	5.22E-08
TR01H007-E22Hh/none-Hi-Kn010G3/L0000-k0	2.17E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0203-k0	4.07E-08
TR01H032-E22Hh/none-Hi-Kn010G3/L0000-k0	5.73E-08
TR01H006-E22Hh/none-Hi-Kn010G3/L0000-k0	2.30E-08
TR01H013-E22Hh/none-Hi-Kn010G3/L0000-k0	4.94E-08
TR01H050-E22Hh/none-Hi-Kn010G3/L0000-k0	5.76E-08
TR01H067-E22Hh/none-Hi-Kn010G3/L0200-k0	6.03E-08
TR01H015-E22Hh/none-Hi-Kn010G3/L0000-k0	6.13E-08
hCE115HA-E22Hh/L0000-k0/GL4-E22Hh/H0000-E22L	6.16E-08
hCE115HA-E22stHh/none-Hi-stKn010G3/L0000-k0	5.17E-08
TR01H069-E22Hh/none-Hi-Kn010G3/L0000-k0	7.11E-08
TR01H015-E22Hh/none-Hi-Kn010G3/TR01L003-k0	6.34E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0202-k0	6.19E-08
TR01H067-E22Hh/none-Hi-Kn010G3/L0201-k0	5.93E-08
TR01H020-E22Hh/none-Hi-Kn010G3/L0000-k0	6.48E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0011-k0	5.95E-08

Antibody name	KD (Human CD3)
hCE115HA-E22Hh/-Hi-Kn010G3/L0000-k0	1.43E-07
TR01H082-E22Hh/none-Hi-Kn010G3/TR01L018-k0	4.72E-08
TR01H015-E22Hh/none-Hi-Kn010G3/TR01L005-k0	6.53E-08
TR01H052-E22Hh/none-Hi-Kn010G3/L0000-k0	6.27E-08
TR01H036-E22Hh/none-Hi-Kn010G3/L0000-k0	6.50E-08
TR01H067-E22Hh/none-Hi-Kn010G3/L0203-k0	4.79E-08
TR01H030-E22Hh/none-Hi-Kn010G3/L0000-k0	6.54E-08
TR01H015-E22Hh/none-Hi-Kn010G3/TR01L001-k0	6.56E-08
TR01H100-E22Hh/none-Hi-Kn010G3/L0011-k0	6.25E-08
TR01H029-E22Hh/none-Hi-Kn010G3/L0000-k0	6.70E-08
TR01H019-E22Hh/none-Hi-Kn010G3/L0000-k0	6.85E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0000-k0	7.37E-08
TR01H018-E22Hh/none-Hi-Kn010G3/L0000-k0	6.93E-08
TR01H027-E22Hh/none-Hi-Kn010G3/L0000-k0	6.95E-08
TR01H049-E22Hh/none-Hi-Kn010G3/L0000-k0	6.79E-08
TR01H066-E22Hh/none-Hi-Kn010G3/L0000-k0	6.02E-08
TR01H091-E22Hh/none-Hi-Kn010G3/L0011-k0	6.67E-08
rCE115H-E22Hh/none-Hi-Kn010G3/L0000-k0	8.00E-08
TR01H015-E22Hh/none-Hi-Kn010G3/TR01L002-k0	7.14E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0226-k0	8.01E-08
TR01H067-E22Hh/none-Hi-Kn010G3/TR01L018-k0	5.26E-08
TR01H093-E22Hh/none-Hi-Kn010G3/L0011-k0	6.60E-08
TR01H067-E22Hh/none-Hi-Kn010G3/L0215-k0	7.41E-08
TR01H015-E22Hh/none-Hi-Kn010G3/TR01L004-k0	7.34E-08
TR01H107-E22Hh/none-Hi-Kn010G3/L0011-k0	6.91E-08
TR01H105-E22Hh/none-Hi-Kn010G3/L0011-k0	6.95E-08
TR01H090-E22Hh/none-Hi-Kn010G3/L0011-k0	6.95E-08
TR01H108-E22Hh/none-Hi-Kn010G3/L0011-k0	6.98E-08
TR01H094-E22Hh/none-Hi-Kn010G3/L0011-k0	7.00E-08
TR01H109-E22Hh/none-Hi-Kn010G3/L0011-k0	7.06E-08
TR01H056-E22Hh/none-Hi-Kn010G3/L0000-k0	7.32E-08
TR01H031-E22Hh/none-Hi-Kn010G3/L0000-k0	7.55E-08
TR01H022-E22Hh/none-Hi-Kn010G3/L0000-k0	7.58E-08
TR01H092-E22Hh/none-Hi-Kn010G3/L0011-k0	7.21E-08
TR01H067-E22Hh/none-Hi-Kn010G3/L0000-k0	7.15E-08
TR01H067-E22Hh/none-Hi-Kn010G3/L0011-k0	7.18E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0248-k0	7.89E-08
TR01H009-E22Hh/none-Hi-Kn010G3/L0000-k0	3.15E-08
TR01H023-E22Hh/none-Hi-Kn010G3/L0000-k0	7.94E-08
TR01H096-E22Hh/none-Hi-Kn010G3/L0011-k0	7.47E-08
TR01H040-E22Hh/none-Hi-Kn010G3/TR01L007-k0	6.82E-08
TR01H054-E22Hh/none-Hi-Kn010G3/L0000-k0	7.79E-08
TR01H021-E22Hh/none-Hi-Kn010G3/L0000-k0	8.05E-08
TR01H103-E22Hh/none-Hi-Kn010G3/L0011-k0	7.72E-08
TR01H099-E22Hh/none-Hi-Kn010G3/L0011-k0	7.74E-08
rCE115H-E22Hh/none-Hi-Kn010G3/L0000vk1-k0	8.52E-08
TR01H101-E22Hh/none-Hi-Kn010G3/L0011-k0	7.87E-08
TR01H053-E22Hh/none-Hi-Kn010G3/L0000-k0	8.23E-08
TR01H035-E22Hh/none-Hi-Kn010G3/L0000-k0	8.49E-08
TR01H067-E22Hh/none-Hi-Kn010G3/TR01L015-k0	8.64E-08
TR01H104-E22Hh/none-Hi-Kn010G3/L0011-k0	8.26E-08
TR01H075-E22Hh/none-Hi-Kn010G3/L0000-k0	9.88E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0227-k0	1.01E-07
TR01H102-E22Hh/none-Hi-Kn010G3/L0011-k0	8.54E-08
TR01H034-E22Hh/none-Hi-Kn010G3/L0000-k0	9.11E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0222-k0	1.01E-07
rCE115H-E22Hh/rCE115L-k0/GL4-E22Hh/H0000-E22L	9.37E-08
TR01H015-E22Hh/none-Hi-Kn010G3/TR01L006-k0	9.30E-08
TR01H040-E22Hh/none-Hi-Kn010G3/L0246-k0	9.28E-08
TR01H097-E22Hh/none-Hi-Kn010G3/L0011-k0	8.76E-08
TR01H011-E22Hh/none-Hi-Kn010G3/L0000-k0	3.71E-08
TR01H010-E22Hh/none-Hi-Kn010G3/L0000-k0	3.73E-08
TR01H095-E22Hh/none-Hi-Kn010G3/L0011-k0	9.09E-08
TR01H082-E22Hh/none-Hi-Kn010G3/TR01L020-k0	1.06E-07
TR01H098-E22Hh/none-Hi-Kn010G3/L0011-k0	9.14E-08
TR01H082-E22Hh/none-Hi-Kn010G3/TR01L017-k0	1.09E-07
TR01H040-E22Hh/none-Hi-Kn010G3/L0247-k0	1.00E-07
rCE115H-E22Hh/none-Hi-Kn010G3/rCE115L-k0	1.24E-07
TR01H004-E22Hh/-Hi-Kn010G3/L0000-k0	1.35E-07
TR01H067-E22Hh/none-Hi-Kn010G3/L0222-k0	7.63E-08
rCE115H-E22Hh/-Hi-Kn010G3/rCE115L-k0	1.38E-07
TR01H008-E22Hh/none-Hi-Kn010G3/L0000-k0	4.22E-08
TR01H070-E22Hh/none-Hi-Kn010G3/L0000-k0	1.20E-07
TR01H106-E22Hh/none-Hi-Kn010G3/L0011-k0	1.00E-07
TR01H024-E22Hh/none-Hi-Kn010G3/L0000-k0	1.08E-07
CE115HA124-E22Hh/-Hi-Kn010G3/L0000-k0	1.43E-07
TR01H040-E22Hh/none-Hi-Kn010G3/L0249-k0	1.11E-07
TR01H082-E22Hh/none-Hi-Kn010G3/L0271-k0	6.82E-08
TR01H057-E22Hh/none-Hi-Kn010G3/L0000-k0	1.12E-07
TR01H058-E22Hh/none-Hi-Kn010G3/L0000-k0	1.15E-07
TR01H068-E22Hh/none-Hi-Kn010G3/L0000-k0	1.01E-07
TR01H082-E22Hh/none-Hi-Kn010G3/L0270-k0	7.42E-08
TR01H082-E22Hh/none-Hi-Kn010G3/L0272-k0	7.44E-08
hCE115HA-E22Hh/none-Hi-Kn010G3/L0000-k0	1.24E-07
TR01H082-E22Hh/none-Hi-Kn010G3/L0268-k0	1.36E-07
hCE115HAA-E22Hh/none-Hi-Kn010G3/L0000-k0	1.08E-07
TR01H067-E22Hh/none-Hi-Kn010G3/L0226-k0	1.32E-07
TR01H067-E22Hh/none-Hi-Kn010G3/L0248-k0	1.39E-07

(3-2) Modifying the affinity of a humanized anti-GPC3 antibody

First, point mutations were introduced into CDR1, CDR2, and CDR3 of the anti-human GPC3 bispecific antibody sequence produced in Reference Example 2, H0000-ERY27_HK (SEQ ID NO: 54), to prepare modified antibodies. Next, the affinity of these modified antibodies for soluble human GPC3 was determined. Combining sites that have an affinity-enhancing effect yielded modified antibodies having the affinities shown in Table 13.

Table 13

Antibody name	KD (Human GPC3)
H0610-G1dh/none-Hi-Kn010G3/L0000-k0	3.97E-09
H0610-G1dh/none-Hi-Kn010G3/L0222-k0	1.40E-13
H0610-G1dh/none-Hi-Kn010G3/L0258-k0	3.52E-13
GCH054-G1dh/none-Hi-Kn010G3/L0262-k0	5.25E-13
GCH060-G1dh/none-Hi-Kn010G3/L0222-k0	6.42E-13
H0610-G1dh/none-Hi-Kn010G3/L0246-k0	1.21E-12
GCH057-G1dh/none-Hi-Kn010G3/L0222-k0	1.85E-12
GCH054-G1dh/none-Hi-Kn010G3/L0249-k0	3.61E-12
GCH055-G1dh/none-Hi-Kn010G3/L0222-k0	3.90E-12
GCH094-G1dh/none-Hi-Kn010G3/L0246-k0	4.12E-12
H0610-G1dh/none-Hi-Kn010G3/L0249-k0	6.86E-12
H0610-G1dh/none-Hi-Kn010G3/TR01L017-k0	8.27E-12
H0610-G1dh/none-Hi-Kn010G3/L0265-k0	8.70E-12
H0610-G1dh/none-Hi-Kn010G3/L0261-k0	1.07E-11
GCH065-G1dh/none-Hi-Kn010G3/L0262-k0	1.18E-11
GCH056-G1dh/none-Hi-Kn010G3/L0262-k0	1.19E-11
H0610-G1dh/none-Hi-Kn010G3/L0268-k0	1.69E-11
H0610-G1dh/none-Hi-Kn010G3/TR01L020-k0	2.24E-11
GCH054-G1dh/none-Hi-Kn010G3/L0246-k0	3.15E-11
GCH054-G1dh/none-Hi-Kn010G3/L0222-k0	3.15E-11
GCH073-G1dh/none-Hi-Kn010G3/L0201-k0	3.50E-11
H0610-G1dh/none-Hi-Kn010G3/L0248-k0	5.55E-11
GCH065-G1dh/none-Hi-Kn010G3/L0201-k0	7.74E-11
H0610-G1dh/none-Hi-Kn010G3/L0226-k0	9.30E-11
H0610-G1dh/none-Hi-Kn010G3/L0093-k0	1.06E-10
GCH098-G1dh/none-Hi-Kn010G3/L0201-k0	1.11E-10
H0610-G1dh/none-Hi-Kn010G3/L0267-k0	1.79E-10
H0610-G1dh/none-Hi-Kn010G3/L0228-k0	2.02E-10
H0610-G1dh/none-Hi-Kn010G3/L0262-k0	2.11E-10
H0610-G1dh/none-Hi-Kn010G3/L0266-k0	2.13E-10
H0610-G1dh/none-Hi-Kn010G3/L0264-k0	2.19E-10
H0610-G1dh/none-Hi-Kn010G3/L0224-k0	2.43E-10
H0610-G1dh/none-Hi-Kn010G3/L0167-k0	2.11E-10
CF115HA251-E22Hn/L0000-k0/GL4-E22Hk/H0610-E22L	2.36E-10
TR01H015-E22Hn/L0000-k0/GL4-E22Hk/H0610-E22L	2.63E-10
CF115HA236-E22Hn/GLS3108-k0/GL4-E22Hk/H0610-E22L	2.67E-10
H0610-G1dh/none-Hi-Kn010G3/L0259-k0	3.34E-10
H0610-G1dh/none-Hi-Kn010G3/L0227-k0	4.08E-10
GCH065-G1dh/none-Hi-Kn010G3/L0272-k0	3.93E-10
H0610-G1dh/none-Hi-Kn010G3/L0269-k0	4.59E-10
H0610-G1dh/none-Hi-Kn010G3/L0223-k0	4.75E-10
TR01H002-E22Hn/GLS3108-k0/GL4-E22Hk/H0610-E22L	4.75E-10
GCH054-G1dh/none-Hi-Kn010G3/L0212-k0	5.17E-10
H0610-G1dh/none-Hi-Kn010G3/L0208-k0	5.30E-10
H0610-G1dh/none-Hi-Kn010G3/L0263-k0	5.64E-10
H0610-G1dh/none-Hi-Kn010G3/L0231-k0	5.89E-10
H0610-G1dh/none-Hi-Kn010G3/L0143-k0	5.73E-10
GCH055-G1dh/none-Hi-Kn010G3/L0212-k0	6.14E-10
H0610-G1dh/none-Hi-Kn010G3/L0211-k0	6.47E-10
H0610-G1dh/none-Hi-Kn010G3/L0238-k0	6.37E-10
H0610-G1dh/none-Hi-Kn010G3/L0214-k0	6.57E-10
H0610-G1dh/none-Hi-Kn010G3/L0243-k0	6.49E-10
GCH025-G1dh/none-Hi-Kn010G3/L0204-k0	6.70E-10
GCH054-G1dh/none-Hi-Kn010G3/TR01L016-k0	7.63E-10
H0610-G1dh/none-Hi-Kn010G3/L0168-k0	6.99E-10
GCH094-G1dh/none-Hi-Kn010G3/L0271-k0	6.92E-10
GCH054-G1dh/none-Hi-Kn010G3/TR01L019-k0	8.71E-10
H0610-G1dh/none-Hi-Kn010G3/L0234-k0	7.78E-10
GCH098-G1dh/none-Hi-Kn010G3/L0011-k0	8.02E-10
H0610-G1dh/none-Hi-Kn010G3/L0204-k0	7.27E-10
H0610-G1dh/none-Hi-Kn010G3/L0240-k0	8.48E-10
H0610-G1dh/none-Hi-Kn010G3/L0239-k0	8.74E-10
H0610-G1dh/none-Hi-Kn010G3/L0212-k0	9.94E-10
GCH065-G1dh/none-Hi-Kn010G3/L0011-k0	8.84E-10
H0610-G1dh/none-Hi-Kn010G3/L0200-k0	1.04E-09
H0610-G1dh/none-Hi-Kn010G3/L0124-k0	9.72E-10
GCH073-G1dh/none-Hi-Kn010G3/L0011-k0	9.10E-10
H0610-G1dh/none-Hi-Kn010G3/TR01L016-k0	1.08E-09
GCH054-G1dh/none-Hi-Kn010G3/L0201-k0	1.08E-09
H0610-G1dh/none-Hi-Kn010G3/L0090-k0	1.12E-09
H0610-G1dh/none-Hi-Kn010G3/L0209-k0	1.12E-09
H0610-G1dh/none-Hi-Kn010G3/L0201-k0	1.13E-09
H0610-G1dh/none-Hi-Kn010G3/L0161-k0	9.73E-10
H0610-G1dh/none-Hi-Kn010G3/L0206-k0	8.65E-10
H0610-G1dh/none-Hi-Kn010G3/L0186-k0	1.08E-09
H0610-G1dh/none-Hi-Kn010G3/TR01L019-k0	1.15E-09
H0610-G1dh/none-Hi-Kn010G3/L0085-k0	1.17E-09
GCH055-G1dh/none-Hi-Kn010G3/L0200-k0	1.13E-09
H0610-G1dh/none-Hi-Kn010G3/L0154-k0	1.01E-09
H0610-G1dh/none-Hi-Kn010G3/L0229-k0	1.20E-09
GCH054-G1dh/none-Hi-Kn010G3/L0200-k0	1.18E-09
GCH094-G1dh/none-Hi-Kn010G3/L0201-k0	1.17E-09

Antibody name	KD (Human GPC3)
H0610-G1dh/none-Hi-Kn010G3/L0000-k0	3.97E-09
H0610-G1dh/none-Hi-Kn010G3/L0205-k0	1.01E-09
GCH099-G1dh/none-Hi-Kn010G3/L0201-k0	1.29E-09
H0610-G1dh/none-Hi-Kn010G3/L0242-k0	1.19E-09
GCH056-G1dh/none-Hi-Kn010G3/L0201-k0	1.16E-09
H0610-G1dh/none-Hi-Kn010G3/L0213-k0	1.25E-09
GCH060-G1dh/none-Hi-Kn010G3/L0200-k0	1.34E-09
GCH065-G1dh/none-Hi-Kn010G3/L0000-k0	1.41E-09
GCH100-G1dh/none-Hi-Kn010G3/L0201-k0	1.37E-09
H0610-G1dh/none-Hi-Kn010G3/L0015-k0	1.31E-09
H0610-G1dh/none-Hi-Kn010G3/L0151-k0	1.25E-09
H0610-G1dh/none-Hi-Kn010G3/L0237-k0	1.31E-09
H0610-G1dh/none-Hi-Kn010G3/L0220-k0	1.36E-09
H0610-G1dh/none-Hi-Kn010G3/L0155-k0	1.28E-09
GCH055-G1dh/none-Hi-Kn010G3/L0215-k0	1.52E-09
H0610-G1dh/none-Hi-Kn010G3/L0202-k0	1.22E-09
GCH056-G1dh/none-Hi-Kn010G3/L0215-k0	1.59E-09
H0610-G1dh/none-Hi-Kn010G3/L0012-k0	1.55E-09
GCH054-G1dh/none-Hi-Kn010G3/L0215-k0	1.62E-09
H0610-G1dh/none-Hi-Kn010G3/L0215-k0	1.64E-09
GCH098-G1dh/none-Hi-Kn010G3/L0000-k0	1.77E-09
H0610-G1dh/none-Hi-Kn010G3/L0125-k0	1.71E-09
GCH057-G1dh/none-Hi-Kn010G3/L0215-k0	1.83E-09
H0610-G1dh/none-Hi-Kn010G3/L0217-k0	1.79E-09
H0610-G1dh/none-Hi-Kn010G3/L0014-k0	1.82E-09
H0610-G1dh/none-Hi-Kn010G3/L0216-k0	1.86E-09
TR01H015-E2702GsKsc/GCH019-E2704sEpsc/L0000-k0	1.64E-09
H0610-G1dh/none-Hi-Kn010G3/TR01L015-k0	2.16E-09
H0610-G1dh/none-Hi-Kn010G3/TR01L018-k0	2.17E-09
H0610-G1dh/none-Hi-Kn010G3/L0218-k0	1.99E-09
H0610-G1dh/none-Hi-Kn010G3/L0000vk1-k0	2.16E-09
H0610-G1dh/none-Hi-Kn010G3/L0160-k0	2.12E-09
H0610-G1dh/none-Hi-Kn010G3/L0047-k0	2.23E-09
GCH073-G1dh/none-Hi-Kn010G3/L0000-k0	2.00E-09
GCH054-G1dh/none-Hi-Kn010G3/TR01L015-k0	2.45E-09
H0610-G1dh/none-Hi-Kn010G3/L0219-k0	2.28E-09
GCH094-G1dh/none-Hi-Kn010G3/L0272-k0	2.10E-09
H0610-G1dh/none-Hi-Kn010G3/L0149-k0	2.18E-09
GCH054-G1dh/none-Hi-Kn010G3/TR01L018-k0	2.59E-09
GCH054-G1dh/none-Hi-Kn010G3/L0203-k0	2.48E-09
H0610-G1dh/none-Hi-Kn010G3/L0122-k0	2.42E-09
H0610-G1dh/none-Hi-Kn010G3/L0134-k0	2.53E-09
H0610-G1dh/none-Hi-Kn010G3/L0152-k0	2.36E-09
H0610-G1dh/none-Hi-Kn010G3/L0203-k0	2.11E-09
H0610-G1dh/none-Hi-Kn010G3/L0075-k0	2.85E-09
H0610-G1dh/none-Hi-Kn010G3/L0038-k0	2.75E-09
H0610-G1dh/none-Hi-Kn010G3/L0011-k0	2.76E-09
H0610-G1dh/none-Hi-Kn010G3/L0157-k0	2.60E-09
H0610-G1dh/none-Hi-Kn010G3/L0145-k0	2.66E-09
H0610-G1dh/none-Hi-Kn010G3/TR01L010-k0	2.92E-09
H0610-G1dh/none-Hi-Kn010G3/L0009-k0	2.99E-09
GCH099-G1dh/none-Hi-Kn010G3/L0011-k0	2.78E-09
H0610-G1dh/none-Hi-Kn010G3/L0006-k0	3.04E-09
H0610-G1dh/none-Hi-Kn010G3/L0173-k0	2.83E-09
H0610-G1dh/none-Hi-Kn010G3/L0127-k0	3.12E-09
H0610-G1dh/none-Hi-Kn010G3/L0082-k0	3.43E-09
H0610-G1dh/none-Hi-Kn010G3/L0064-k0	3.37E-09
H0610-G1dh/none-Hi-Kn010G3/L0008-k0	3.30E-09
H0610-G1dh/none-Hi-Kn010G3/L0013-k0	3.35E-09
H0610-G1dh/none-Hi-Kn010G3/L0140-k0	3.38E-09
H0610-G1dh/none-Hi-Kn010G3/L0039-k0	3.41E-09
GCH043-G1dh/none-Hi-Kn010G3/L0000-k0	3.74E-09
H0610-G1dh/none-Hi-Kn010G3/TR01L008-k0	3.48E-09
H0610-G1dh/none-Hi-Kn010G3/L0148-k0	3.28E-09
GCH062-G1dh/none-Hi-Kn010G3/L0000-k0	3.73E-09
H0610-G1dh/none-Hi-Kn010G3/L0163-k0	3.38E-09
H0610-G1dh/none-Hi-Kn010G3/L0233-k0	3.55E-09
H0610-G1dh/none-Hi-Kn010G3/L0230-k0	4.00E-09
GCH006-G1dh/none-Hi-Kn010G3/L0000-k0	4.06E-09
H0610-G1dh/none-Hi-Kn010G3/L0032-k0	3.72E-09
H0610-G1dh/none-Hi-Kn010G3/L0181-k0	3.51E-09
H0610-G1dh/none-Hi-Kn010G3/TR01L009-k0	3.81E-09
H0610-G1dh/none-Hi-Kn010G3/L0141-k0	3.86E-09
H0610-G1dh/none-Hi-Kn010G3/L0079-k0	4.23E-09
GCH094-G1dh/none-Hi-Kn010G3/L0270-k0	3.60E-09
GCH066-G1dh/none-Hi-Kn010G3/L0000-k0	4.29E-09
GCH064-G1dh/none-Hi-Kn010G3/L0000-k0	4.14E-09
H0610-G1dh/none-Hi-Kn010G3/L0066-k0	4.20E-09
GCH027-G1dh/none-Hi-Kn010G3/L0000-k0	3.83E-09
H0610-G1dh/none-Hi-Kn010G3/L0003-k0	4.01E-09
H0610-G1dh/none-Hi-Kn010G3/L0042-k0	4.27E-09
H0610-G1dh/none-Hi-Kn010G3/TR01L011-k0	4.02E-09

Antibody name	KD (Human GPC3)
H0610-G1dh/none-Hi-Kn010G3/L0000-k0	3.97E-08
GCH015-G1dh/none-Hi-Kn010G3/L0000-k0	4.14E-09
H0610-G1dh/none-Hi-Kn010G3/L0175-k0	3.84E-09
GCH100-G1dh/none-Hi-Kn010G3/L0011-k0	3.81E-09
GCH014-G1dh/none-Hi-Kn010G3/L0000-k0	4.20E-09
GCH053-G1dh/none-Hi-Kn010G3/L0000-k0	4.05E-09
rCE115HA-E22Hh/L0000-k0/GL4-E22Hh/H0000-E22L	4.28E-08
GCH094-G1dh/none-Hi-Kn010G3/L0011-k0	3.88E-09
GCH045-G1dh/none-Hi-Kn010G3/L0000-k0	4.63E-09
H0610-G1dh/none-Hi-Kn010G3/TR01L012-k0	4.25E-09
H0610-G1dh/none-Hi-Kn010G3/L0115-k0	4.34E-09
H0610-G1dh/none-Hi-Kn010G3/L0044-k0	4.57E-09
H0610-G1dh/none-Hi-Kn010G3/L0107-k0	4.38E-09
H0610-G1dh/none-Hi-Kn010G3/L0007-k0	4.39E-09
GCH013-G1dh/none-Hi-Kn010G3/L0000-k0	4.44E-09
H0610-G1dh/none-Hi-Kn010G3/L0045-k0	4.66E-09
GCH010-G1dh/none-Hi-Kn010G3/L0000-k0	4.12E-09
GCH040-G1dh/none-Hi-Kn010G3/L0000-k0	4.80E-09
H0610-G1dh/none-Hi-Kn010G3/L0002-k0	4.43E-09
H0610-G1dh/none-Hi-Kn010G3/L0016-k0	4.44E-09
GCH007-G1dh/none-Hi-Kn010G3/L0000-k0	4.93E-09
GCH042-G1dh/none-Hi-Kn010G3/L0000-k0	4.89E-09
rCE115H-E22Hh/rCE115L-k0/GL4-E22Hh/H0000-E22L	4.57E-09
H0610-G1dh/none-Hi-Kn010G3/L0120-k0	4.54E-09
H0610-G1dh/none-Hi-Kn010G3/L0065-k0	4.79E-09
GCH016-G1dh/none-Hi-Kn010G3/L0000-k0	4.59E-09
GCH035-G1dh/none-Hi-Kn010G3/L0000-k0	4.94E-09
GCH039-G1dh/none-Hi-Kn010G3/L0000-k0	4.95E-09
GCH099-G1dh/none-Hi-Kn010G3/L0000-k0	4.24E-09
H0610-G1dh/none-Hi-Kn010G3/L0041-k0	4.85E-09
GCH019-G1dh/none-Hi-Kn010G3/L0000-k0	4.38E-09
GCH029-G1dh/none-Hi-Kn010G3/L0000-k0	5.01E-09
GCH056-G1dh/none-Hi-Kn010G3/L0011-k0	4.31E-09
H0610-G1dh/none-Hi-Kn010G3/L0147-k0	4.38E-09
GCH034-G1dh/none-Hi-Kn010G3/L0000-k0	5.09E-09
GCH003-G1dh/none-Hi-Kn010G3/L0000-k0	5.20E-09
H0610-G1dh/none-Hi-Kn010G3/L0139-k0	4.78E-09
H0610-G1dh/none-Hi-Kn010G3/L0089-k0	5.24E-09
H0610-G1dh/none-Hi-Kn010G3/L0113-k0	4.82E-09
H0610-G1dh/none-Hi-Kn010G3/L0180-k0	4.48E-09
GCH005-G1dh/none-Hi-Kn010G3/L0000-k0	5.32E-09
GCH067-G1dh/none-Hi-Kn010G3/L0000-k0	5.24E-09
H0610-G1dh/none-Hi-Kn010G3/L0187-k0	4.92E-09
H0610-G1dh/none-Hi-Kn010G3/L0043-k0	5.14E-09
H0610-G1dh/none-Hi-Kn010G3/L0117-k0	4.92E-09
GCH061-G1dh/none-Hi-Kn010G3/L0000-k0	5.13E-09
GCH022-G1dh/none-Hi-Kn010G3/L0000-k0	4.92E-09
H0610-G1dh/none-Hi-Kn010G3/L0091-k0	5.43E-09
GCH023-G1dh/none-Hi-Kn010G3/L0000-k0	4.94E-09
H0610-G1dh/none-Hi-Kn010G3/L0062-k0	5.28E-09
H0610-G1dh/none-Hi-Kn010G3/L0136-k0	5.04E-09
H0610-G1dh/none-Hi-Kn010G3/TR01L003-k0	5.08E-09
H0610-G1dh/none-Hi-Kn010G3/L0069-k0	5.32E-09
H0610-G1dh/none-Hi-Kn010G3/L0123-k0	5.08E-09
GCH025-G1dh/none-Hi-Kn010G3/L0000-k0	5.05E-09
GCH100-G1dh/none-Hi-Kn010G3/L0000-k0	5.39E-09
H0610-G1dh/none-Hi-Kn010G3/L0046-k0	5.45E-09
H0610-G1dh/none-Hi-Kn010G3/L0144-k0	4.84E-09
GCH026-G1dh/none-Hi-Kn010G3/L0000-k0	5.17E-09
H0610-G1dh/none-Hi-Kn010G3/L0138-k0	5.24E-09
GCH058-G1dh/none-Hi-Kn010G3/L0000-k0	5.03E-09
H0610-G1dh/none-Hi-Kn010G3/L0129-k0	5.28E-09
GCH032-G1dh/none-Hi-Kn010G3/L0000-k0	5.74E-09
H0610-G1dh/none-Hi-Kn010G3/TR01L005-k0	5.37E-09
GCH012-G1dh/none-Hi-Kn010G3/L0000-k0	5.40E-09
GCH055-G1dh/none-Hi-Kn010G3/L0000-k0	5.60E-09
H0610-G1dh/none-Hi-Kn010G3/L0104-k0	5.90E-09
GCH059-G1dh/none-Hi-Kn010G3/L0000-k0	5.70E-09
GCH054-G1dh/none-Hi-Kn010G3/L0000-k0	5.30E-09
GCH008-G1dh/none-Hi-Kn010G3/L0000-k0	5.55E-09
H0610-G1dh/none-Hi-Kn010G3/L0232-k0	5.38E-09
H0610-G1dh/none-Hi-Kn010G3/L0126-k0	5.62E-09
GCH094-G1dh/none-Hi-Kn010G3/L0000-k0	5.89E-09
H0610-G1dh/none-Hi-Kn010G3/L0132-k0	5.65E-09
H0610-G1dh/none-Hi-Kn010G3/L0106-k0	5.66E-09
GCH054-G1dh/none-Hi-Kn010G3/L0011-k0	5.25E-09
H0610-G1dh/none-Hi-Kn010G3/L0109-k0	5.70E-09
H0610-G1dh/none-Hi-Kn010G3/L0063-k0	6.03E-09
GCH068-G1dh/none-Hi-Kn010G3/L0000-k0	6.23E-09
GCH057-G1dh/none-Hi-Kn010G3/L0000-k0	5.61E-09
H0610-G1dh/none-Hi-Kn010G3/L0137-k0	5.87E-09

(3-3) Modification of pI by point mutations

In commercial production of bispecific antibodies, a high level of purity is required. When using ion-exchange chromatography, modifying the molecular isoelectric point (pI) has been reported to be effective (PLoS One. 2013;8(2):e57479). Therefore, point mutations for pI modifications were introduced into CDR1, CDR2, and CDR3 of the humanized anti-human GPC3 antibody sequence produced in Reference Example 2, H0000-ERY27_HK (SEQ ID NO: 54), to prepare modified antibodies. Next, the affinity of these modified antibodies for soluble human GPC3 was determined.

As a result, amino acid modifications that can lower the pI while maintaining the affinity for human GPC3 were found to be amino acids at positions 19, 43, 53, and 61 according to Kabat numbering.

Combination of sites showing effects of maintaining the affinity for human GPC3 and lowering the pI yielded antibodies having the affinities and pI values shown in Table 14.

Table 14

Antibody name (homomeric antibody)	Calculated pI value (homomeric antibody)	Antibody name (single-arm antibody)	human GPC3 KD (single-arm antibody)	Mutation sites based on H0610-E2704sEpsc
H0610-E2704sEpsc/L0000-k0	7.8	H0610-G1dh/none-Hi-Kn010G3/L0000-k0	4.16E-09	-
GCH054-E2704sEpsc/L0011-k0	6.2	GCH054-G1dh/none-Hi-Kn010G3/L0011-k0	5.25E-09	K19T/Q43E/P52aG/K53E/G55P/Q61E
GCH065-E2704sEpsc/L0011-k0	6.4	GCH065-G1dh/none-Hi-Kn010G3/L0011-k0	8.84E-10	K19T/Q43E/P52aG/K53P/G55P/Q61E
GCH094-E2704sEpsc/L0011-k0	6.2	GCH094-G1dh/none-Hi-Kn010G3/L0011-k0	4.54E-09	K19T/I37V/P40A/Q43E/I48M/P52aG/K53E/G55P/Q61E

(3-4) Modifying the extracellular matrix-binding ability by point mutation

It has been reported that non-specific binding to the extracellular matrix (ECM) and such may have effects on pharmacokinetics (MAbs. 2012 Nov-Dec;4(6):753-60). Therefore, the ECM-binding ability of the modified antibodies obtained in the Reference Examples was determined by the method described in Reference Example 8. As a result, the humanized anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibody, GPC3_ERY27_hCE115 (SEQ ID NOs: 54, 55, and 56), were confirmed to have high ECM-binding abilities. Therefore, any of the point mutations examined in Reference Examples 3-1, 3-2, and 3-3 for the humanized anti-human CD3 ϵ chain antibody sequence hCE115HA-ERY27_HE (SEQ ID NO: 55) was investigated to be a combination for reducing the ECM-binding ability. As a result, amino acids at positions 11, 16, 52a, 53, 98, and 100 by Kabat numbering were found to contribute to the maintenance of affinity for CD3 ϵ and to have influence on the reduction of the ECM-binding ability, and antibodies with a reduced ECM-binding ability in comparison to that of an antibody variant of the humanized anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibody, GPC3_ERY27_hCE115, were obtained (Table 15).

Table 15

Antibody name	ECM binding ratio (standard = 1)
GPC3_ERY22_CE115 (rCE115H-E22Hh/rCE115L-k0/GL4-E22Hk/H0000-E22L)	4.0
GPC3_ERY27 (hCE115HA-E22Hh/L0000-k0/GL4-E22Hk/H0000-E22L)	50.9
CE115HA236-E22Hh/GLS3108-k0/GL4-E22Hk/H0610-E22L	429.9
CE115HA236-E22Hh/GLS3108-k0/GL4-E22Hk/H0000-E22L	414.8
CE115HA251-E22Hh/L0000-k0/GL4-E22Hk/H0000-E22L	346.9
CE115HA251-E22Hh/L0000-k0/GL4-E22Hk/H0610-E22L	334.4
TR01H002-E22Hh/GLS3108-k0/GL4-E22Hk/H0610-E22L	301.1
TR01H002-E22Hh/GLS3108-k0/GL4-E22Hk/H0000-E22L	216.9
TR01H015-E22Hh/L0000-k0/GL4-E22Hk/H0610-E22L	185.7
TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0208-k0	50.4
CE115HA122-E22Hh/L0000-k0/GL4-E22Hk/H0000-E22L	47.0
TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0211-k0	15.5
TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0206-k0	15.4
TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0209-k0	7.4
rCE115H-E22Hh/rCE115L-k0/GL4-E22Hk/H0610-E22L	4.6
TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0204-k0	4.4
TR01H067-E2702GsKsc/GCH054-E2704sEpsc/L0212-k0	3.3
TR01H113-E2702GsKsc/GCH065-E2704sEpsc/L0011-k0	2.5
TR01H082-E2702GsKsc/GCH065-E2704sEpsc/L0011-k0	1.7
TR01H113-E2702GsKsc/GCH094-E2704sEpsc/L0011-k0	1.6
rCE115H-E22Hh/rCE115L-k0/L0000-E22Hk/H0610-E22L	1.4
TR01H084-E2702GsKsc/GCH065-E2704sEpsc/L0011-k0	1.3
TR01H084-E2702GsKsc/GCH094-E2704sEpsc/L0011-k0	1.2
TR01H082-E2702GsKsc/GCH094-E2704sEpsc/L0201-k0	1.1
TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0000-k0	0.8
TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0201-k0	0.8
TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0203-k0	0.8
TR01H082-E2702GsKsc/GCH094-E2704sEpsc/L0011-k0	0.7
TR01H109-E2702GsKsc/GCH065-E2704sEpsc/L0011-k0	0.7
TR01H067-E2702GsKsc/GCH054-E2704sEpsc/L0222-k0	0.6
TR01H067-E2702GsKsc/GCH054-E2704sEpsc/L0201-k0	0.5
TR01H109-E2702GsKsc/GCH094-E2704sEpsc/L0011-k0	0.4
TR01H113-E2702sKsc/GCH065-E2704sEpsc/L0011-k0	0.3
MRAH-G1d/MRAL-k0(standard)	1

(3-5) Modifying the binding ability to the SuRe™ ligand by point mutations

- 5 An example where the binding of an antibody to Protein A depends on its variable region sequence (VH3) is known (J Biomol Tech. 2011 Jul; 22(2):50-2). In the Protein A purification of the humanized anti-human CD3ε chain and anti-human GPC3 bispecific antibody, removal of the homomeric anti-CD3 antibody is important for suppressing non-specific reactions *via* CD3. Therefore, it is considered desirable to suppress the binding of the homomeric
- 10 anti-CD3 antibody to Protein A. Presumably, the SuRe™ ligand will be used in commercial production, and thus point mutations for SuRe™ ligand binding were introduced into CDR2 of the humanized anti-CD3 antibody H-chain variants, TR01H082-E2702GsKsc and

TR01H084-E2702GsKsc (SEQ ID NO: 398 and 399), to prepare modified antibodies. The binding ability of these modified antibodies to the SuRe™ ligand was determined by the method described in Reference Example 9. As a result, amino acids at positions 19, 57, and 59 by Kabat numbering were found to contribute to the maintenance of the affinity for CD3ε and to have influence on the Sure™ ligand-binding ability, and antibodies with a reduced Sure™ ligand-binding ability in comparison to that of TR01H082-E2702GsKsc/L0011-k0 (SEQ ID NOs: 398 and 410) or TR01H084-E2702GsKsc/L0011-k0 (SEQ ID NOs: 399 and 410) were obtained (Table 16).

Table 16

Antibody name	SuRe™ binding (RU)	Mutation sites based on CE115HA000
TR01H084-E2702GsKsc/L0011-k0	5065.8	R16G/A52aD/N53Q/D72A/L78I/G98A/Y100G/A102I
TR01H082-E2702GsKsc/L0011-k0	4469.2	V11L/A52aD/N53Q/G98A/Y100G
TR01H090-E2702GsKsc/L0011-k0	3606.3	V11L/R16G/A52aD/N53Q/G98A/Y100G
TR01H093-E2702GsKsc/L0011-k0	2459.7	V11L/A52aD/N53Q/K64Q/G98A/Y100G
TR01H094-E2702GsKsc/L0011-k0	2351.9	V11L/A52aD/N53Q/K64S/G98A/Y100G
TR01H114-E2702GsKsc/L0011-k0	1485.5	R16G/A52aD/N53Q/T57S/D72A/L78I/G98A/Y100G/A102I
TR01H092-E2702GsKsc/L0011-k0	1159.5	V11L/A52aD/N53Q/K64A/G98A/Y100G
TR01H100-E2702GsKsc/L0011-k0	383.0	V11L/A52aD/N53Q/T57S/G98A/Y100G
TR01H111-E2702GsKsc/L0011-k0	50.7	R16G/R19K/A52aD/N53Q/D72A/L78I/G98A/Y100G/A102I
TR01H110-E2702GsKsc/L0011-k0	29.5	R19K/A52aD/N53Q/G98A/Y100G
TR01H091-E2702GsKsc/L0011-k0	27.5	V11L/R19K/A52aD/N53Q/G98A/Y100G
TR01H091-E2702GsKsc/L0011-k0	15.0	V11L/R19K/A52aD/N53Q/G98A/Y100G
TR01H112-E2702GsKsc/L0011-k0	8.8	R16G/A52aD/N53Q/T57Q/D72A/L78I/G98A/Y100G/A102I
TR01H113-E2702GsKsc/L0011-k0	7.0	R16G/A52aD/N53Q/Y59V/D72A/L78I/G98A/Y100G/A102I
TR01H096-E2702GsKsc/L0011-k0	2.7	V11L/A52aD/N53Q/T57G/G98A/Y100G
TR01H109-E2702GsKsc/L0011-k0	2.2	V11L/A52aD/N53Q/Y59V/G98A/Y100G
TR01H098-E2702GsKsc/L0011-k0	1.6	V11L/A52aD/N53Q/T57P/G98A/Y100G
TR01H107-E2702GsKsc/L0011-k0	1.4	V11L/A52aD/N53Q/Y59Q/G98A/Y100G
TR01H103-E2702GsKsc/L0011-k0	1.4	V11L/A52aD/N53Q/Y59G/G98A/Y100G
TR01H104-E2702GsKsc/L0011-k0	1.0	V11L/A52aD/N53Q/Y59I/G98A/Y100G
TR01H105-E2702GsKsc/L0011-k0	0.8	V11L/A52aD/N53Q/Y59L/G98A/Y100G
TR01H099-E2702GsKsc/L0011-k0	0.6	V11L/A52aD/N53Q/T57Q/G98A/Y100G
TR01H102-E2702GsKsc/L0011-k0	0.5	V11L/A52aD/N53Q/Y59F/G98A/Y100G
TR01H101-E2702GsKsc/L0011-k0	0.5	V11L/A52aD/N53Q/T57V/G98A/Y100G
TR01H108-E2702GsKsc/L0011-k0	0.4	V11L/A52aD/N53Q/Y59T/G98A/Y100G
TR01H097-E2702GsKsc/L0011-k0	0.1	V11L/A52aD/N53Q/T57L/G98A/Y100G
TR01H106-E2702GsKsc/L0011-k0	0.0	V11L/A52aD/N53Q/Y59P/G98A/Y100G
TR01H095-E2702GsKsc/L0011-k0	-0.2	V11L/A52aD/N53Q/T57F/G98A/Y100G

(3-6) Production of optimized bispecific antibodies by combining point mutations that lead to improvement of various properties

Optimized modified antibodies can be produced by combining the point mutations that lead to improvement of various properties as described in Reference Examples 3-1 to 3-5. As examples of such modified antibodies, the antibodies described in Table 17 were produced, and they were subjected to the T-cell-dependent cellular cytotoxicity (TDCC) evaluation using methods similar to those of Reference Example 1. The results are shown in Figs. 15 to 20. As a result, optimized humanized anti-human CD3ε chain and anti-human GPC3 bispecific

antibodies showing a T-cell-dependent cellular cytotoxicity equivalent to or greater than that of GPC3_ERY22_rCE115 prior to humanization were obtained.

Table 17

Sample number in TDCC assay, and abbreviation of antibody name in drug efficacy evaluation	
Sample number in TDCC assay	Abbreviation of antibody name
1	GPC3_ERY22_CE115 (rCE115H-E22Hh/rCE115L-k0/GL4-E22Hk/H0000-E22L)
2	GPC3_ERY27 (hCE115HA-E22Hh/L0000-k0/GL4-E22Hk/H0000-E22L)
3	CE115HA251-E22Hh/L0000-k0/GL4-E22Hk/H0000-E22L
4	CE115HA236-E22Hh/GLS3108-k0/GL4-E22Hk/H0000-E22L
5	TR01H002-E22Hh/GLS3108-k0/GL4-E22Hk/H0000-E22L
6	CE115HA122-E22Hh/L0000-k0/GL4-E22Hk/H0000-E22L
7	rCE115H-E22Hh/rCE115L-k0/L0000-E22Hk/H0610-E22L
8	rCE115H-E22Hh/rCE115L-k0/GL4-E22Hk/H0610-E22L
13	TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0000-k0
14	TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0201-k0
15	TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0203-k0
16	TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0204-k0
17	TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0206-k0
18	TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0208-k0
19	TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0209-k0
20	TR01H040-E2702GsKsc/H0610-E2704sEpsc/L0211-k0
21	rCE115H-E2702GsKsc/H0610-E2704sEpsc/L0000-k0
22	TR01H061-E2702GsKsc/H0610-E2704sEpsc/L0000-k0
23	TR01H068-E2702GsKsc/H0610-E2704sEpsc/L0000-k0
24	TR01H071-E2702GsKsc/H0610-E2704sEpsc/L0000-k0
25	TR01H067-E2702GsKsc/GCH054-E2704sEpsc/L0201-k0
26	TR01H067-E2702GsKsc/GCH054-E2704sEpsc/L0212-k0
27	TR01H067-E2702GsKsc/GCH054-E2704sEpsc/L0222-k0
28	TR01H067-E2702GsKsc/GCH054-E2704sEpsc/L0000-k0
29	TR01H082-E2702GsKsc/GCH094-E2704sEpsc/L0201-k0
30	TR01H082-E2702GsKsc/GCH094-E2704sEpsc/L0011-k0
31	TR01H084-E2702GsKsc/GCH094-E2704sEpsc/L0011-k0
32	TR01H084-E2702GsKsc/GCH065-E2704sEpsc/L0011-k0
33	TR01H082-E2702GsKsc/GCH065-E2704sEpsc/L0011-k0
34	TR01H109-E2702GsKsc/GCH094-E2704sEpsc/L0011-k0
35	TR01H109-E2702GsKsc/GCH065-E2704sEpsc/L0011-k0
36	TR01H113-E2702GsKsc/GCH094-E2704sEpsc/L0011-k0
37	TR01H113-E2702GsKsc/GCH065-E2704sEpsc/L0011-k0
38	TR01H113-E2702sKsc/GCH065-E2704sEpsc/L0011-k0

Reference Examples 3-1 to 3-6 showed that the following amino acid residues, for example, are important for maintaining the properties of the optimized anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibodies showing a T-cell-dependent cellular cytotoxicity equivalent to or greater than that of GPC3_ERY22_rCE115 prior to humanization.

In anti-human CD3 ϵ chain antibodies, the examples are Leu at position 11, Gly at position 16, Asp at position 52a, Gln at position 53, Ala at position 72, Ile at position 78, Ala at position 98, Gly at position 100, and Ile at position 102. In anti-human GPC3 antibodies, the

examples are Thr at position 19, Glu at position 43, Gly at position 52a, Pro or Glu at position 53, Pro at position 55, and Glu at position 61. Furthermore, in common antibody L chains, the examples are Pro at position 25, Pro at position 27a, Pro at position 27b, Ile at position 33, Gln at position 34, Arg or Trp at position 56, and Tyr at position 89. (All positions are indicated by
5 Kabat numbering).

[Reference Example 4] Evaluation of the *in vivo* efficacy

Some of the above-described antibodies were evaluated for their *in vivo* efficacy using tumor-bearing models.

10 Evaluation of the *in vivo* efficacy was carried out on representative antibodies from among those shown in Table 17, which have been confirmed to have cytotoxic activities from the *in vitro* assay described in Reference Example 3-6. In the *in vivo* efficacy evaluation, any influence caused by differences in the microenvironment due to tumor aggregate formation on the evaluation results was taken into consideration. Therefore, two types of human cancer cell
15 lines having different sensitivities to the antibody drug efficacy, i.e., PC-10 and NCI-H446, were used for the evaluation, even though the GPC3 expression levels of these cell lines were nearly equal. The cell lines were transplanted into the NOD scid mice, and the NOD scid mice with confirmed tumor establishment were subjected to injection of T cells grown by *in vitro* culturing of human PBMCs. The mice (referred to as a T-cell injected model) were treated by
20 administration of the optimized anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibodies.

More specifically, in drug efficacy tests of the optimized anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibodies using the PC-10 T-cell injected model, the tests below were performed. T cells were expansively cultured using PBMCs separated from blood
25 collected from healthy volunteers and T cell activation/ expansion kit/ human (MACS Miltenyi biotec). The human cancer cell line PC-10 (1×10^7 cells) was mixed with MatrigelTM Basement Membrane Matrix (BD), and transplanted to the inguinal subcutaneous region of NOD scid mice (CLEA Japan, female, 6W). The day of transplantation was defined as day 0. On the day before transplantation, the anti-asialo-GM1 antibody (Wako Pure Chemicals) was administered
30 intraperitoneally to the mice at 0.2 mg/mouse. On days 13 to 15 after the transplantation, the mice were separated into groups according to their body weight and tumor size, and the anti-asialo-GM1 antibody was administered again intraperitoneally to the mice at 0.2 mg/mouse. On the following day, T cells obtained by the aforementioned expansive culturing were transplanted intraperitoneally at 3×10^7 cells/mouse. Four hours after T-cell transplantation, the
35 optimized anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibodies were administered intravenously through the caudate vein at 1 mg/kg. The optimized anti-human

CD3 ϵ chain and anti-human GPC3 bispecific antibodies were administered only once.

As a result, anti-tumor activities were more clearly observed in the optimized anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibody-administered group than in the solvent-administered group (Fig. 21a, b).

5 Drug efficacy tests for the optimized anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibodies on the NCI-H446 T-cell injected model were performed by similar methods. The optimized anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibodies were administered once intravenously through the caudate vein at 5 mg/kg against NCI-H446.

10 As a result, anti-tumor activities were more clearly observed in the optimized anti-human CD3 ϵ chain and anti-human GPC3 bispecific antibody-administered group than in the solvent-administered group (Fig. 22a, b).

[Reference Example 5] Production of antibody expression vectors, and antibody expression and purification

15 Amino acid substitutions were introduced by methods known to those skilled in the art such as using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), PCR, or the In-fusion Advantage PCR cloning kit (TAKARA) to construct expression vectors. Nucleotide sequences of the obtained expression vectors were determined by a method known to those skilled in the art. The produced plasmids were transiently introduced into cells of the human embryonic kidney
20 cancer-derived cell line HEK293H (Invitrogen) or FreeStyle293 (Invitrogen) to express antibodies. From the obtained culture supernatants, antibodies were purified using the rProtein A SepharoseTM Fast Flow (GE Healthcare) by a method known to those skilled in the art. Absorbance at 280 nm of the purified antibody solutions was measured using a spectrophotometer, and antibody concentrations were calculated from the determined values
25 using an absorption coefficient calculated by the PACE method (Protein Science 1995; 4: 2411-2423).

[Reference Example 6] The ADCC activity of each test antibody using human peripheral blood mononuclear cells as the effector cell

30 The ADCC activity of each test antibody was determined according to the method below.

Human peripheral blood mononuclear cells (hereinafter referred to as human PBMC) were used as the effector cell to measure the ADCC activity of each test antibody as below.

35 (1) Preparation of a human PBMC solution

From a healthy volunteer (adult male) of Chugai Pharmaceutical Co. Ltd., 50 mL of

peripheral blood was collected using a syringe preloaded with 200 μL of a 1000 unit/mL heparin solution (Novo-Heparin Injection 5000 units, Novo Nordisk). The peripheral blood was diluted two-fold with PBS(-), divided into four aliquots, and added into a Leucosep lymphocyte separation tube (Greiner Bio-one) that had been loaded with 15 mL of Ficoll-Paque PLUS and subjected to centrifugation in advance. This separation tube containing aliquots of peripheral blood was centrifuged at 2150 rpm for ten minutes at room temperature, and then the mononuclear cell fraction was collected. The cells in each fraction were washed once with Dulbecco's Modified Eagle's Medium (SIGMA) containing 10% FBS (hereinafter referred to as 10% FBS/D-MEM) and then suspended in 10% FBS/D-MEM at a cell density of 5×10^6 cells/mL. After incubation in an incubator at 37°C for one hour, the cells were washed once with 10% FBS/D-MEM, and the cells were suspended in 10% FBS/D-MEM to produce a cell density of 2×10^5 cells/mL. The cell suspension was subjected to the experiment below as the target cell.

(2) Chromium release assay (ADCC activity)

The ADCC activity was evaluated from the specific chromium release rate according to the chromium release method. First, antibody solutions prepared at each concentration (0, 0.004, 0.04, 0.4, 4, and 40 $\mu\text{g/mL}$) were added to a 96-well U-bottomed plate at 50 μL per well. Next, the target cells were seeded at 50 μL per well (1×10^4 cells/well), and this was allowed to stand at room temperature for 15 minutes. The human PBMC solution prepared in (1) was added at 100 μL per well (5×10^5 cells/well), and the plate was left to stand in a 5% carbon dioxide gas incubator at 37°C for four hours, followed by centrifugation. The radioactivity of 100 μL of culture supernatant in each well of the plate was measured using a gamma counter. The specific chromium release rate was determined based on the following equation:

$$\text{Specific chromium release rate (\%)} = (A - C) \times 100 / (B - C)$$

In this equation, A represents the mean value of radioactivity (cpm) of 100 μL of culture supernatant in each well; B represents the mean value of radioactivity (cpm) of 100 μL of culture supernatant in the well where 100 μL of a 2% aqueous NP-40 solution (Nonidet P-40, Nacalai Tesque) and 50 μL of 10% FBS/D-MEM had been added to the target cells; and C represents the mean value of radioactivity (cpm) of 100 μL of culture supernatant in the well where 150 μL of 10% FBS/D-MEM had been added to the target cells. The examinations were performed in triplicate and the mean values and standard deviations of the specific chromium release rates (%) in the above-mentioned examination reflecting the ADCC activity were calculated for each of the test antibodies.

[Reference Example 7] Assessment of T_m of the modified antibodies by differential scanning

fluorimetry

In this examination, the T_m (thermal denaturation temperature) value of the modified antibodies was assessed by differential scanning fluorimetry using Rotor-Gene Q (QIAGEN). It has been reported that this method has a favorable correlation with T_m assessment using a differential scanning calorimeter widely known as a method for evaluating thermal stability of antibodies (Journal of Pharmaceutical Science 2010; 4: 1707-1720).

The 5000x-concentrated SYPROTM orange (Molecular Probes) was diluted with PBS (Sigma), and then admixed with the antibody solutions to prepare measurement samples. Twenty- μ L aliquots of each sample were placed into measurement tubes, and the temperature was increased from 30 °C to 99 °C at a temperature elevation rate of 240°C/hr. Changes in fluorescence accompanying the temperature elevation were detected at 470 nm (excitation wavelength) / 555 nm (fluorescence wavelength).

The data were analyzed using the Rotor-Gene Q Series software (QIAGEN) to calculate the temperature at which fluorescence transition was observed, and this temperature was defined as the T_m .

[Reference Example 8] Assessment of the ECM-binding ability

The assessment was carried out according to the method described in WO2012093704. Specifically, BD Matrigel (BD Biosciences, #356237) was prepared at 2 mg/mL using TBS (Takara, #T903), and this was dispensed into a 96-well measurement plate (Meso Scale Discovery, #L15XB-3(High Bind)) at 5 μ L per well and then allowed to stand overnight in a cool place. Then, 150 μ L of an ECL blocking buffer (PBS containing 0.05% Tween20, 0.5% BSA, and 0.01% sodium azide) was dispensed into each well of the plate, and this was allowed to stand at room temperature for two hours or more.

A goat anti-human IgG(γ) (Invitrogen, #628400) was ruthenium-labeled with MSD SULFO-TAG NHS Ester (Meso Scale Discovery, #R91AN-2) by following the attached instructions. This was diluted in an ECL dilution buffer (PBS containing 0.01% Tween20, 0.1% BSA, and 0.01% sodium azide) to have a final concentration of 2 μ g/mL. Furthermore, the standard antibody and the test antibodies were diluted in PBS-T (PBS containing 0.05% Tween 20 and 0.01% sodium azide) to have a final concentration of 3 μ g/mL.

To a 96-well reaction plate (Thermo scientific, Nunc #145399), 10 μ L of the ECL dilution buffer, 20 μ L of the standard antibody and test antibody (3 μ g/mL), and 30 μ L of the ruthenium-labeled antibody (2 μ g/mL) were added sequentially, and this was allowed to react for one hour at room temperature with stirring in the dark.

The ECL blocking buffer was removed from the 96-well measurement plate by tilting, 50 μ L of the sample solution from the 96-well reaction plate was added, and this was allowed to

stand in the dark at room temperature for one hour. This was followed by removal of the sample solution from the 96-well measurement plate by tilting, and immediately after addition of 150 μ L of 2x T buffer (4x MSD Read Buffer T (Meso Scale Discovery) diluted two-fold using the ECL dilution buffer), ECL measurements were taken. SECTOR Imager 2400 (Meso Scale
5 Discovery) was used for taking the measurements.

Analyses were carried out by dividing the fluorescence intensity of the test antibody by the fluorescence intensity of the standard antibody to calculate and compare the intensities by defining the value for the standard antibody to be 1.

10 [Reference Example 9] Assessment of the SuReTM ligand-binding ability

The ability to bind to the SuReTM ligand was assessed by using BiacoreTM-T200 (GE Healthcare Japan). HBS-EP+ (GE Healthcare Japan) was used for the running buffer, and an amine coupling kit (GE Healthcare Japan) was used to covalently bind the Mab Select SuReTM Ligand (GE Healthcare Japan) to the CM5 chip (carboxymethyl dextran-coated chip). The
15 antibody used as the analyte was prepared at 5 μ g/mL using HBS-EP+. Measurements were carried out by first injecting the 5- μ g/mL antibody solution at a flow rate of 10 μ L/min for 3 minutes, then switching to HBS-EP+, and measuring the response (RU) after allowing the flow to continue for 0.5 minutes. After completion of the measurements, the sensor chip was
20 regenerated by washing with 10 mM Gly-HCl at pH 1.5. For the control flow cell, a similar experiment was performed without covalent bonding of the ligand to the chip, and the affinity for the SuReTM ligand was analyzed by taking the difference between the responses (RU).

Sequences corresponding to the SEQ ID NOs mentioned in the Reference Examples are shown in the Table below.

Table 18

SEQ ID NO: Name	SEQ ID NO: Name	SEQ ID NO: Name
1 GPC3 nucleotide sequence (NM_001164617.1)	81 TR01H018	154 TR01H099
2 GPC3 amino acid sequence (NP_001158089.1)	82 TR01H019	155 TR01H100
3 Signal sequence	83 TR01H020	156 TR01H101
4 T cell receptor α -chain peptide (CAA26636.1)	84 TR01H021	157 TR01H102
5 T cell receptor β -chain peptide (C25777)	85 TR01H022	158 TR01H103
6 T cell receptor γ 1-chain peptide (A26659)	86 TR01H023	159 TR01H104
7 T cell receptor γ 2-chain peptide (AAB63312.1)	87 TR01H024	160 TR01H105
8 T cell receptor δ -chain peptide (AAA61033.1)	88 TR01H025	161 TR01H106
9 CD3 γ -chain nucleotide (NM_000073.2)	89 TR01H026	162 TR01H107
10 CD3 δ -chain nucleotide (NM_000732.4)	90 TR01H027	163 TR01H108
11 CD3 ϵ -chain nucleotide (NM_000733.3)	91 TR01H028	164 TR01H109
12 CD3 γ -chain peptide (NP_000064.1)	92 TR01H029	165 TR01H110
13 CD3 δ -chain peptide (NP_000723.1)	93 TR01H030	166 TR01H111
14 CD3 ϵ -chain peptide (NP_000724.1)	94 TR01H031	167 TR01H112
15~22 Peptide linker	95 TR01H032	168 TR01H113
23 Human Cy1	96 TR01H033	169 TR01H114
24 Human Cy2	97 TR01H034	170 GCH003
25 Human Cy3	98 TR01H035	171 GCH005
26 Human Cy4	99 TR01H036	172 GCH006
27 Fc γ RI nucleotide (NM_000566.3)	100 TR01H037	173 GCH007
28 Fc γ RI peptide (NP_000557.1)	101 TR01H038	174 GCH008
29 Fc γ RIIA nucleotide (BC020823.1)	102 TR01H039	175 GCH010
30 Fc γ RIIA peptide (AAH20823.1)	103 TR01H040	176 GCH012
31 Fc γ RIIB nucleotide (BC146678.1)	104 TR01H041	177 GCH013
32 Fc γ RIIB peptide (AAI46679.1)	105 TR01H042	178 GCH014
33 Fc γ RIIIA nucleotide (BC033678.1)	106 TR01H043	179 GCH015
34 Fc γ RIIIA peptide (AAH33678.1)	107 TR01H044	180 GCH016
35 Fc γ RIIIB nucleotide (BC128562.1)	108 TR01H045	181 GCH019
36 Fc γ RIIIB peptide (AAI28563.1)	109 TR01H046	182 GCH022
37 Fc region (addition of A to the N terminus of RefSeq accession number AAC82527.1)	110 TR01H047	183 GCH023
38 Fc region (addition of A to the N terminus of RefSeq accession number AAB59393.1)	111 TR01H048	184 GCH025
39 Fc region (addition of A to the N terminus of RefSeq accession number AAB59394.1)	112 TR01H049	185 GCH026
40 H0000, GPC3 H-chain variable region	113 TR01H050	186 GCH027
41 GL4, GPC3 L-chain variable region	114 TR01H051	187 GCH029
42 rCE115H, CE115 H-chain variable region	115 TR01H052	188 GCH032
43 rCE115L, CE115 L-chain variable region	116 TR01H053	189 GCH034
44 G1dh	117 TR01H054	190 GCH035
45 ERY22_Hk	118 TR01H055	191 GCH039
46 ERY22_Hh	119 TR01H056	192 GCH040
47 GL4-ERY22_Hk	120 TR01H057	193 GCH042
48 H0000-ERY22_L	121 TR01H058	194 GCH043
49 rCE115H-ERY22_Hh	122 TR01H061	195 GCH045
50 rCE115L-k0	123 TR01H062	196 GCH053
51 hCE115HL (Heavy chain of humanized CE115)	124 TR01H063	197 GCH054
52 hCE115HA (Heavy chain of humanized CE115)	125 TR01H064	198 GCH055
53 L0000 (Light chain of humanized CE115)	126 TR01H065	199 GCH056
54 H0000-ERY27_HK	127 TR01H066	200 GCH057
55 hCE115HA-ERY27_HE	128 TR01H067	201 GCH059
56 L0000-k0	129 TR01H068	202 GCH060
57 E22Hh	130 TR01H069	203 GCH061
58 E22Hk	131 TR01H070	204 GCH062
59 Hi-Kn010G3	132 TR01H071	205 GCH064
60 E2702GsKsc	133 TR01H072	206 GCH065
61 E2704sEpsc	134 TR01H073	207 GCH066
62 E2702sKsc	135 TR01H074	208 GCH067
63 k0	136 TR01H075	209 GCH068
64 CE115HA177	137 TR01H076	210 GCH073
65 CE115HA178	138 TR01H077	211 GCH094
66 CE115HA179	139 TR01H079	212 GCH098
67 CE115HA180	140 TR01H080	213 GCH099
68 hCE115HAa	141 TR01H081	214 GCH100
69 TR01H006	142 TR01H082	215 H0610
70 TR01H007	143 TR01H083	216 L0000vk1
71 TR01H008	144 TR01H084	217 L0002
72 TR01H009	145 TR01H090	218 L0003
73 TR01H010	146 TR01H091	219 L0006
74 TR01H011	147 TR01H092	220 L0007
75 TR01H012	148 TR01H093	221 L0008
76 TR01H013	149 TR01H094	222 L0009
77 TR01H014	150 TR01H095	223 L0011
78 TR01H015	151 TR01H096	224 L0012
79 TR01H016	152 TR01H097	225 L0013
80 TR01H017	153 TR01H098	226 L0014

SEQ ID NO: Name	SEQ ID NO: Name	SEQ ID NO: Name
227 L0015	300 L0202	373 TR01L015
228 L0016	301 L0203	374 TR01L016
229 L0032	302 L0204	375 TR01L017
230 L0038	303 L0205	376 TR01L018
231 L0039	304 L0206	377 TR01L019
232 L0041	305 L0207	378 TR01L020
233 L0042	306 L0208	379 TR01L023
234 L0043	307 L0209	380 TR01L024
235 L0044	308 L0210	381 CE115HA122-E22Hh
236 L0045	309 L0211	382 CE115HA236-E22Hh
237 L0046	310 L0212	383 CE115HA251-E22Hh
238 L0047	311 L0213	384 GCH054-E2704sEpsc
239 L0062	312 L0214	385 GCH065-E2704sEpsc
240 L0063	313 L0215	386 GCH094-E2704sEpsc
241 L0064	314 L0216	387 H0610-E2704sEpsc
242 L0065	315 L0217	388 hCE115HA-E22Hh
243 L0066	316 L0218	389 rCE115H-E22Hh
244 L0069	317 L0219	390 rCE115H-E2702GsKsc
245 L0075	318 L0220	391 TR01H002-E22Hh
246 L0079	319 L0222	392 TR01H015-E22Hh
247 L0082	320 L0223	393 TR01H040-E2702GsKsc
248 L0085	321 L0224	394 TR01H061-E2702GsKsc
249 L0089	322 L0226	395 TR01H067-E2702GsKsc
250 L0090	323 L0227	396 TR01H068-E2702GsKsc
251 L0091	324 L0228	397 TR01H071-E2702GsKsc
252 L0093	325 L0229	398 TR01H082-E2702GsKsc
253 L0104	326 L0230	399 TR01H084-E2702GsKsc
254 L0106	327 L0231	400 TR01H109-E2702GsKsc
255 L0107	328 L0232	401 TR01H113-E2702GsKsc
256 L0109	329 L0233	402 TR01H113-E2702sKsc
257 L0113	330 L0234	403 GL4-E22Hk
258 L0115	331 L0235	404 L0000-E22Hk
259 L0117	332 L0236	405 H0000-E22L
260 L0120	333 L0237	406 H0610-E22L
261 L0122	334 L0238	407 rCE115L-k0
262 L0123	335 L0239	408 GLS3108-k0
263 L0124	336 L0240	409 L0000-k0
264 L0125	337 L0241	410 L0011-k0
265 L0126	338 L0242	411 L0201-k0
266 L0127	339 L0243	412 L0203-k0
267 L0129	340 L0246	413 L0204-k0
268 L0132	341 L0247	414 L0206-k0
269 L0134	342 L0248	415 L0208-k0
270 L0136	343 L0249	416 L0209-k0
271 L0137	344 L0250	417 L0211-k0
272 L0138	345 L0258	418 L0212-k0
273 L0139	346 L0259	419 L0222-k0
274 L0140	347 L0260	420 TR01H001
275 L0141	348 L0261	421 TR01H002
276 L0143	349 L0262	422 TR01H003
277 L0144	350 L0263	423 TR01H004
278 L0145	351 L0264	424 rCE115H
279 L0147	352 L0265	425 CE115HA121
280 L0148	353 L0266	426 CE115HA122
281 L0149	354 L0267	427 CE115HA124
282 L0151	355 L0268	428 CE115HA192
283 L0152	356 L0269	429 CE115HA236
284 L0154	357 L0270	430 CE115HA251
285 L0155	358 L0271	431 CE115HA252
286 L0157	359 L0272	432 E22L
287 L0160	360 TR01L001	
288 L0161	361 TR01L002	
289 L0163	362 TR01L003	
290 L0167	363 TR01L004	
291 L0168	364 TR01L005	
292 L0173	365 TR01L006	
293 L0175	366 TR01L007	
294 L0180	367 TR01L008	
295 L0181	368 TR01L009	
296 L0186	369 TR01L010	
297 L0187	370 TR01L011	
298 L0200	371 TR01L012	
299 L0201	372 TR01L013	

[Reference Example 10] Production of human CD3 gene-substituted mice

(1) Construction of a mouse Cd3 gene region modification vector (Fig. 26A)

A bacterial artificial chromosome (BAC) clone was used, into which a genomic region where the mouse CD3 ϵ , CD3 δ , and CD3 γ genes are positioned had been cloned. A loxP sequence was inserted at the position approximately 3.5 kb 5' upstream of the gene region encoding mouse Cd3 ϵ in this BAC, and the genome region further upstream was removed leaving approximately 3.1 kb. At that time, the loxP sequence was introduced together with neomycin-resistance (neo) gene cassette and insertion was conducted by homologous recombination using a Red/ET system (GeneBridges). In that case, from among the *Escherichia coli* clones that grew in a kanamycin-supplemented medium, clones for which polymerase chain reaction (PCR) method resulted in correct amplification were selected. Next, loxP sequence and Rox sequences were placed at 3' downstream of the Cd3 γ gene on the BAC. More specifically, the loxP sequence and Rox sequences were introduced along with hygromycin-resistance (Hyg) gene cassette, and insertion was conducted by homologous recombination using a Red/ET system. In that case, from among the *Escherichia coli* clones that grew in a hygromycin-supplemented medium, clones in which the loxP sequence and Rox sequences were inserted as expected were selected by PCR method. Next, the genomic region 3' downstream of the Hyg gene cassette was removed leaving approximately 3.4 kb.

(2) Introduction of a mouse Cd3 gene region modification vector into mouse embryonic stem cells (ES cells) (Fig. 26A)

The above-mentioned mouse Cd3 gene region modification vector was introduced into mouse ES cells (C57BL/6N mouse-derived cells) via electroporation, and after selective culturing with G418, drug-resistant clones were obtained. From these clones, screening for homologous recombinants was performed by a PCR method. For electroporation, 60 μ g of the mouse Cd3 gene region modification vector was linearized with NotI or the NotI-untreated circular vector was extracted with phenol/chloroform, precipitated with ethanol, and then dissolved in PBS.

ES cells used in screening were cultured on a 96-well plate and washed twice using 200 μ l of PBS solution per well. Then, the cells were treated at 55°C for two hours after adding a cell lysis buffer having the following composition (5 μ l of 10x LA buffer II (TAKARA LA for Taq), 5 μ l of 25 mM MgCl₂, 5 μ l of 5% NP-40, 2 μ l of proteinase K (TAKARA, 20 mg/ml), and 33 μ l of distilled water), and subsequently treated at 95°C for 15 minutes to inactivate proteinase K, to thereby serve as PCR samples.

The PCR reaction mixture was made up of 1 μ l of the sample, 2.5 μ l of 10x LA buffer II, 2.5 μ l of 25 mM MgCl₂, 4 μ l of dNTP (2.5 mM), 0.1 μ l each of the primers (50 μ M each), 0.25

μl of LA Taq (TAKARA), and 14.55 μl of distilled water (25 μl in total). The PCR conditions included preheating at 94°C for two minutes, 35 cycles of an amplification cycle of 98°C for ten seconds and 68°C for 4 minutes 30 seconds, and additional heating at 68°C for five minutes.

The following primers were used. The primers were HygF1474 which was positioned within the Hyg gene cassette as a forward primer, and g4989R which was positioned as a reverse primer at the mouse genomic region on the 3' downstream side of the 3' homology arm in the mouse Cd3 gene modification vector (see Fig. 27). In samples of the ES cells in which homologous recombination occurred, an approximately 4-kb band was amplified. HygF1474 (forward) 5'-TATCAGAGCTTGGTTGACGG-3' (SEQ ID NO: 436); and g4989R (reverse) 5'-ACTCGTTGTGGCTTAGAAGCAGTAACAATACC-3' (SEQ ID NO: 437). Furthermore, clones from which amplification signals were obtained using the above-mentioned primer set were subjected to validation using a different primer set. More specifically, e27248F was positioned as a forward primer at the mouse genomic region on the 5' upstream side of the 5' homology arm in the mouse Cd3 gene modification vector, and Neo0635R was positioned as a reverse primer within the Neo gene cassette. In samples of ES cells in which homologous recombination occurred, an approximately 4-kb band was amplified. e27248F (forward) 5'-ACTGTAATCCTAGTACTTAGGAGGCTGAGG-3' (SEQ ID NO: 438); and Neo0635R (reverse) 5'-AATCCATCTTGTTC AATGGCCGATCC-3' (SEQ ID NO: 439).

(3) Construction of a human CD3 gene region introduction vector (Fig. 26B)

A BAC clone was used, into which a genomic region where the human CD3ε, CD3δ, and CD3γ genes are positioned had been cloned. A loxP sequence was inserted at 5' upstream of the gene region encoding human CD3ε in this BAC. At that time, the loxP sequence was introduced along with Hyg gene cassette, and insertion was conducted by homologous recombination using a Red/ET system (GeneBridges). In that case, from among the *Escherichia coli* clones that grew in a hygromycin-supplemented medium, clones for which PCR method resulted in correct amplification were selected. Next, at 3' downstream of the human CD3γ gene in the BAC, puromycin-resistance (Puro) gene flanked on both ends by Frt sequences was introduced together with Neo gene cassette to position a Rox sequence further downstream, and insertion was conducted by homologous recombination using a Red/ET system. In that case, from among the *Escherichia coli* clones that grew in a kanamycin-supplemented medium, clones in which the Frt sequences, the Puro gene, the Rox sequence, and the Neo gene were inserted as expected were selected by PCR method.

(4) Introduction of a human CD3 gene region introduction vector and a recombinase expression vector into Cd3 gene region-modified mouse ES cells

The human CD3 gene region introduction vector, a Cre recombinase expression vector, and a Dre recombinase expression vector were introduced via electroporation into ES cell clones (1D4, 5H1, 6I5, and 3A5) in which the loxP sequences and Rox sequences were correctly inserted at the targeted sites of the mouse Cd3 gene region in the above-mentioned step; and after selective culturing with puromycin, the grown ES cell clones were genotyped.

First, PCR screening was performed for selection of clones in which recombination between the loxP sequences and between the Rox sequences placed at the mouse Cd3 gene region took place by the action of Cre and Dre, and the genomic region from Cd3 ϵ to Cd3 γ was deleted. The ES cells used in screening were cultured on a 96-well plate, washed twice using 200 μ l of PBS per well, and treated at 55°C for two hours after adding a cell lysis buffer having the following composition (5 μ l of 10x LA buffer II (TAKARA LA for Taq), 5 μ l of 25 mM MgCl₂, 5 μ l of 5% NP-40, 2 μ l of proteinase K (TAKARA, 20 mg/mL), and 33 μ l of distilled water), and subsequently treated at 95°C for 15 minutes to inactivate proteinase K, to thereby serve as PCR samples.

The PCR reaction mixture was made up of 1 μ l of the sample, 2.5 μ l of 10x LA buffer II, 2.5 μ l of 25 mM MgCl₂, 4 μ l of dNTP (2.5 mM), 0.1 μ l each of the primers (50 μ M each), 0.25 μ l of LA Taq (TAKARA), and 14.55 μ l of distilled water (25 μ l in total). The PCR conditions included preheating at 94°C for two minutes, 35 cycles of an amplification cycle of 98°C for ten seconds and 68°C for 4 minutes 30 seconds, and additional heating at 68°C for five minutes.

The following primers were used. The primers were e30230F which was positioned as a forward primer at the genomic region on the 5' upstream side of the mouse Cd3 ϵ gene, and g1439R which was positioned as a reverse primer at the genomic region on the 3' downstream side of the mouse Cd3 γ gene (see Fig. 28A). In samples of the ES cells in which the Cd3 gene region was deleted, an approximately 0.7-kb band was amplified. e30230F (forward) 5'-TAGCAGCCTTCAGATGAAGAGGTAGGACTC-3' (SEQ ID NO: 440); and g1439R (reverse) 5'-TTGATGTGCCACCTCACTGCTGCACTGG-3' (SEQ ID NO: 441).

PCR screening was performed for selecting clones in which the human CD3 gene region was introduced from the ES cell clones deficient in the mouse Cd3 gene region. The PCR samples that were used for detecting the deletion of the mouse Cd3 gene region were subjected to the screening. The PCR reaction mixture was made up of 1 μ l of the sample, 2.5 μ l of 10x LA buffer II, 2.5 μ l of 25 mM MgCl₂, 4 μ l of dNTP (2.5 mM), 0.1 μ l each of the primers (50 μ M each), 0.25 μ l of LA Taq (TAKARA), and 14.55 μ l of distilled water (25 μ L in total). The PCR conditions included preheating at 94°C for two minutes, 35 cycles of an amplification cycle of 94°C for 30 seconds, 58°C for one minute, and 72°C for five minutes, and additional heating at 72°C for five minutes. The following primers were used. The primers were hCD3 ϵ _5arm_F2 which was positioned as a forward primer at the genomic region on the 5' upstream side of the

human CD3 ϵ gene, and hCD3 ϵ _ex2_R2 which was positioned as a reverse primer within the second exon of the human CD3 ϵ gene (see Fig. 28B). In samples of the ES cells in which the human CD3 gene region was introduced, an approximately 5.5-kb band was amplified.

hCD3 ϵ _5arm_F2 (forward) 5'-AACTGACAATGGGACATCAGCTGA-3' (SEQ ID NO: 442);
 5 and hCD3 ϵ _ex2_R2 (reverse) 5'-ATGGGACTGTTACTTTACTAAGAT-3' (SEQ ID NO: 443).

(5) Production of mouse Cd3 gene-deficient and human CD3 gene-introduced mice

The homologous recombinant ES clones were suspended by trypsin treatment, and washed with the ES cell medium. Female BALB/c mice which were subjected to
 10 superovulation treatment by administering 5 IU of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) intraperitoneally at 48-hour intervals were crossed with male mice of the same strain. The day when a plug was confirmed in a female mouse was regarded as day 0.5. On gestation day 3.5, blastocyst-stage embryos collected by perfusing the
 15 uterus were used as host embryos, in which 10 to 15 of the ES cells were injected. The embryos after the injection were transferred into the uterus of ICR recipient females on Day 2.5 pseudopregnancy, and their offspring were obtained 17 days later. Screening based on the coat color of the offspring obtained by injection of the ES cells to the blastocysts, yielded chimeric mice having a mixture of the recombinant ES cells (black) and the host blastocyst-derived cells (albino). After sexual maturation, the male chimeric mice were crossed with
 20 C57BL/6N-female mice, and transmission of the knock-in allele to the next generation was confirmed by a PCR method using the genomic DNA extracted from the tissues of the second-generation mice as the template. PCR was performed by the above-mentioned method used for screening of the ES cells. As a result, individuals from which the human CD3 gene region-specific 5.5-kb signal and the mouse Cd3 gene region deficiency-specific 0.7-kb signal
 25 were detected were obtained, and the human CD3 gene region allele and the mouse Cd3 gene region-deficient allele were confirmed to be transmitted to these individuals. Furthermore, breeding of mice having the above-described genotype yielded mouse individuals whose mouse Cd3 gene region is homozygously deleted and which have the human CD3 gene region, that is, human CD3 gene region-substituted mice were obtained. Transgenic mice in which human
 30 CD3 ϵ alone had been introduced (hereinafter, hCD3 ϵ Tg mice) were produced according to the report by Wang *et al.* (Wang *et al.* (1994) PNAS. 91:9402-9406), and they were examined as comparisons in the later experiments.

(6) Thymus weights and spleen weights of human CD3 gene-substituted mice

35 Spleen and thymus were collected from mice (12 to 14-week old, male) and the tissue weights were measured. As shown in Fig. 29, the thymus of the human CD3-substituted mice

did not show gross abnormalities. Tissue weight per body weight was calculated for analysis. The body weights and tissue weights (spleen and thymus) were measured for four male mice in each group, and represented as graphs. The tissue weight per body weight ratios were calculated, the values obtained for each individual are plotted by a black dot, and the mean value is shown by a column (Fig. 30). Regarding spleen weight, increasing trend was observed in the Cd3 gene-deficient mice as compared to mice of other genotypes, but no remarkable differences were observed. On the other hand, regarding thymus weight, the Cd3 gene-deficient mice showed decrease down to one third or so as compared to that of the wild-type. In the human CD3 gene-substituted mice produced by introducing a human CD3 gene into the Cd3 gene-deficient mice, recovery of thymus weight was observed, and particularly in the individuals of line no. 1C3, thymus weight was recovered even to the level equivalent to that of the wild-type mice. As reported by Wang *et al.*, thymic atrophy was observed in hCD3 ϵ Tg mice (Wang *et al.* (1994) PNAS. 91:9402-9406).

(7) Confirmation of expressions of human CD3 and mouse Cd3 in the respective lines of human CD3 gene-substituted mice

- Confirmation by RT-PCR method using hemocyte RNA -

Expressions of human CD3 ϵ , human CD3 δ , human CD3 γ , mouse Cd3 ϵ , mouse Cd3 δ , and mouse Cd3 γ were analyzed by RT-PCR using hemocyte RNA. Using a Catrimox-14 RNA Isolation Kit (TaKaRa Bio), total RNA was prepared from blood collected from the dorsal metatarsal vein or the abdominal vena cava. A 1 μ g portion each of the total RNAs was used as a template to synthesize cDNAs by performing reverse transcription reactions with a SuperScript III First Strand cDNA Synthesis Kit (Invitrogen) using Oligo dT (20) primers. Human CD3 ϵ , human CD3 δ , human CD3 γ , mouse Cd3 ϵ , mouse Cd3 δ , and mouse Cd3 γ were detected by performing PCR using the synthesized cDNAs as templates. Primers for the protein coding regions were designed to detect the expression of all of the genes. Human CD3 ϵ was detected using the combination of forward primer E0333F (5'-AAGAAATGGGTGGTATTACACAGACACC-3' (SEQ ID NO: 444)) and reverse primer E0912R (5'-TGGGCCAGCGGGAGGCAGTGTTCTCCAGAGG-3' (SEQ ID NO: 445)). Human CD3 δ was detected using the combination of forward primer D0092F (5'-TAGTTCGGTGACCTGGCTTTATCTACTGG-3' (SEQ ID NO: 446)) and reverse primer D0685R (5'-ATGGCTGCTTCTAGAAGCCACCAGTCTCAGG-3' (SEQ ID NO: 447)). Human CD3 γ was detected using the combination of forward primer G0048F (5'-TGCTCCACGCTTTTGCCGGAGGACAG-3' (SEQ ID NO: 448)) and reverse primer G0666R (5'-TAGGAGGAGAACACCTGGACTACTC-3' (SEQ ID NO: 449)). On the other hand, mouse Cd3 ϵ was detected using the combination of forward primer e0065F

(5'-AGCATTCTGAGAGGATGCGGTGGAACAC-3' (SEQ ID NO: 450)) and reverse primer e0699R (5'-TGCTCGGAGGGCTGGATCTGGGTCCACAG-3' (SEQ ID NO: 451)). Mouse Cd3 δ was detected using the combination of forward primer d055F (5'-TCATCCTGTGGCTTGCCTCTATTTGTTGC-3' (SEQ ID NO: 452)) and reverse primer d651R (5'-TTGCTATGGCACTTTGAGAAACCTCCATC-3' (SEQ ID NO: 453)). Mouse Cd3 γ was detected using the combination of forward primer g080F (5'-AATACTTCTACTGGAGAAGCAAAGAG-3' (SEQ ID NO: 454)) and reverse primer g316R (5'-TAGTTGCATTTAGAGGACTTATTATGC-3' (SEQ ID NO: 455)).

The composition of the PCR reaction solution (25 μ l in total) was made up of 1 μ l of the sample, 2.5 μ l of 10x Ex buffer, 2 μ l of dNTP (2.5 mM), 0.1 μ l each of the primers (50 μ M each), 0.25 μ l of Ex Taq (TAKARA), and 19.05 μ l of distilled water. The PCR conditions for human CD3 δ , human CD3 γ , mouse Cd3 δ , and mouse Cd3 γ included preheating at 94°C for two minutes, 35 cycles of an amplification cycle of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for two minutes, and additional heating at 72°C for five minutes. For human CD3 ϵ and mouse Cd3 ϵ , the PCR conditions included preheating at 94°C for two minutes, 40 cycles of an amplification cycle of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for two minutes, and additional heating at 72°C for five minutes. PCR primers were designed so that the detected amplification products of human CD3 ϵ , human CD3 δ , and human CD3 γ will be 580 bp, 594 bp, and 620 bp, respectively, and those of mouse Cd3 ϵ , mouse Cd3 δ , and mouse Cd3 γ will be 635 bp, 597 bp, and 237 bp, respectively.

In the Cd3 gene-deficient mice, the respective mouse Cd3 molecule-derived PCR signals were not detected. Only human CD3 ϵ , human CD3 δ , and human CD3 γ were detected, and none of mouse Cd3 ϵ , mouse Cd3 δ , and mouse Cd3 γ was detected from the samples derived from lines 1C3 and 8I12 of the above-mentioned lines among the human CD3 gene-substituted mouse lines (line nos. 1C3, 3B1, 8I12, and 2A4) produced by introducing the human CD3 gene region to the Cd3 gene-deficient mice (Fig. 31). From the samples derived from wild-type mice, human CD3 ϵ , human CD3 δ , and human CD3 γ were not detected, and mouse Cd3 ϵ , mouse Cd3 δ , and mouse Cd3 γ were detected (Fig. 31). These results confirmed that mice expressing human CD3 ϵ , CD3 δ , and CD3 γ instead of mouse Cd3 ϵ , Cd3 δ , and Cd3 γ were obtained as designed. Line 4HH3 in Fig. 31 was analyzed in an individual in which the mouse Cd3 allele is a wild-type and the human CD3 gene has been introduced, and the respective human CD3 molecules and the respective mouse Cd3 molecules are both detected. Subsequently, it was cross-bred with Cd3-deficient mice to establish a mouse Cd3 allele-deficient and human CD3 gene-expressing line.

- Analysis by immunohistological staining -

The tissue distribution was examined using the anti-CD3 antibody as the primary antibody. CD3 staining was not observed in any of the tissues from the Cd3-deficient mice, while CD3-specific staining equivalent to that of wild-type mice was observed for the human CD3-substituted mice produced by introducing the human CD3 genes to the Cd3-deficient mice.

5 More specifically, specific staining was observed in the T cell zones in the thymus (Fig. 32A) and spleen (Fig. 32B). In all tissues, staining was observed only in the T cell zone, similarly to the wild-type mice. Furthermore, staining was not observed in the Cd3 gene-deficient mice, indicating that staining in the human CD3 gene-substituted mice was due to the expression of the introduced human CD3 genes. Furthermore, the detection of CD3s in the major organs was the

10 same as in the wild-type, and ectopic staining was not observed (Table 19).

Table 19

hCD3, mCD3KO1G mouse		IACUC 14-074																					
Organs	Line	mCD3ko.hCD3TG										hCD3ε TG				mCD3KO				C57BL/6N			
		#4H3		#8112		#1C3		#3-1-78		#195		None		None		None		None					
		008-130	010-163	003-91	003-86	003-85	003-86	001-60	168	169	97	195	790	001-63	001-64	001-67	B6-01	B6-02	B6-03	B6-01	B6-02	B6-03	
Findings	Animal No.	♀	♀	♂	♂	♂	♂	♂	♀	♀	♂	♂	♂	♀	♀	♂	♀	♀	♂	♀	♀	♂	
IHC Staining	Gender	Date of IHC Staining: A, 2014.6.19; B, 2014.6.25																					
Thymus																							
Atrophy		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, cortex		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, medulla		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Other tissues		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Mesentery		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Atrophy		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, paracortex		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, follicle		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, medulla		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Other tissues		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Ileum		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Atrophy of GALT		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, GALT		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, lamina propria		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Other tissues		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Spleen		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Atrophy		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, PALS		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, follicle		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, red pulp		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Other tissues		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Liver		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, sinusoid		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Other tissues		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Kidney		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, interstitium		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Other tissues		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Adrenal gland		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, interstitium		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Other tissues		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lung		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, alveolar wall		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Other tissues		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Heart		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Lymphocyte, interstitium		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Other tissues		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Gastrocnemius muscle		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	

Findings: — negative; ±, very slight; ++, moderate; +++, severe
IHC Staining: —, negative; ±, rare; +, occasional; ++, frequent; +++, constant

Findings: - negative; ± very slight; + slight; ++ moderate; +++ severe
IHC Staining: - negative; ± rare; + occasional; ++ frequent; +++ constant

(8) Evaluation of abundance ratio of mature T cells in human CD3 gene-substituted mice

FACS analyses were performed using spleen cells. Spleens were collected from mice (12 to 14-week old, male), and cells were isolated using 70 μ m mesh. Erythrocytes were lysed by adding a hemolytic agent (manufactured by SIGMA). After blocking using an Fc blocking solution, FITC-labeled anti-mouse Cd3 antibody, FITC-labeled anti-human CD3 antibody, APC-labeled anti-mouse Cd4 antibody, and PE-labeled anti-mouse Cd8 antibody were used on 2×10^6 cells, and the respective positive cell counts were analyzed by a flow cytometer. It was revealed that the Cd3 gene-deficient mice nearly completely lack in mature T cells, that is, Cd4 and Cd8 single positive cells, while these cells were present in the human CD3 gene-substituted mice at a ratio equivalent to that in the wild-type.

Abundance ratio of mature T cells

Table 20

Experimental group	Number of samples	mCd3	hCD3	mCd4	mCd8
Human CD3s-substituted mouse #1C3	n=4	ND.	38.8 ($\pm$ 3.1)	19.6 ($\pm$ 0.7)	16.1 ($\pm$ 3.6)
Human CD3s-substituted mouse #4HH3	n=2	ND.	29.8 , 28.9	15.5 , 13.9	17.5 , 16.4
Human CD3s-substituted mouse #8I12	n=4	ND.	31.5 ($\pm$ 5.4)	15.5 ($\pm$ 3.1)	15.3 ($\pm$ 2.7)
hCD3E Tg mouse	n=4	19.5 ($\pm$ 3.76)	13.0 ($\pm$ 1.4)	7.4 ($\pm$ 0.6)	7.8 ($\pm$ 0.8)
Cd3s-deficient mouse	n=4	ND.	ND.	1.8 ($\pm$ 1.3)	2.1 ($\pm$ 0.6)
C57BL/6N	n=4	40.4 ($\pm$ 8.42)	ND.	20.3 ($\pm$ 6.7)	12.7 ($\pm$ 2.1)

The table shows the expression ratios of the respective marker-positive cells with respect to the spleen cells (unit %). The mean from four individuals is shown for each the experimental group, except for human CD3s-substituted mice #4HH3, and the expression ratios of two individuals are shown for line #4HH3. (The standard deviation is shown in parenthesis.) ND: not detected.

[Reference Example 11] Evaluation of immune function of human CD3 gene-substituted mice

(1) Examination of the ability to produce specific antibodies in response to immunization to foreign antigen

For production of specific antibodies against foreign antigens, there must exist functional helper T cells that can bind to antigenic peptides presented together with major histocompatibility complex (MHC) antigens on the surface of antigen-presenting cells such as dendritic cells, and the T cells must have functions of giving instructions to antibody-producing cells to produce appropriate antibodies. Whether the above-mentioned human CD3

gene-substituted mice carry helper T cells having normal functions and produce specific antibodies in response to immunization to foreign antigens was examined. Immunization was carried out using chicken ovalbumin (OVA) as the sensitizing antigen together with Freund's adjuvant. Immunization to OVA was performed twice with a four-week interval. More specifically, the first immunization was performed by subcutaneously applying, 100 μ g of OVA per animal with complete Freund's adjuvant to the dorsal region, and four weeks later, similar immunization was performed by subcutaneously applying the antigen with incomplete Freund's adjuvant to the dorsal region. As human CD3 gene-substituted mice, two lines (line nos. 1C3 and 8I12), each of which is derived from a different modified ES cell clone, were selected, and compared to human CD3 ϵ -overexpressing mice. Furthermore, as controls, wild-type mice and Cd3 gene-deficient mice were selected and similar antigen immunizations were performed.

One week after the second immunization, the animals were subjected to laparotomy under isoflurane anesthesia, and then euthanized by collecting whole blood and allowing bleeding from the abdominal vena cava. Serum was separated from the collected blood, and the concentrations of OVA-specific IgG1 and OVA-specific IgE were measured (Fig. 34).

As a result, neither IgG1 type nor IgE type OVA-specific antibodies were detected from the serum of mouse Cd3-deficient mice, whereas OVA-specific IgG1 and IgE were detected in both lines of the human CD3 gene-substituted mice, and their levels were equivalent to those of wild-type mice. These results showed that human CD3 gene-substituted mice have normal ability to produce antibodies in response to foreign antigen immunization.

Industrial Applicability

The present invention provides anticancer agents and pharmaceutical compositions for use in combination with another anticancer agent, the agents or compositions comprising novel multispecific antigen-binding molecules that maintain the strong anti-tumor activity possessed by BiTE and the excellent safety property of not inducing a cytokine storm or such independently from cancer antigen, and also have long half-lives in blood. Anticancer agents and pharmaceutical compositions that comprise an antigen-binding molecule of the present invention as an active ingredient can target glypican 3-expressing cells and tumor tissues containing these cells and induce cytotoxicity. Administration of a multispecific antigen-binding molecule of the present invention to patients makes it possible to have a desirable treatment which not only has a high level of safety but also a reduced physical burden, and is highly convenient.

CLAIMS

1. An anticancer agent comprising as an active ingredient a bispecific antibody of any one of (a) to (c) below that comprises an antibody variable region having glypican 3-binding activity and
5 an antibody variable region having CD3-binding activity:
- (a) a bispecific antibody in which CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having
10 CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;
- 15 (b) a bispecific antibody in which the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ
20 ID NO: 223; and
- (c) a bispecific antibody which has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and common L chains having at least 80% identity to the amino acid
25 sequence of SEQ ID NO: 410.
2. An anticancer agent comprising as an active ingredient a bispecific antibody that comprises the antibody H chain of SEQ ID NO: 385 having glypican 3-binding activity, the antibody H chain of SEQ ID NO: 402 having CD3-binding activity, and the common L chains of SEQ ID
30 NO: 410.
3. The anticancer agent of claim 1 or 2, wherein the cancer is a glypican 3-positive cancer.
4. The anticancer agent of claim 3, wherein the glypican 3-positive cancer is a cancer in which
35 the number of glypican 3 antigens on cell surface per cell is 100 or more.

5. The anticancer agent of any one of claims 1 to 4, wherein the cancer is any cancer selected from the group consisting of gastric cancer, head and neck cancer, esophageal cancer, lung cancer, liver cancer, ovary cancer, breast cancer, colon cancer, kidney cancer, skin cancer, muscle tumor, pancreas cancer, prostate cancer, testis cancer, uterine cancer, cholangiocarcinoma, 5 Merkel cell carcinoma, bladder cancer, thyroid cancer, schwannoma, adrenal cancer, anus cancer, central nervous system tumor, neuroendocrine tissue tumor, penis cancer, pleura tumor, salivary gland tumor, vulva cancer, thymoma, and childhood cancer.
6. The anticancer agent of any one of claims 1 to 5, which is for treating a patient having cancer 10 that is refractory to treatment with an immune checkpoint inhibitor.
7. A pharmaceutical composition for use in combination with another anticancer agent, the pharmaceutical composition comprising as an active ingredient a bispecific antibody of any one of (a) to (c) below that comprises an antibody variable region having glypican 3-binding activity 15 and an antibody variable region having CD3-binding activity:
- (a) a bispecific antibody in which CDR1, CDR2, and CDR3 comprised in the antibody variable region having glypican 3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 206, 20 respectively; CDR1, CDR2, and CDR3 comprised in the antibody variable region having CD3-binding activity are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 168, respectively; and CDR1, CDR2, and CDR3 comprised in an antibody variable region of a common L chain are sequences having at least 80% identity to the amino acid sequences of the CDR1, CDR2, and CDR3 regions comprised in SEQ ID NO: 223, respectively;
- 25 (b) a bispecific antibody in which the antibody variable region having glypican 3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 206; the antibody variable region having CD3-binding activity is a sequence having at least 80% identity to the amino acid sequence of SEQ ID NO: 168; and an antibody variable region of a common L chain is a sequence having at least 80% identity to the amino acid sequence of SEQ 30 ID NO: 223; and
- (c) a bispecific antibody which has an antibody H chain having glypican 3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 385; an antibody H chain having CD3-binding activity and having at least 80% identity to the amino acid sequence of SEQ ID NO: 402; and common L chains having at least 80% identity to the amino acid 35 sequence of SEQ ID NO: 410.

8. The pharmaceutical composition of claim 7, wherein the bispecific antibody is administered simultaneously with said another anticancer agent.
9. The pharmaceutical composition of claim 7, wherein the bispecific antibody is administered
5 before or after administration of said another anticancer agent.
10. The pharmaceutical composition of any one of claims 7 to 9, wherein said another anticancer agent is a chemotherapeutic agent, a T cell-activating agonist agent, an immune checkpoint inhibitor, or an angiogenic inhibitor.
10
11. The pharmaceutical composition of claim 10, wherein the chemotherapeutic agent is an antimetabolite, a plant alkaloid, or a platinum compound.
12. The pharmaceutical composition of claim 10, wherein the T cell-activating agonist agent is
15 an agonist antibody against TNFRSF.
13. The pharmaceutical composition of claim 10, wherein the immune checkpoint inhibitor is a PD1 antibody, a PDL1 antibody, a TIM3 antibody, or an LAG3 antibody.
- 20 14. The pharmaceutical composition of claim 10, wherein the angiogenic inhibitor is a VEGFR2 antibody.
15. An agent for inducing cytotoxicity, an agent for suppressing cell proliferation, an agent for inhibiting cell proliferation, an agent for activating immune response, an agent for treating cancer,
25 or an agent for preventing cancer, which comprises the pharmaceutical composition of any one of claims 7 to 14.

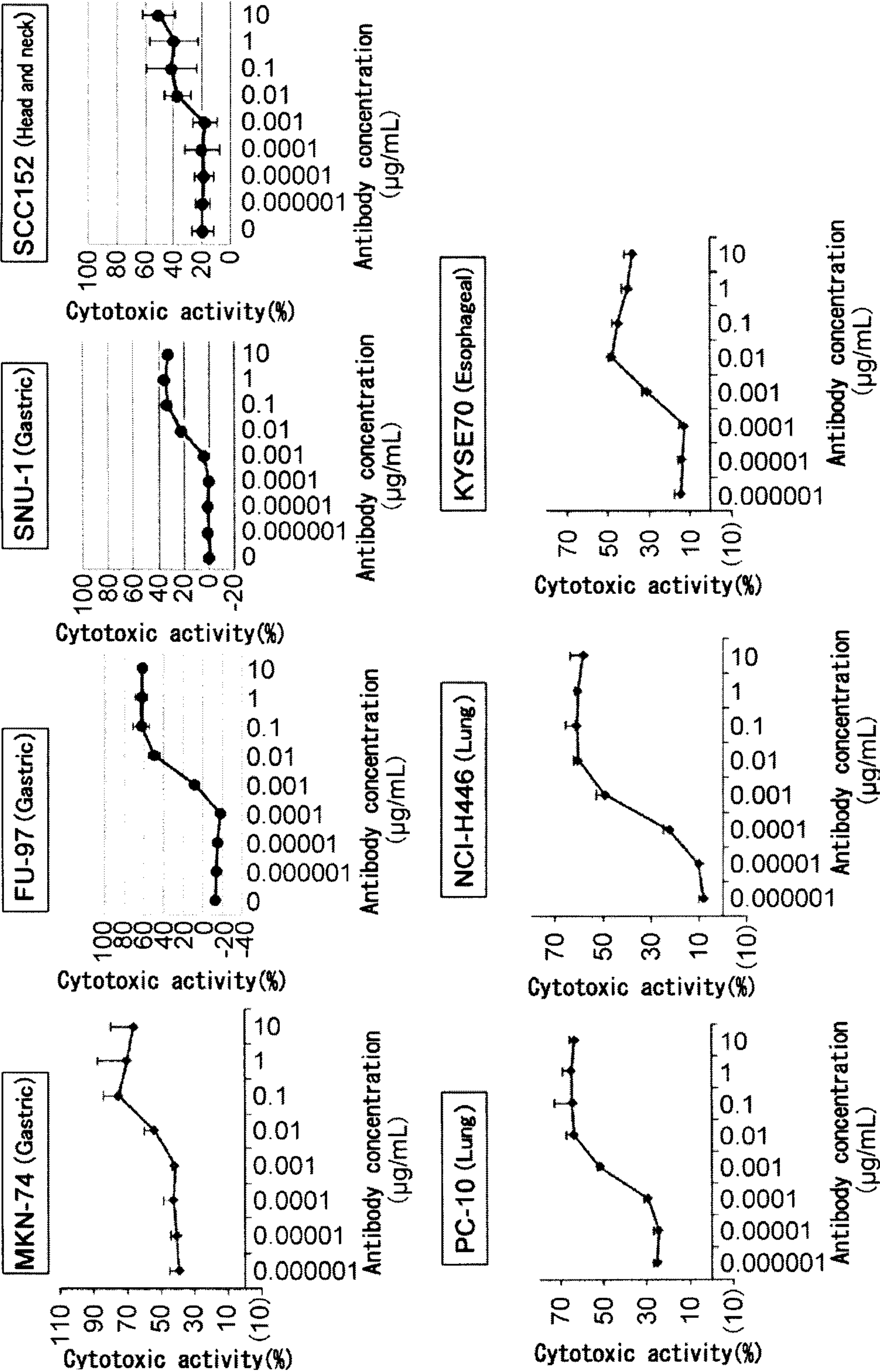


FIG. 1-1

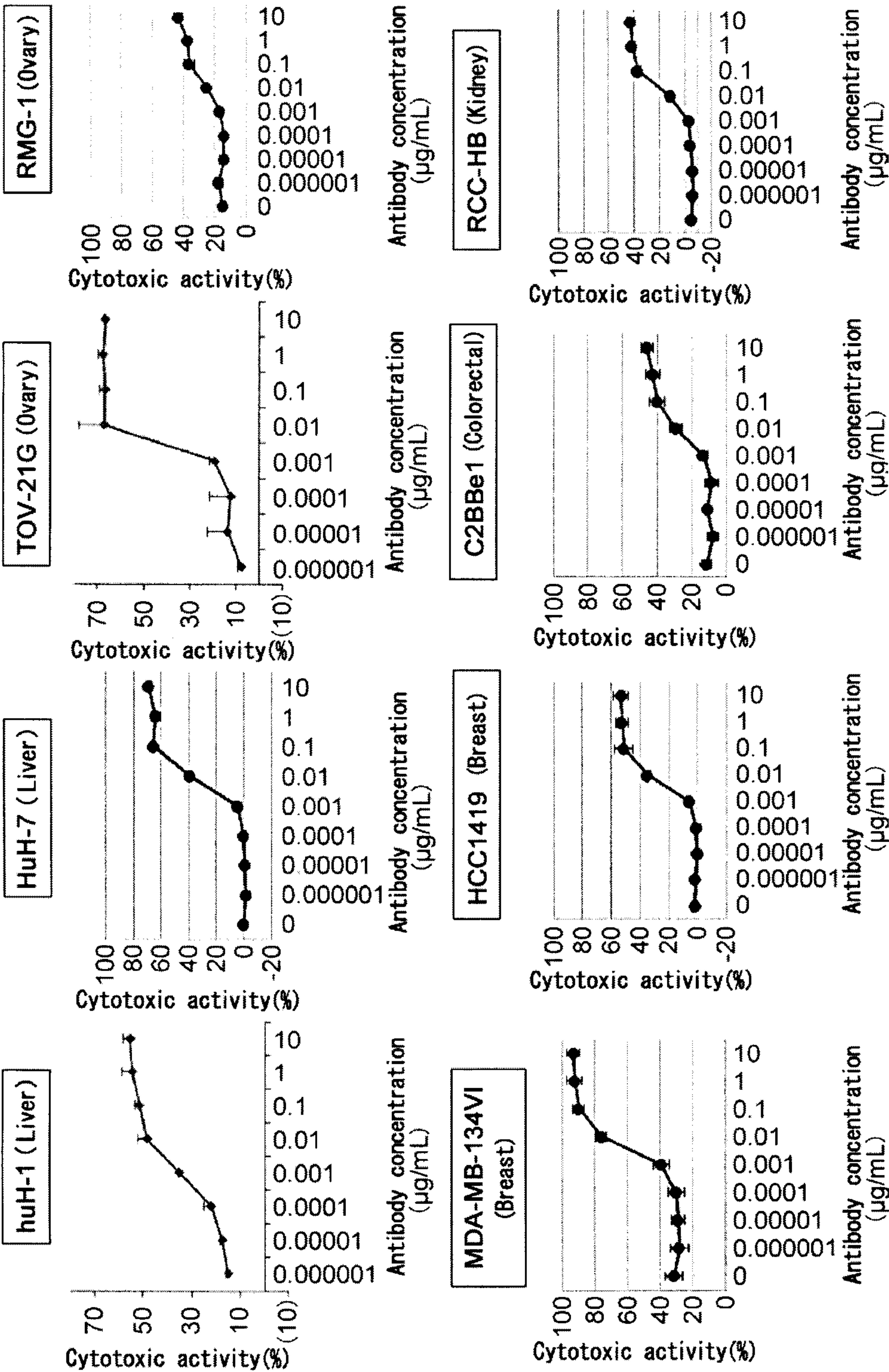


FIG. 1-2

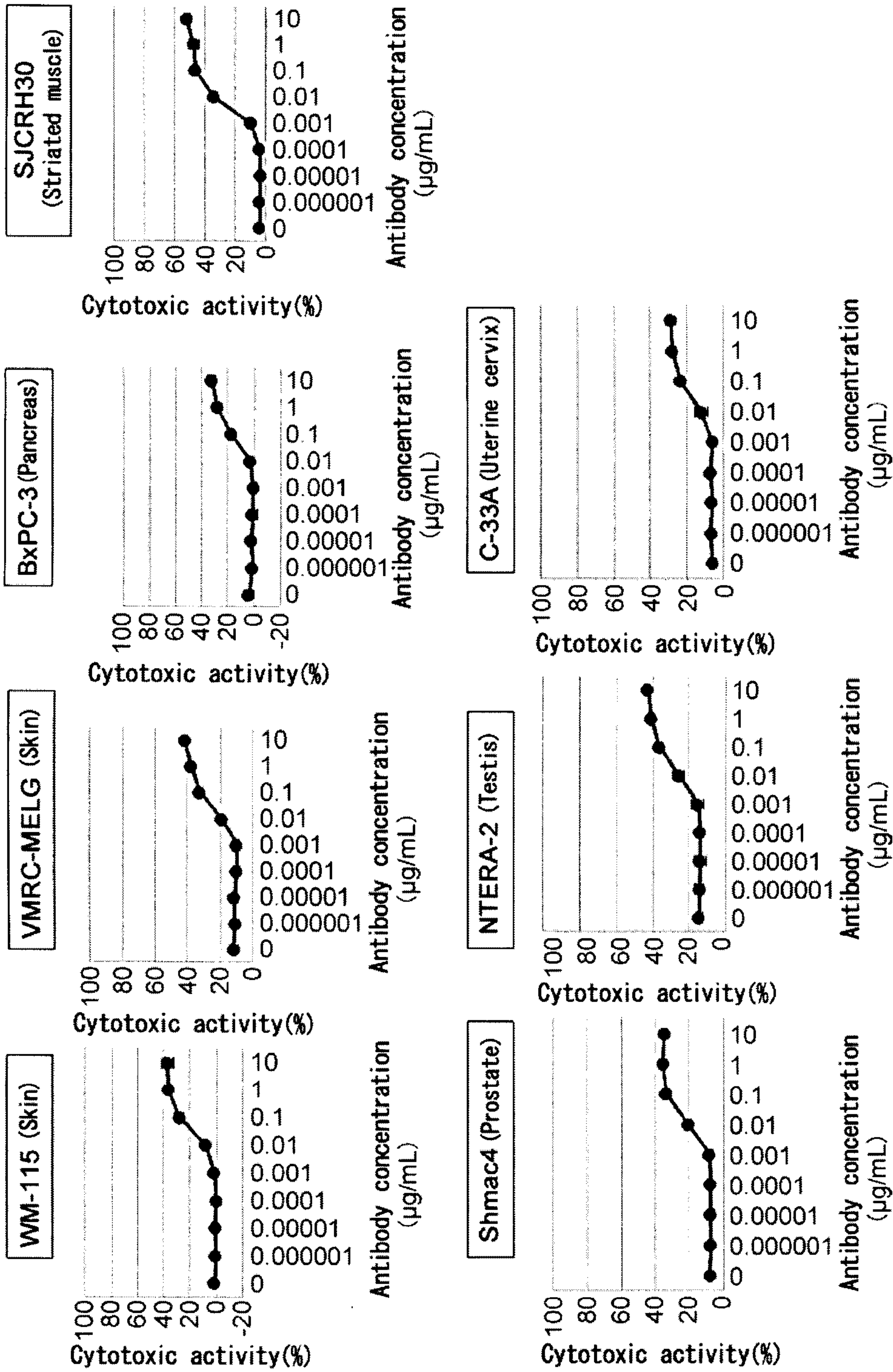


FIG. 1-3

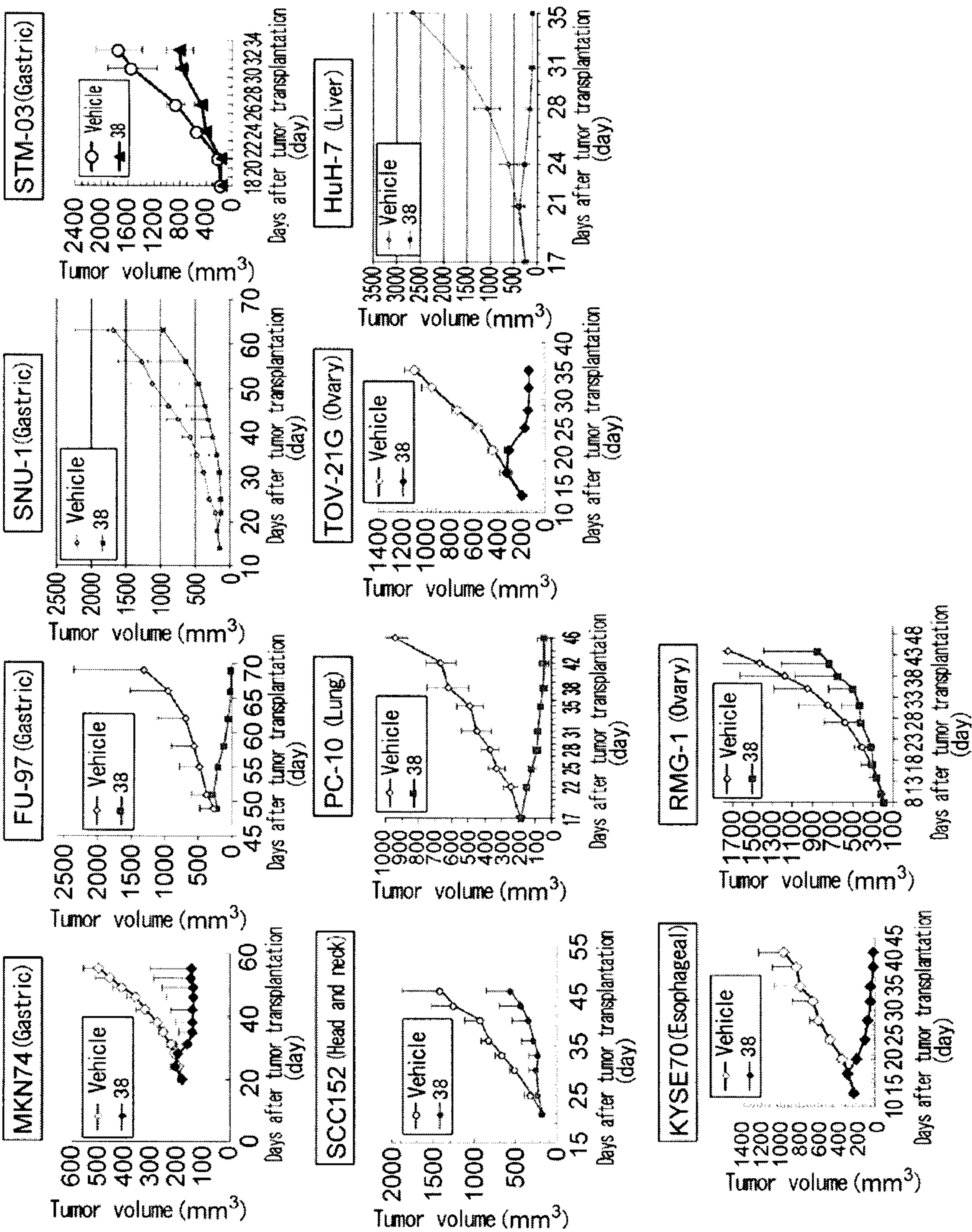
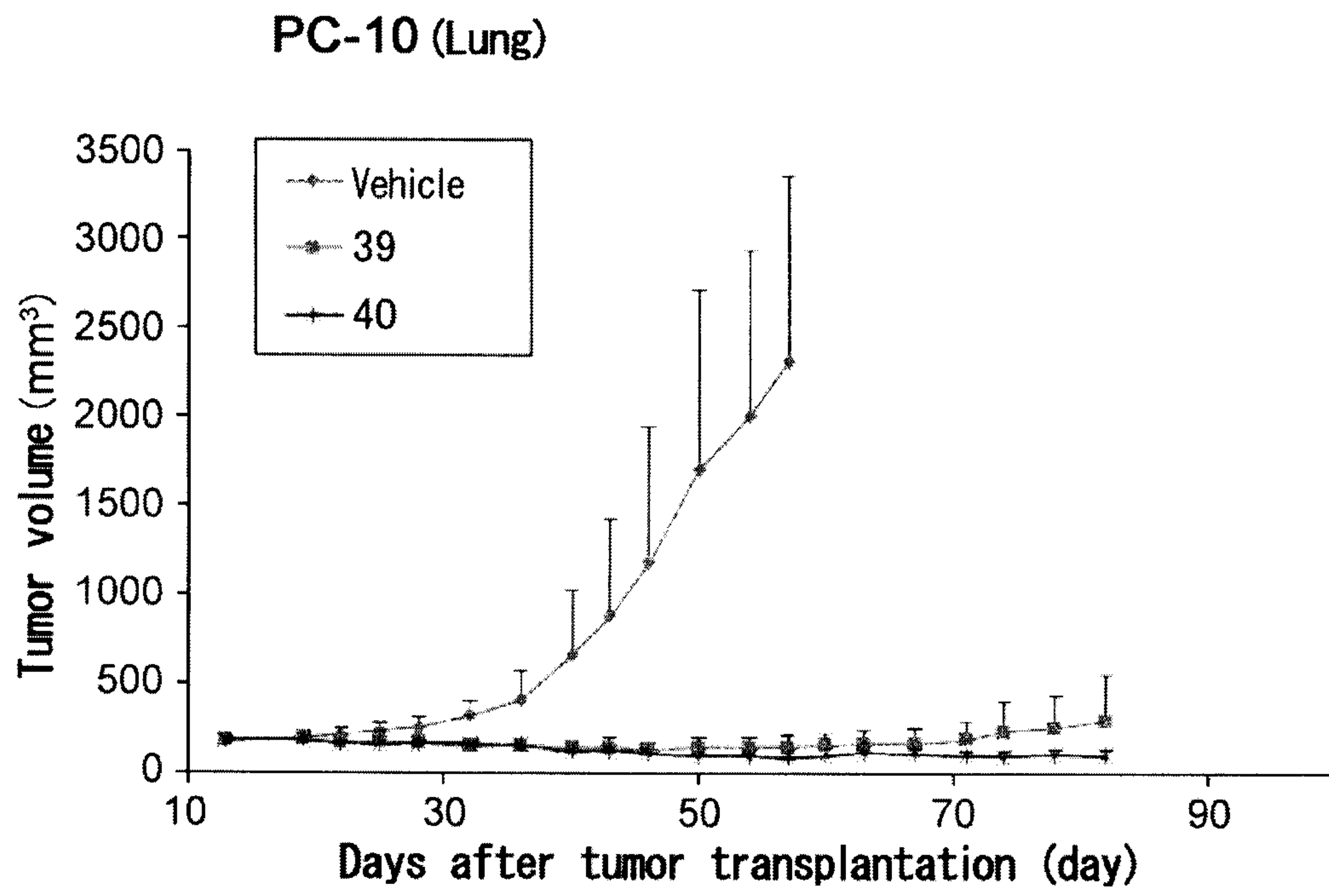
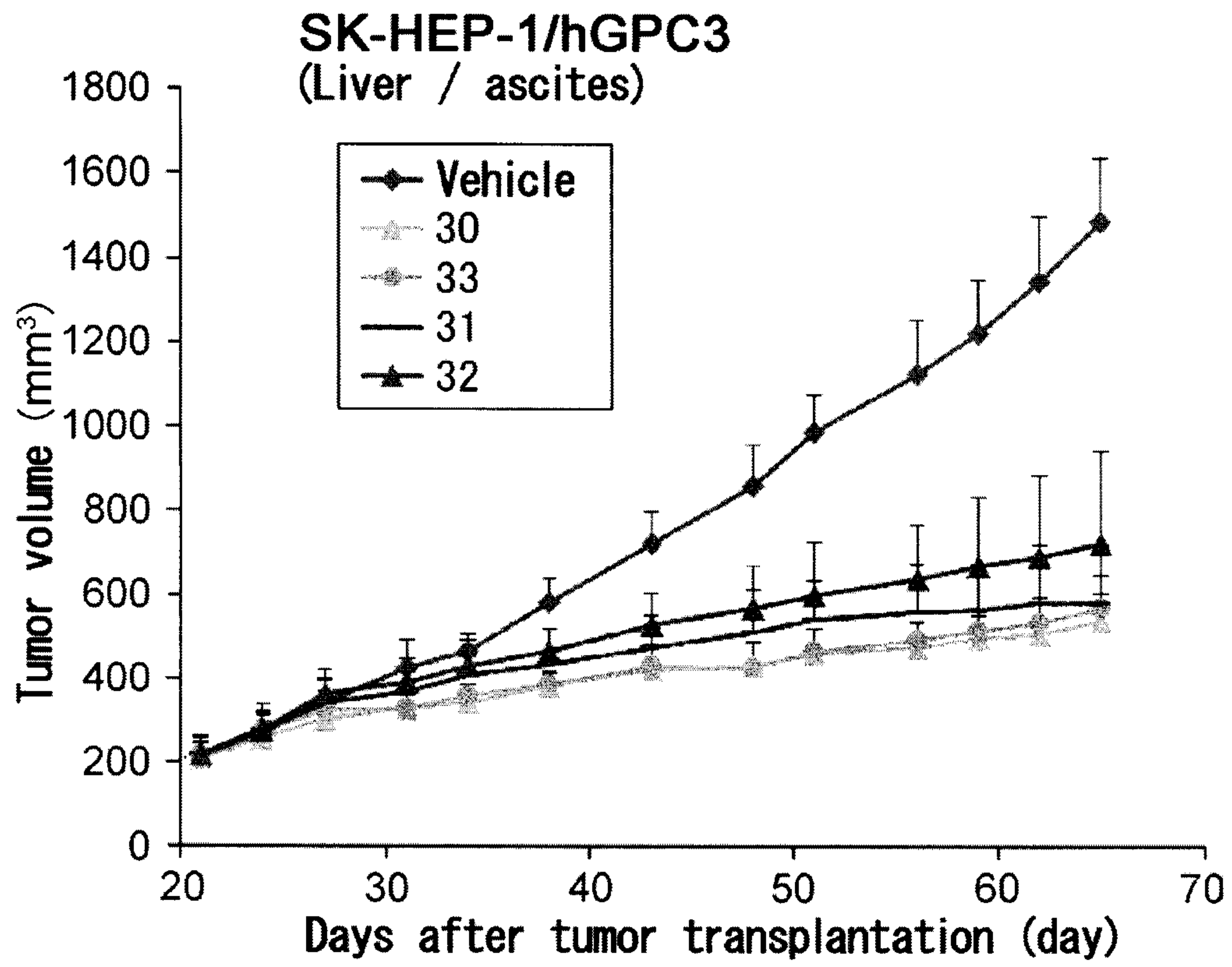
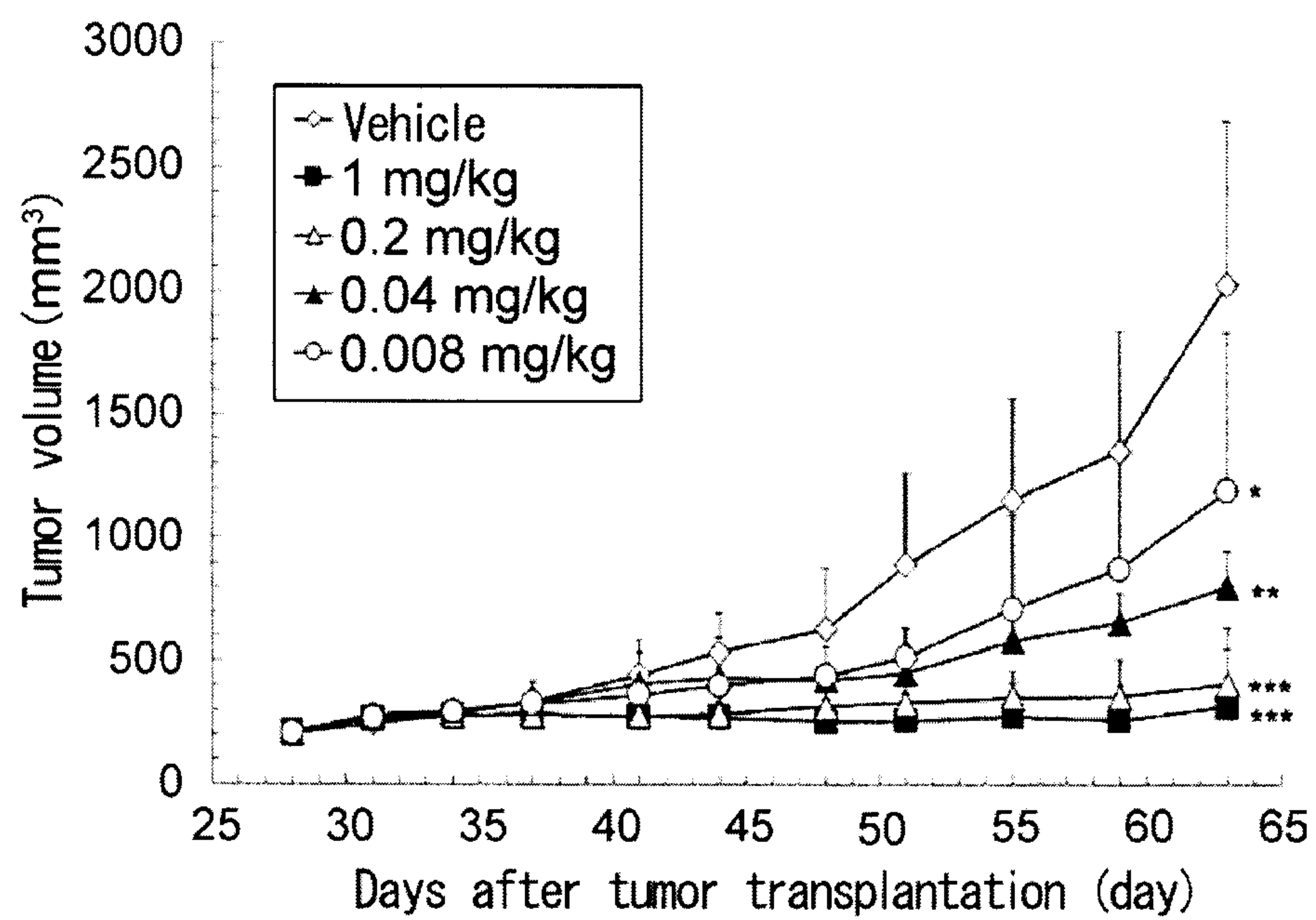
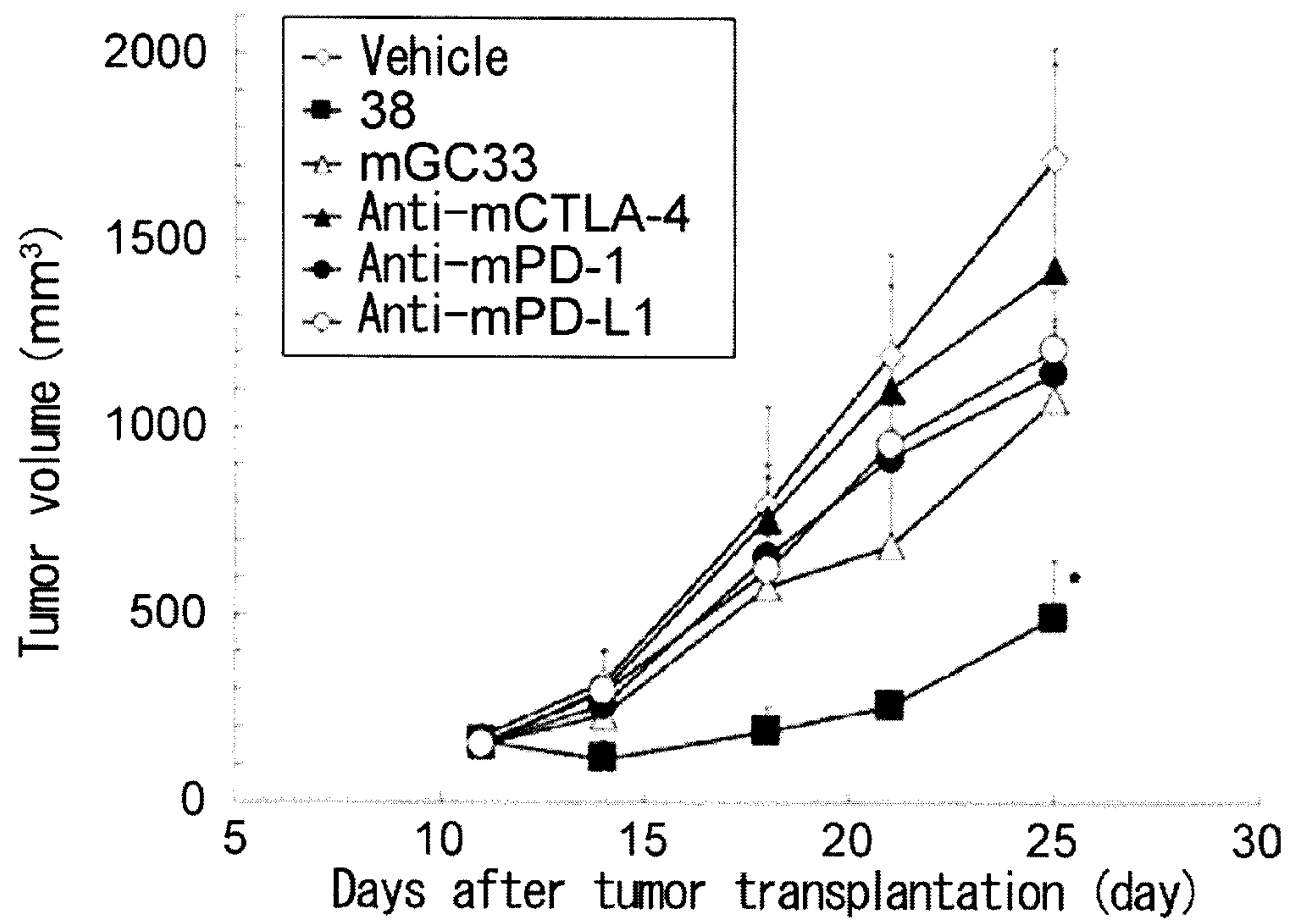


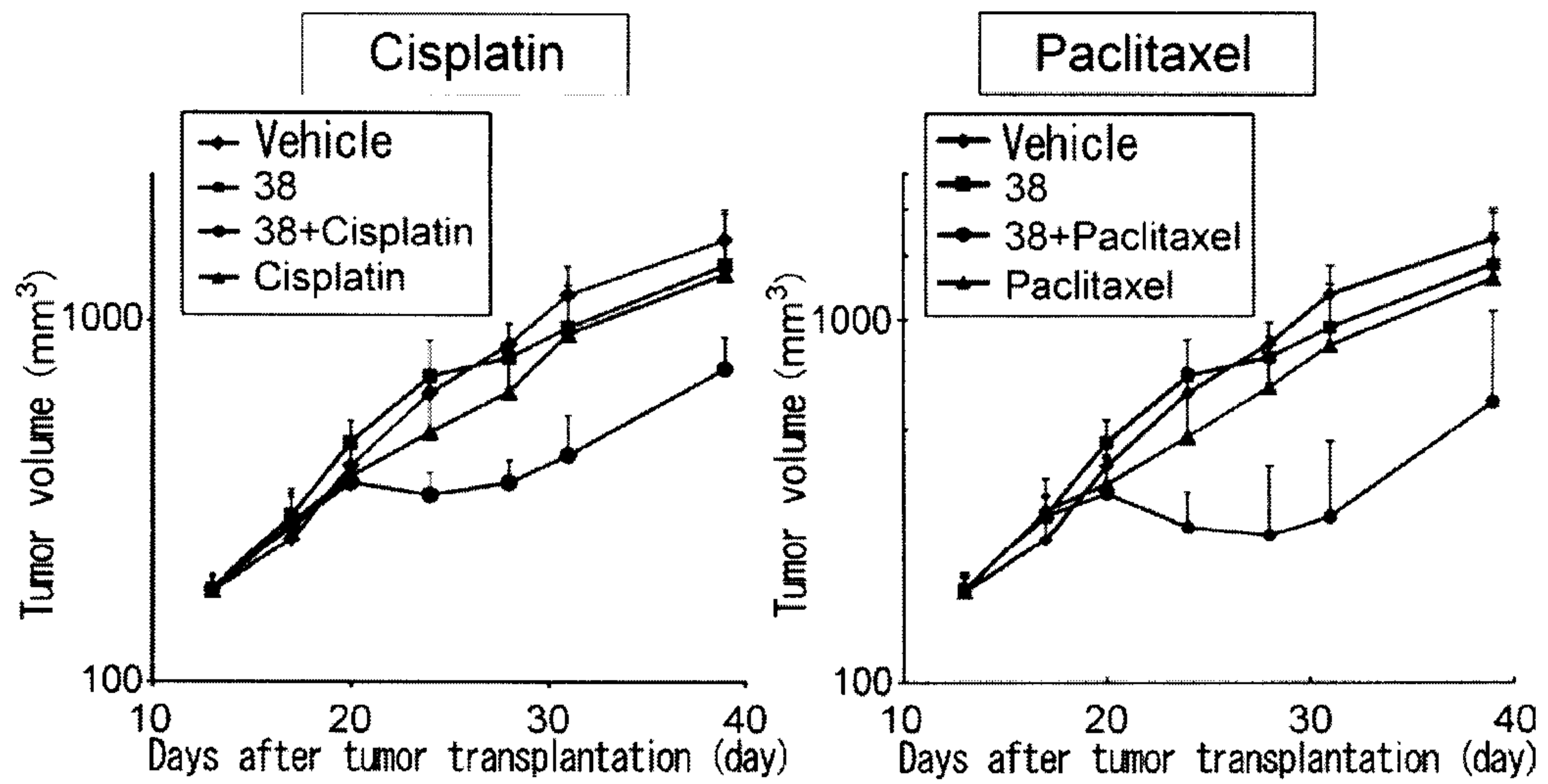
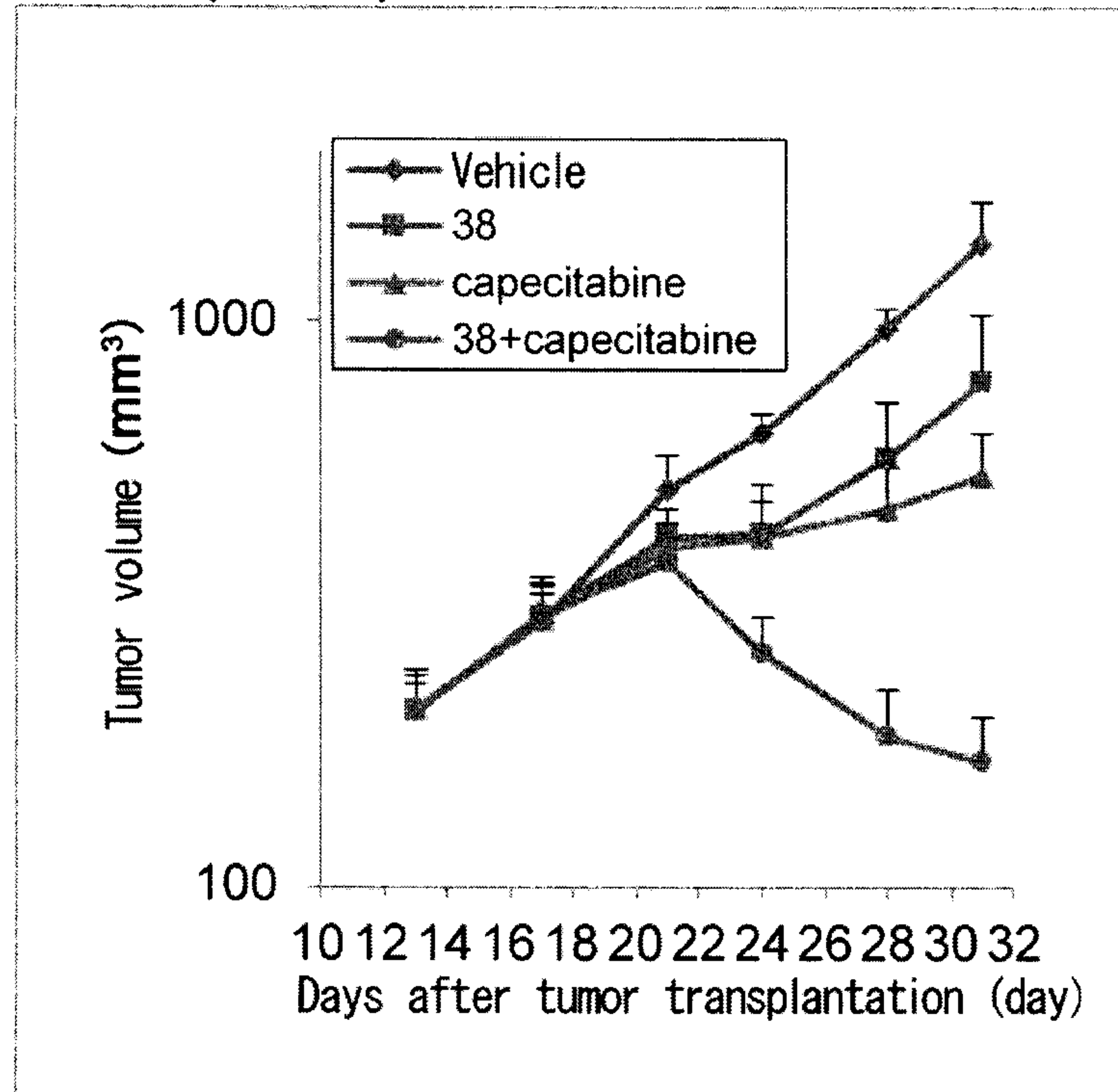
FIG. 2

**FIG. 3**

**FIG. 4**

PC-10 (Lung)**FIG. 5**

LLC1/hGPC3 (Mouse lung)**FIG. 6**

MKN45(Gastric)**FIG. 7**

NCI-H446 (Lung)

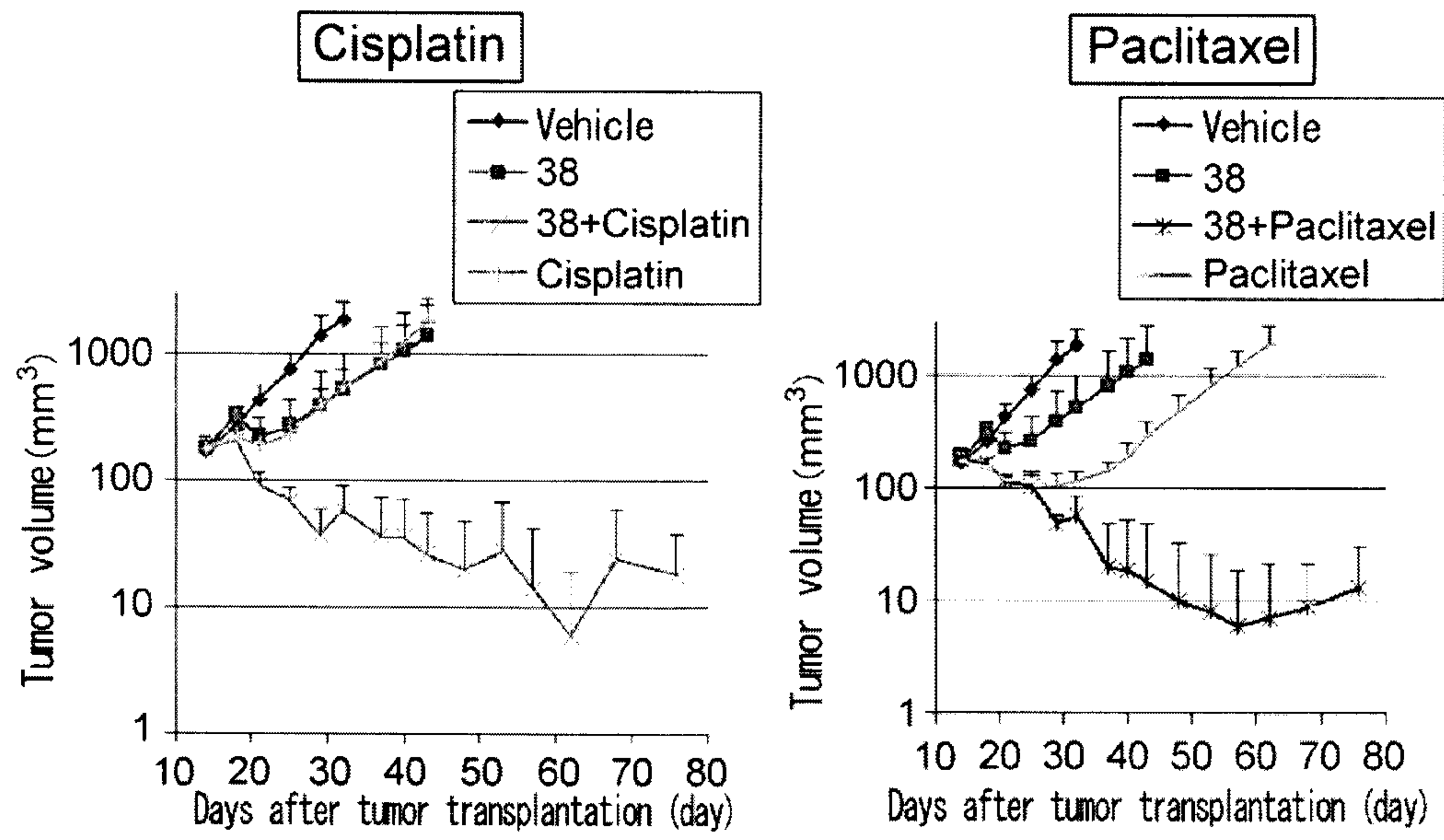


FIG. 8

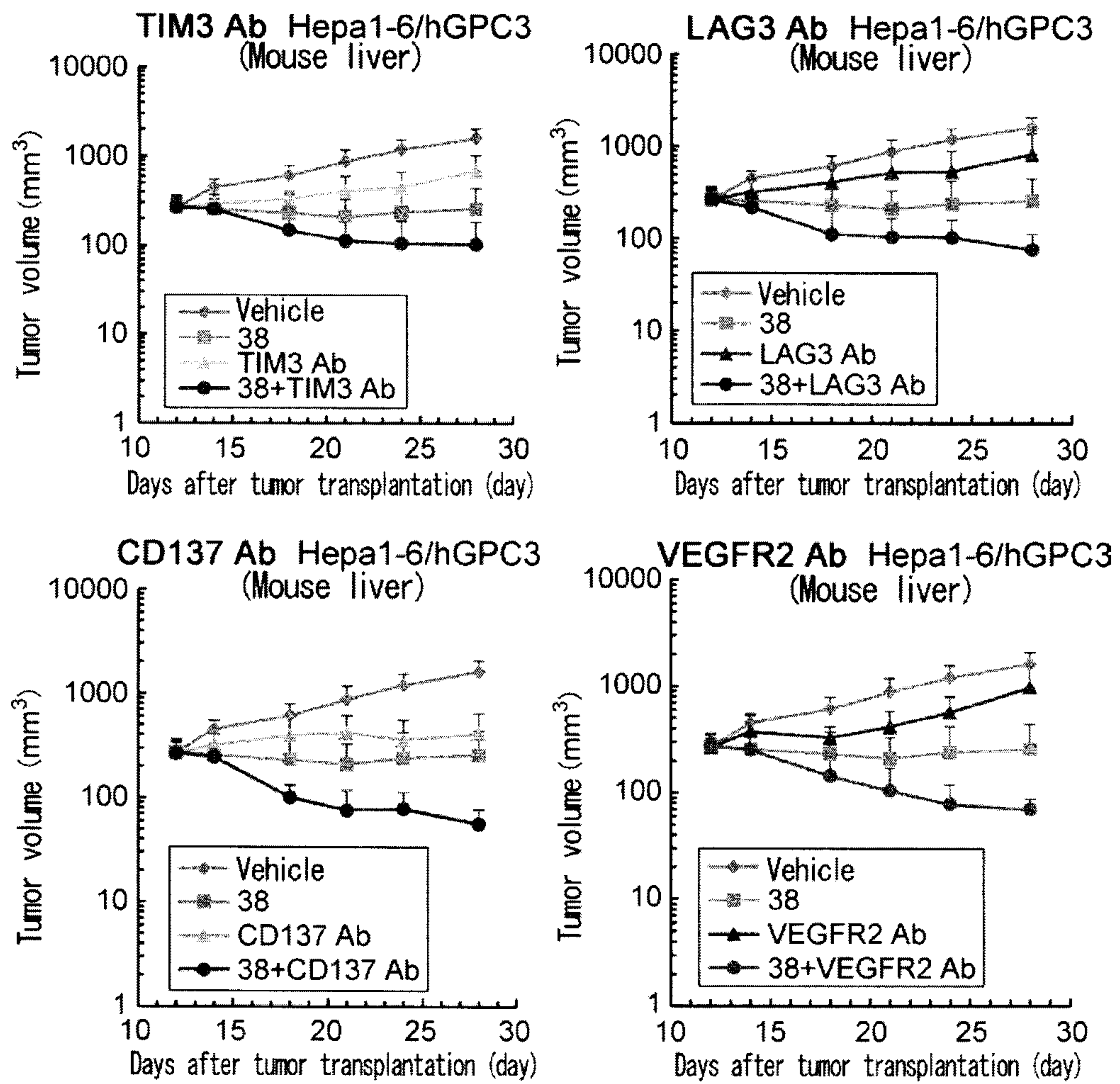
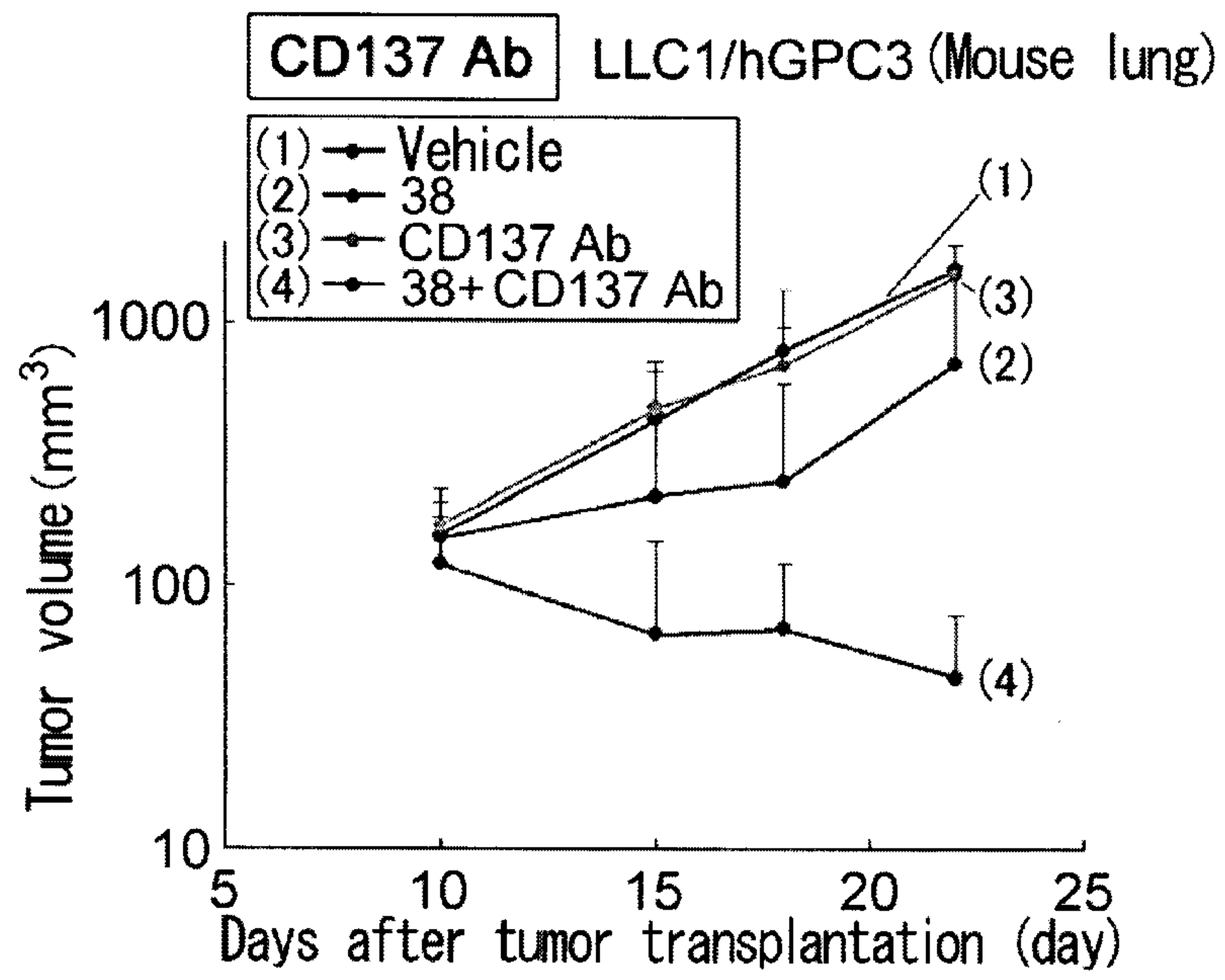


FIG. 9

**FIG. 10**

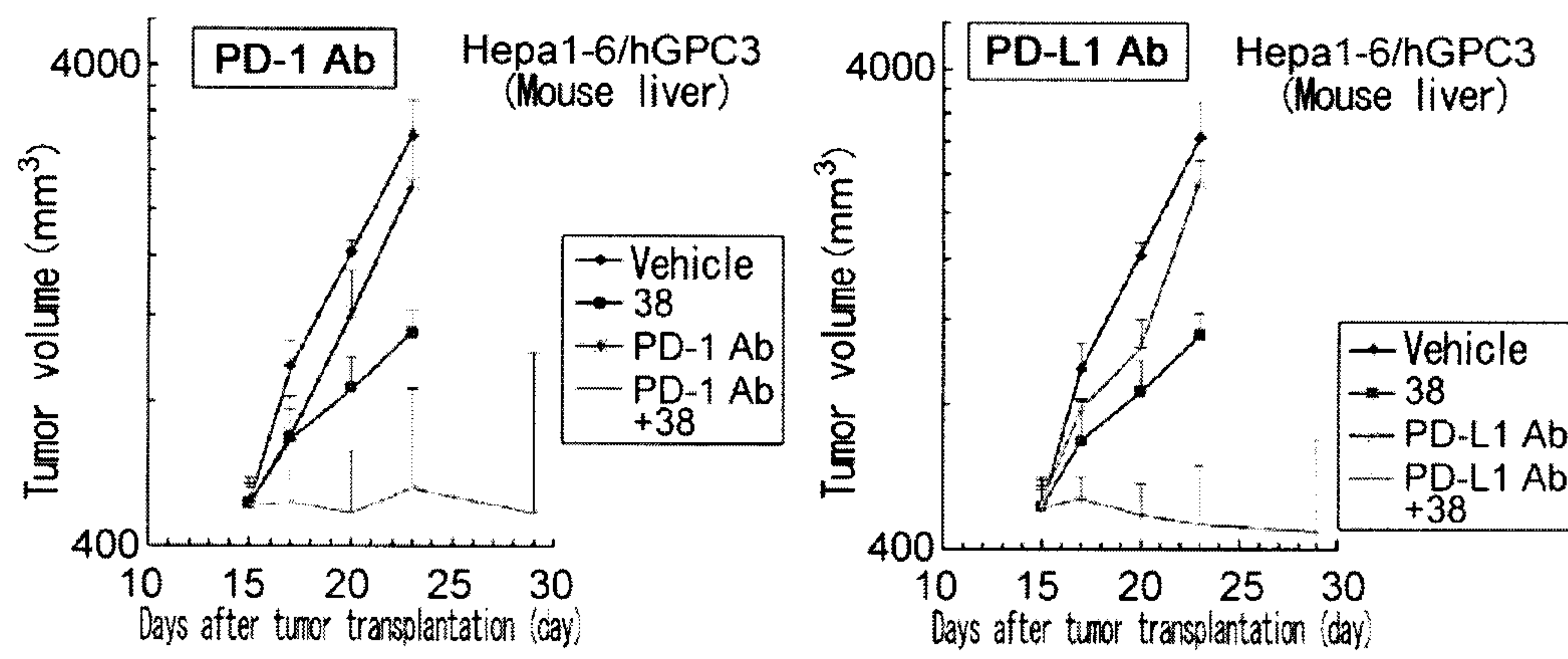
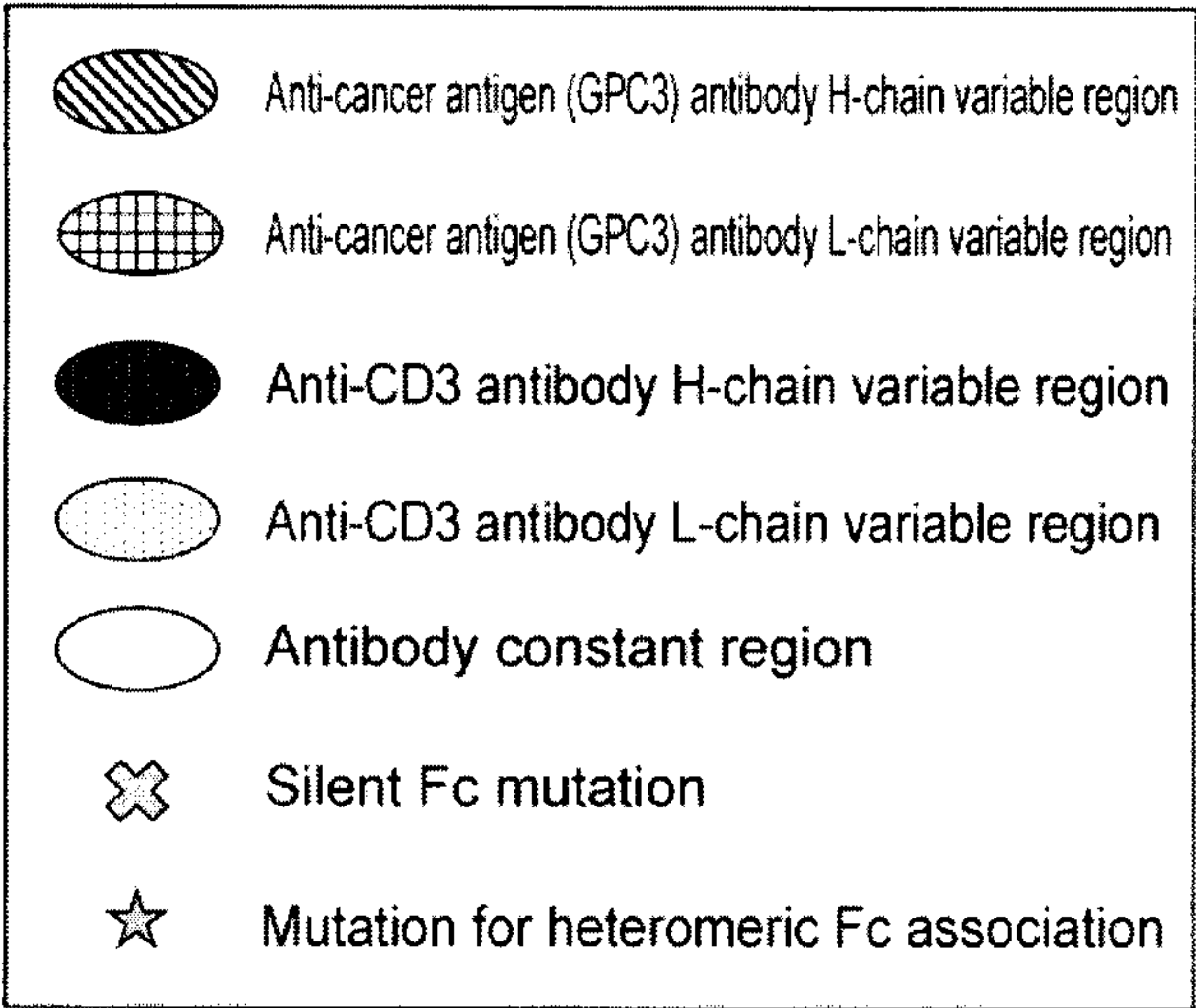
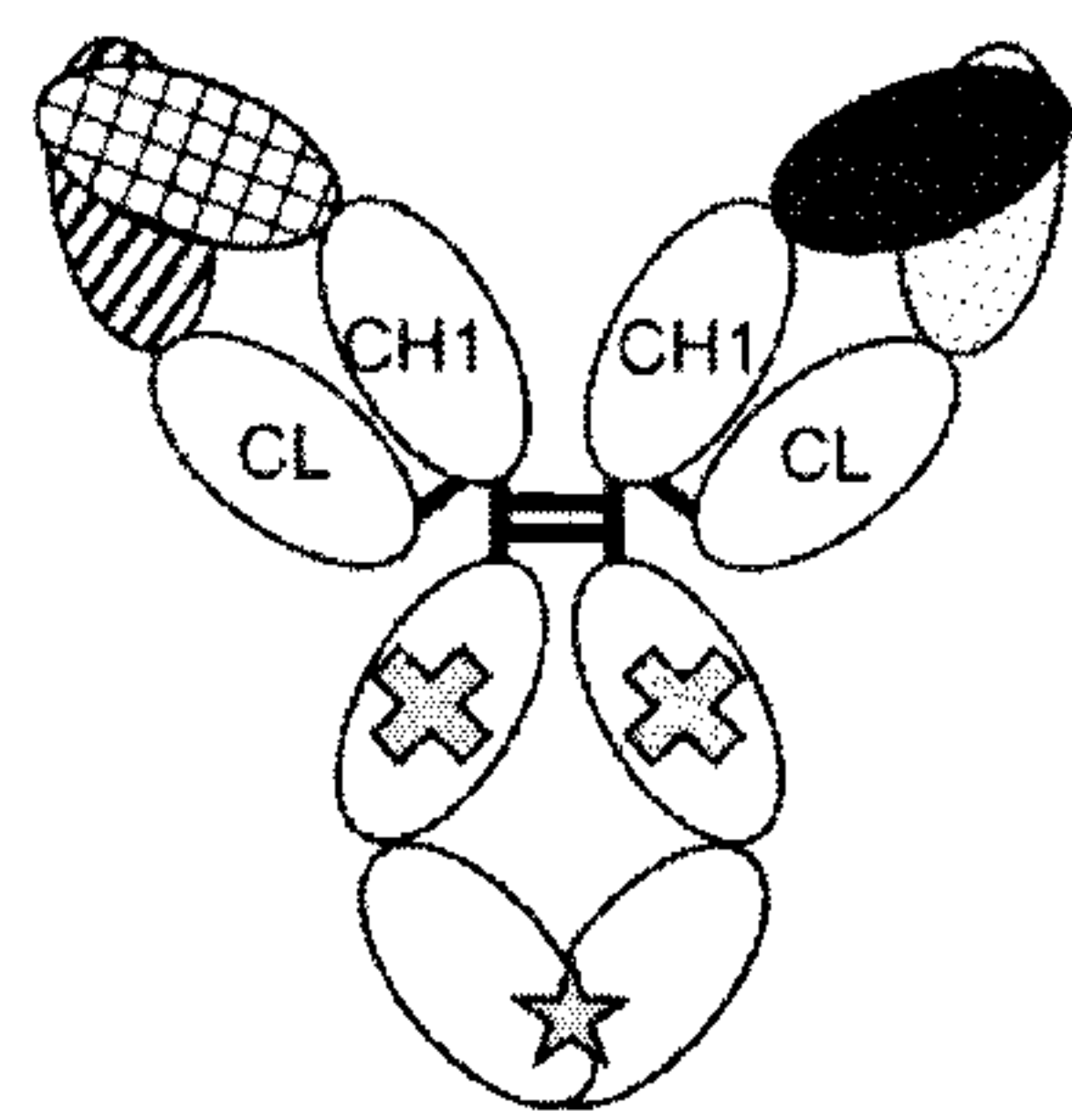


FIG. 11

a ERY22



b ERY27

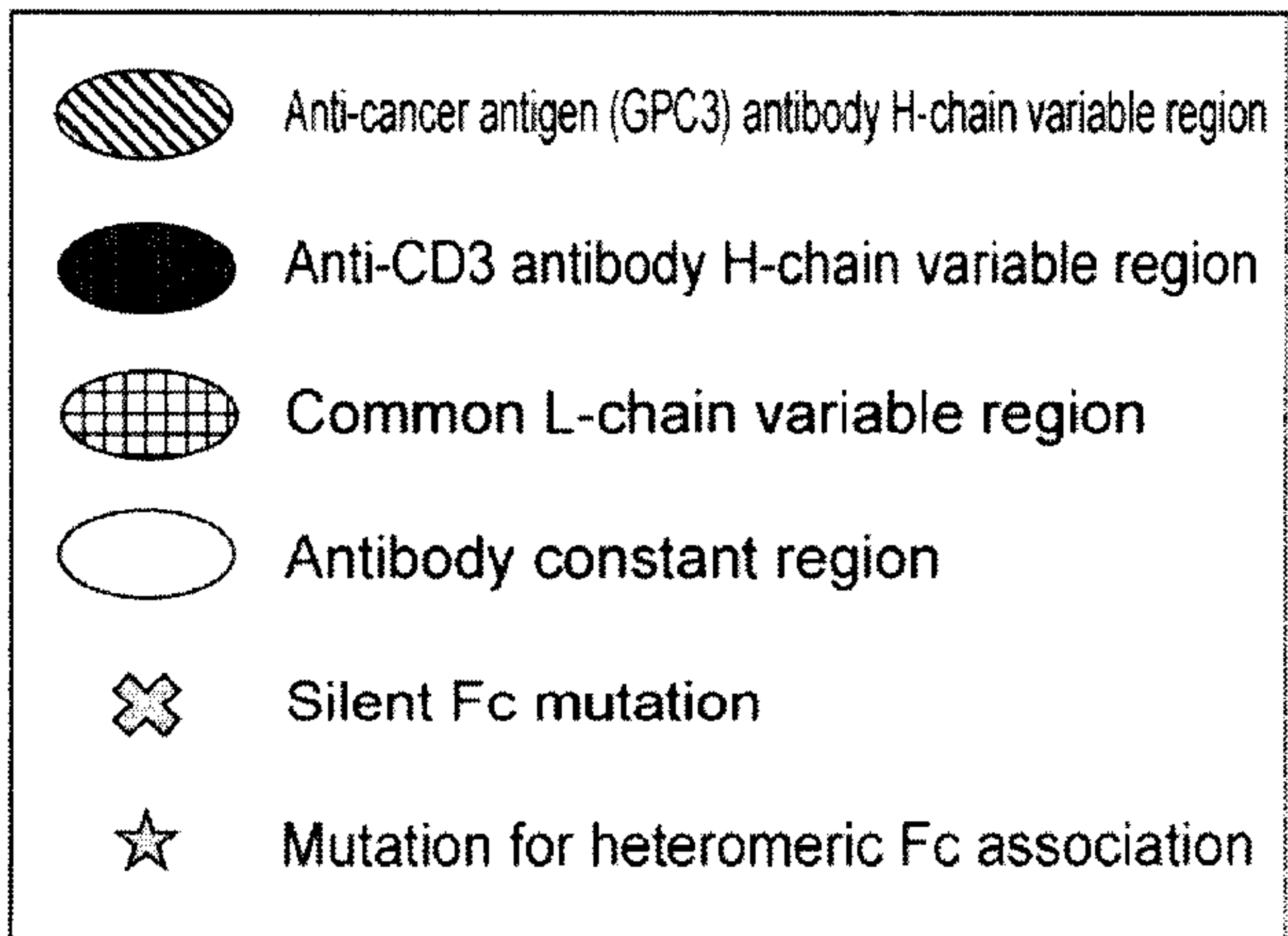
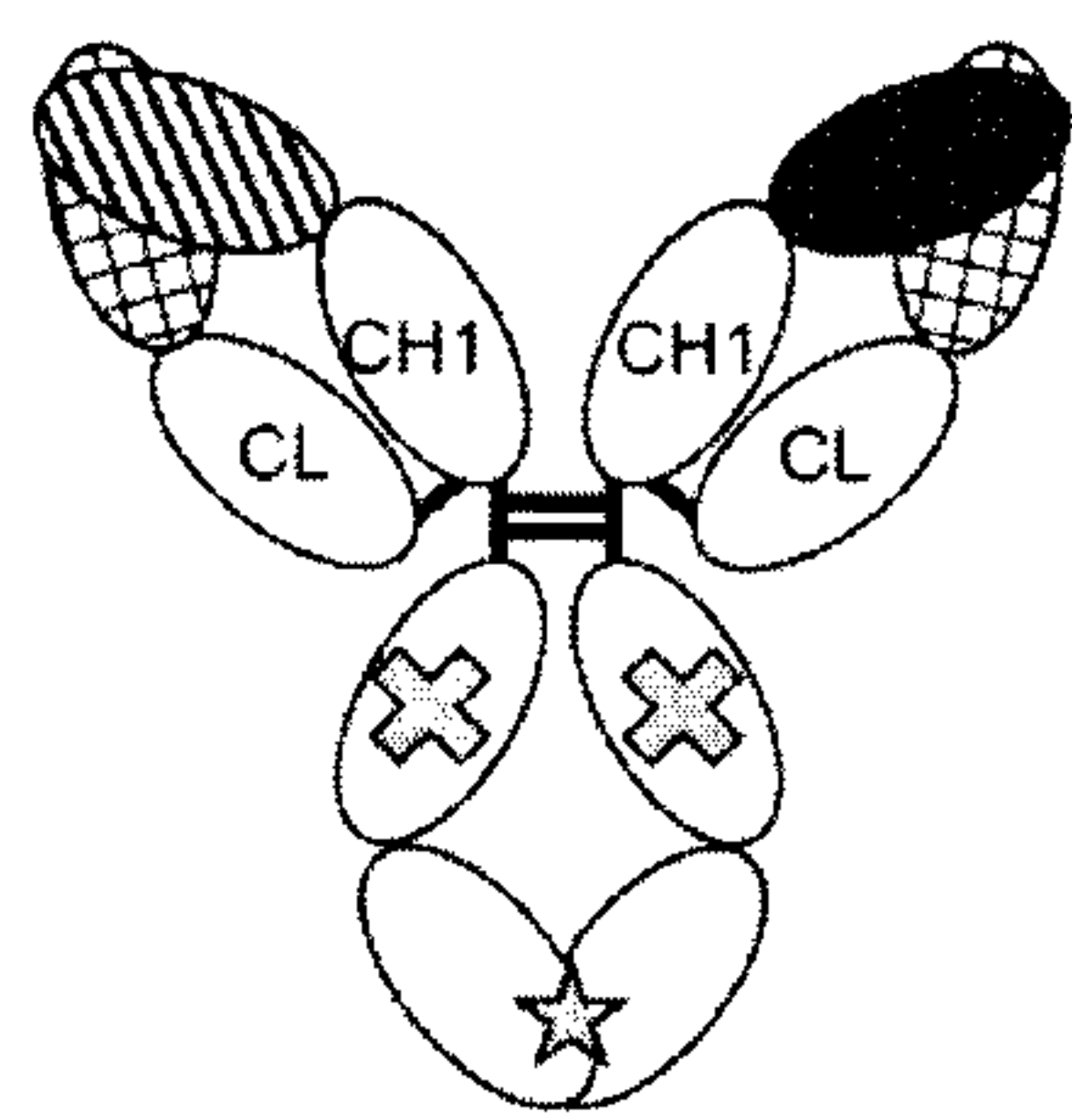


FIG. 12

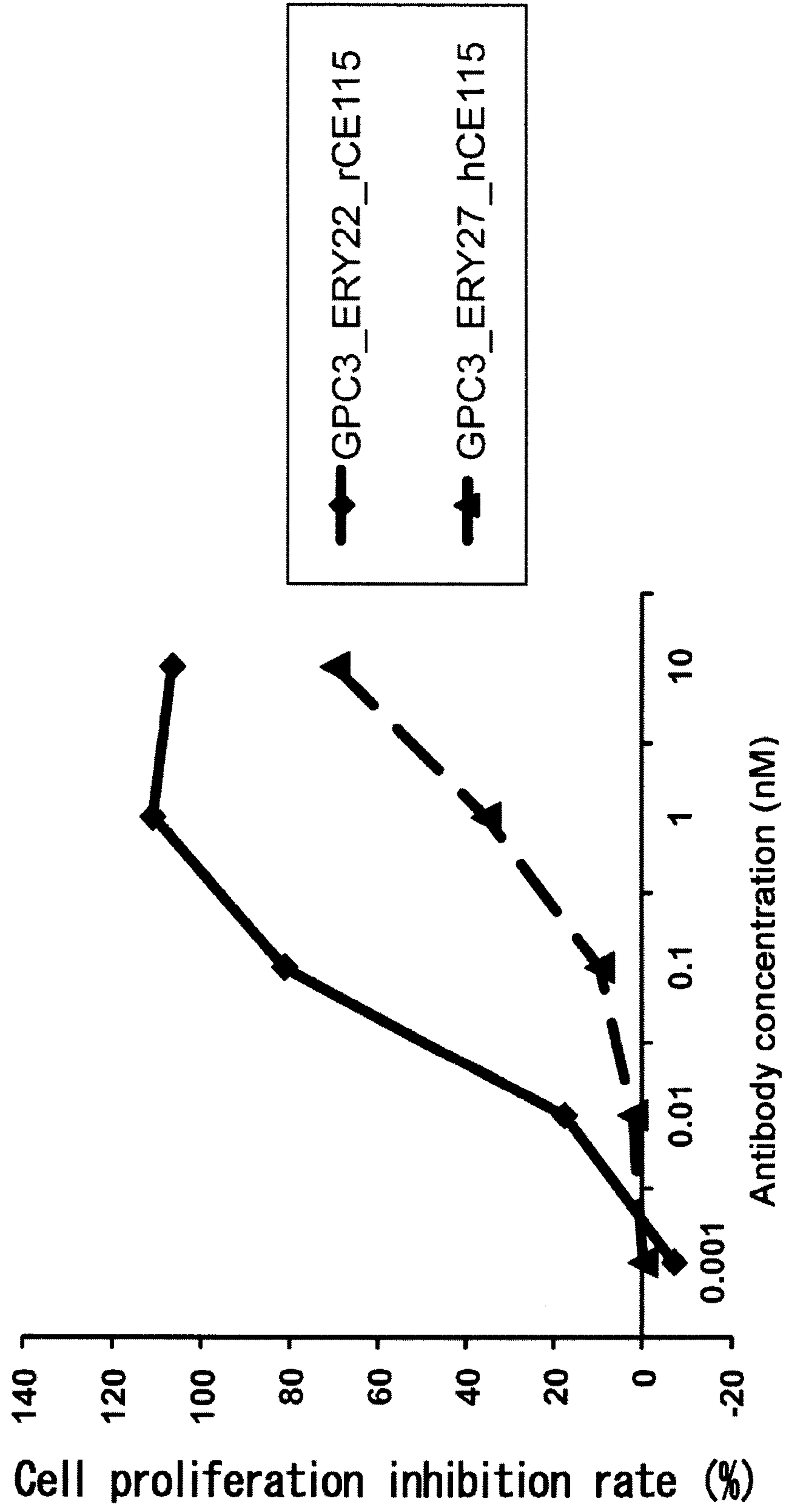


FIG. 13

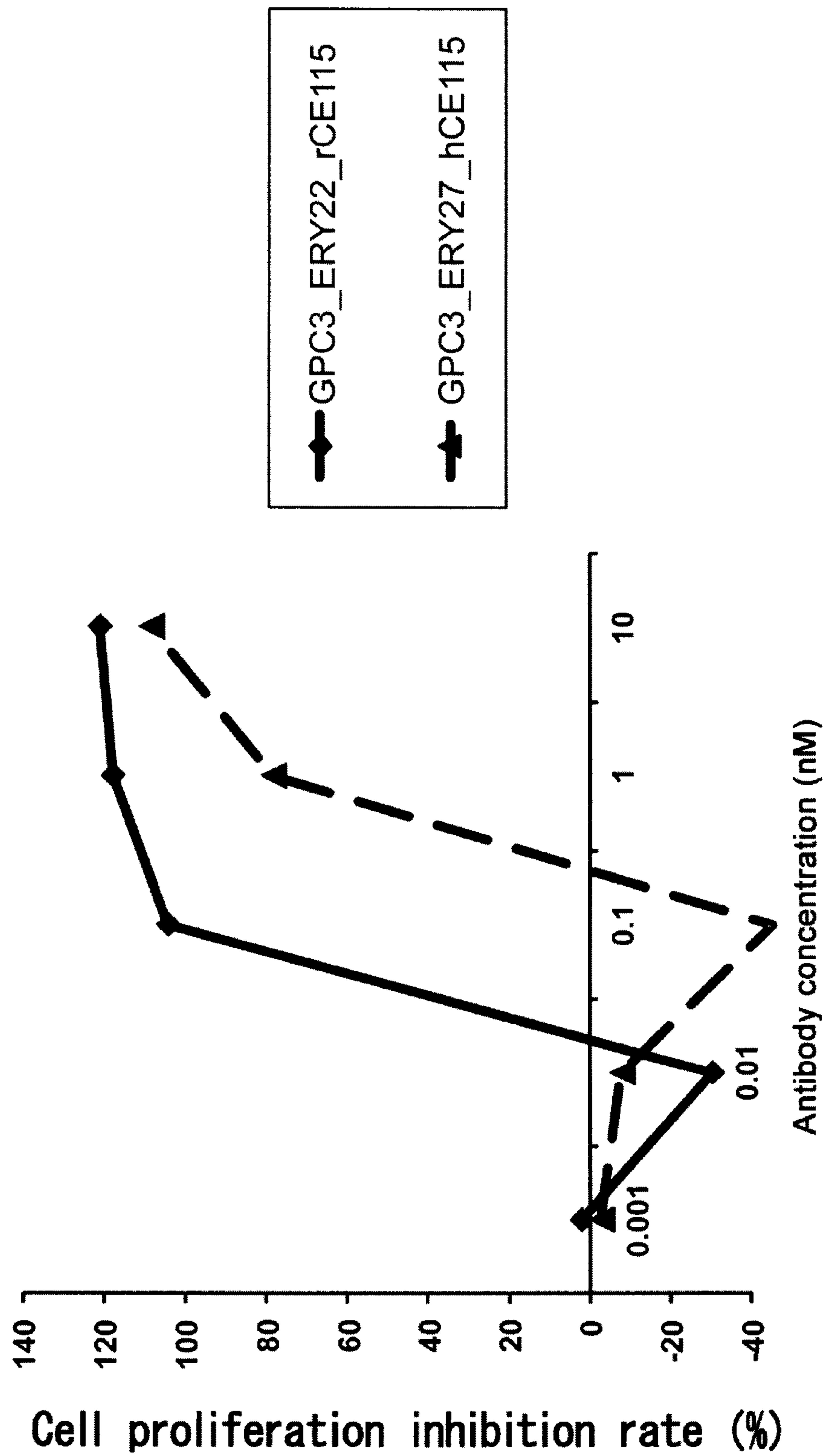


FIG. 14

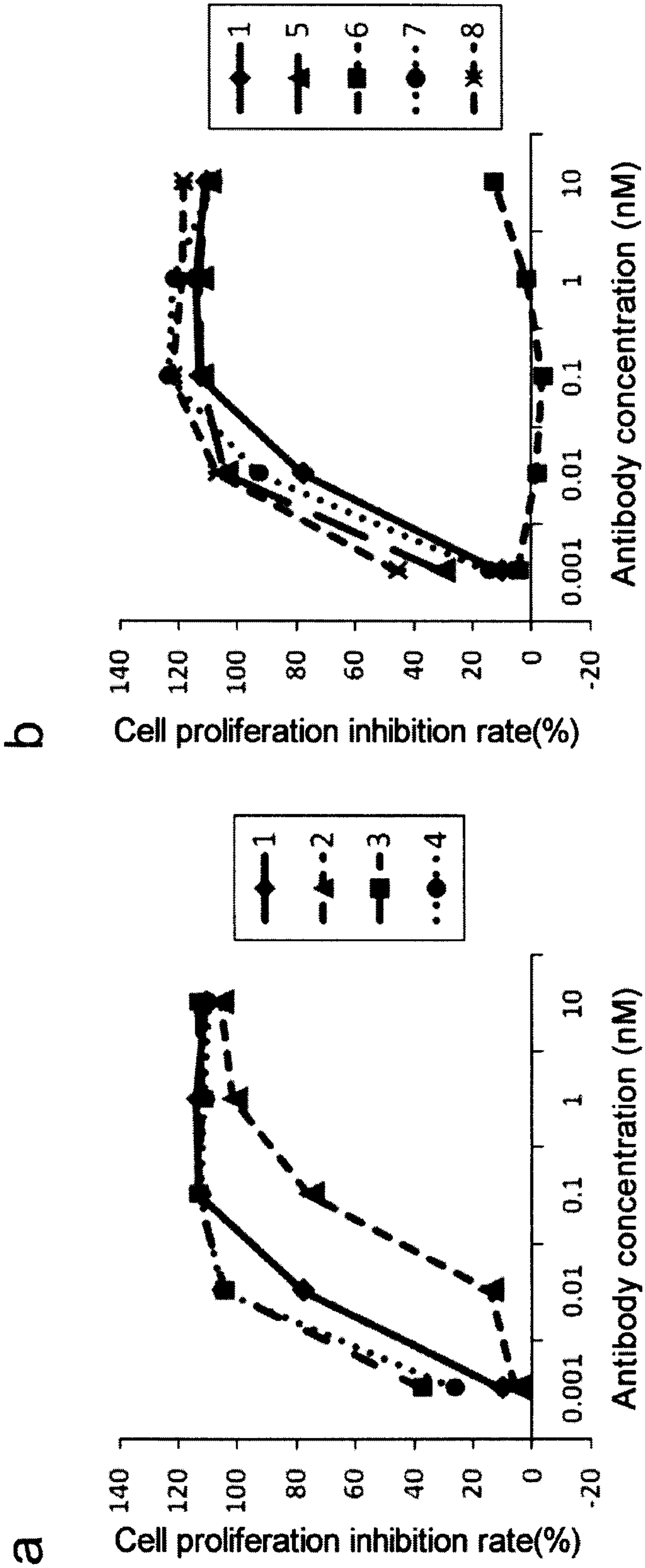


FIG. 15

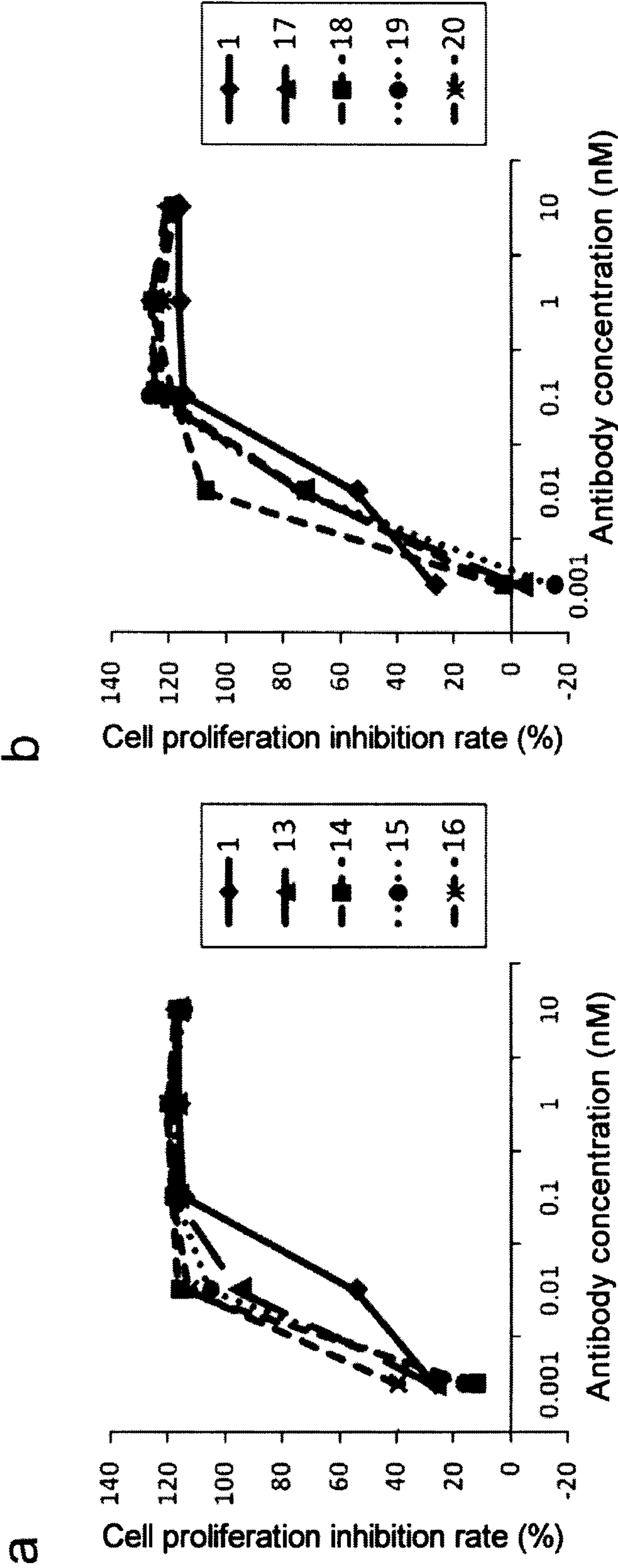


FIG. 16

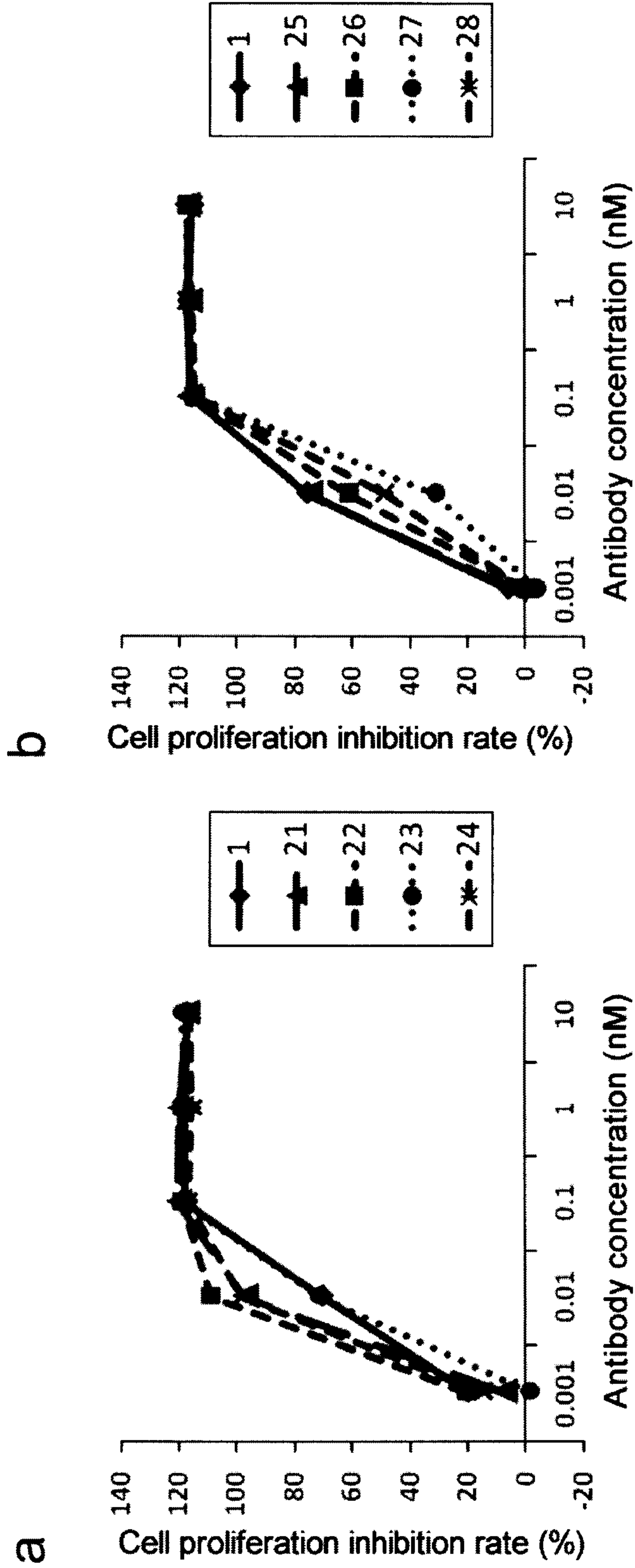


FIG. 17

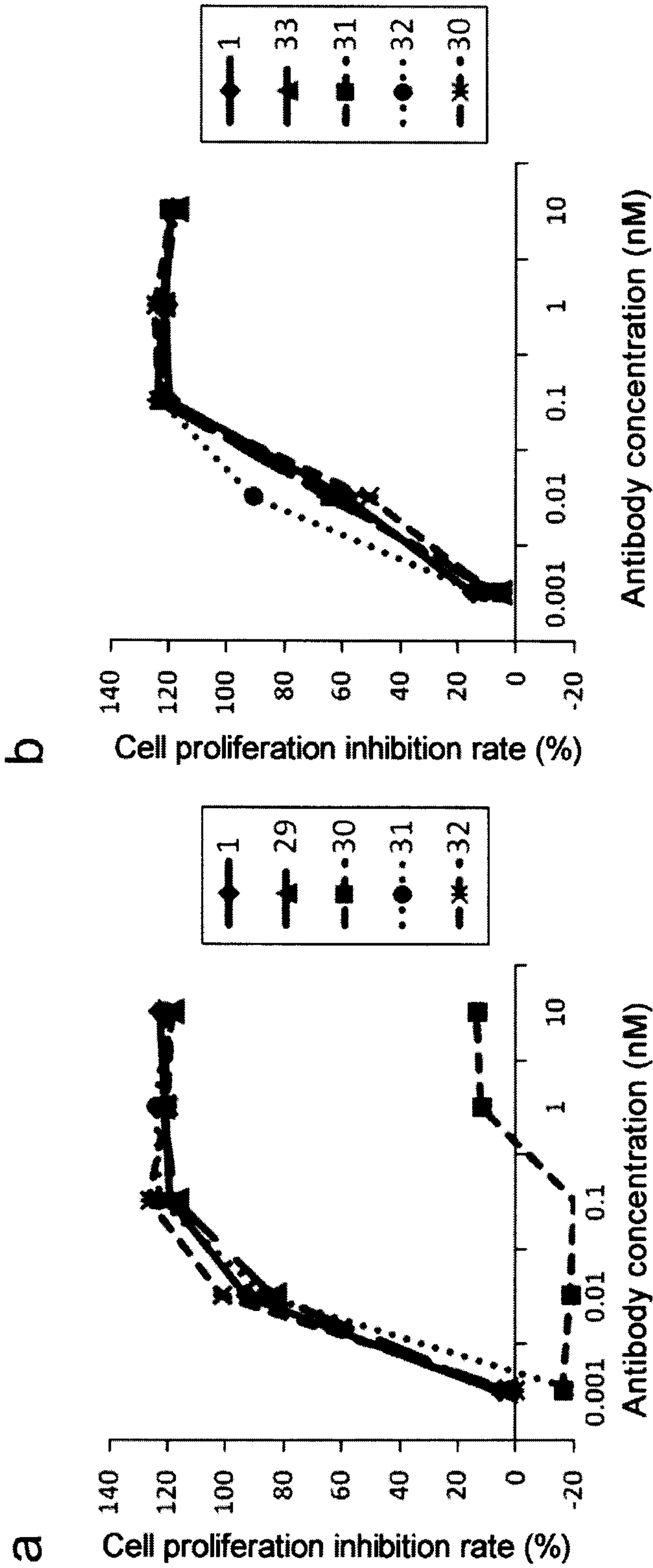


FIG. 18

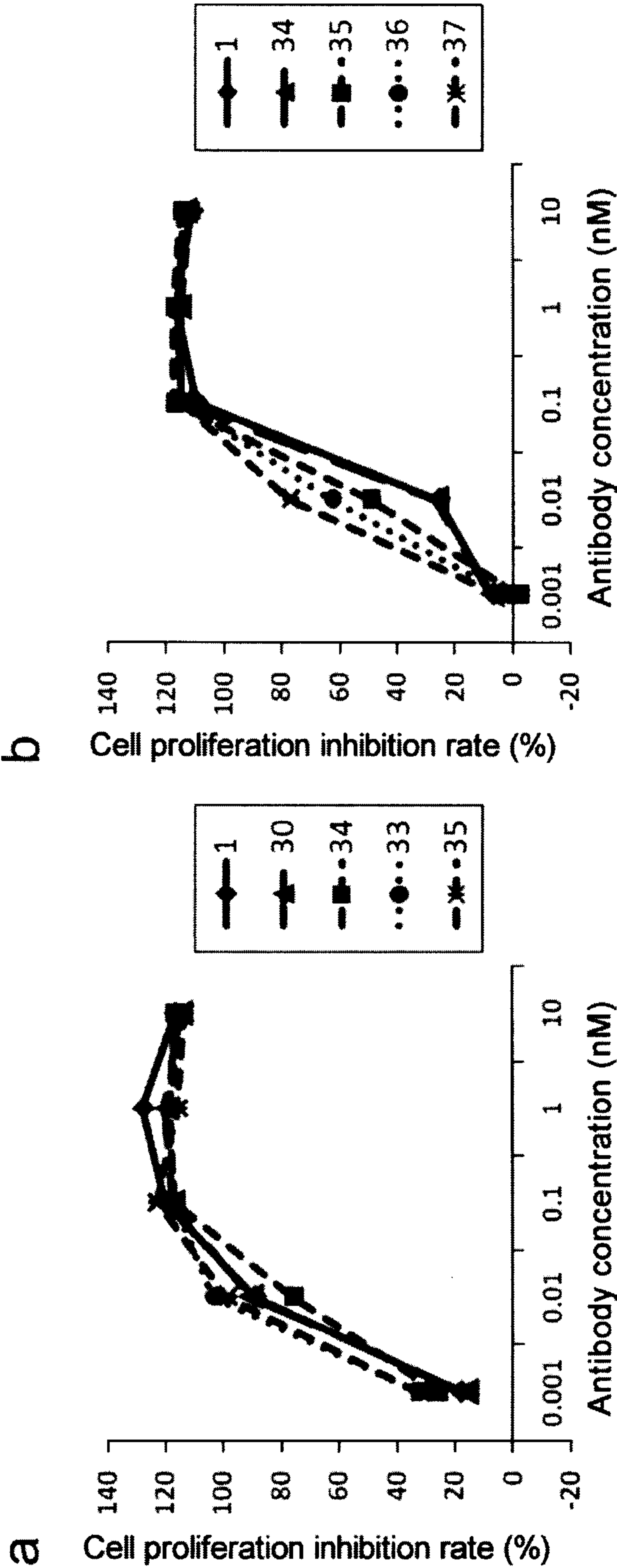


FIG. 19

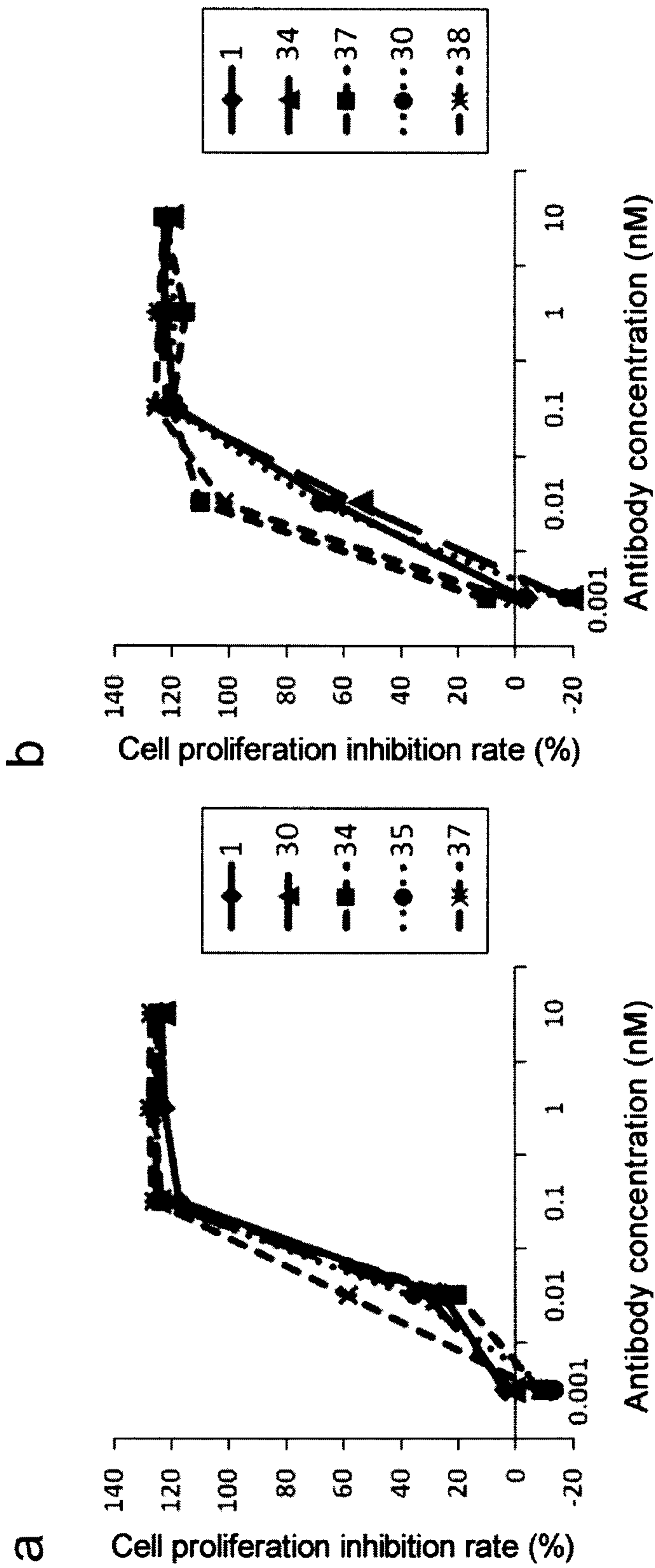


FIG. 20

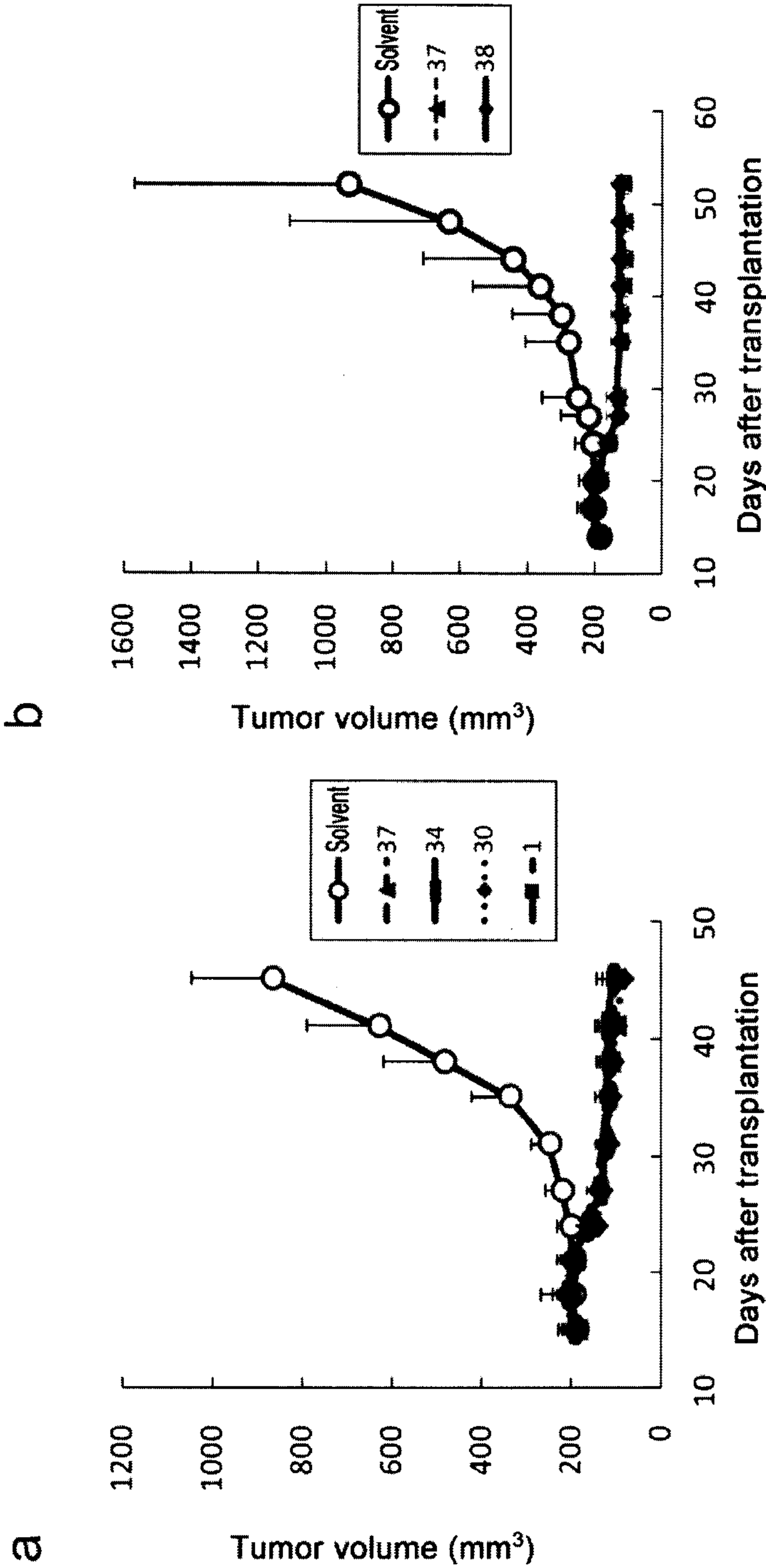


FIG. 21

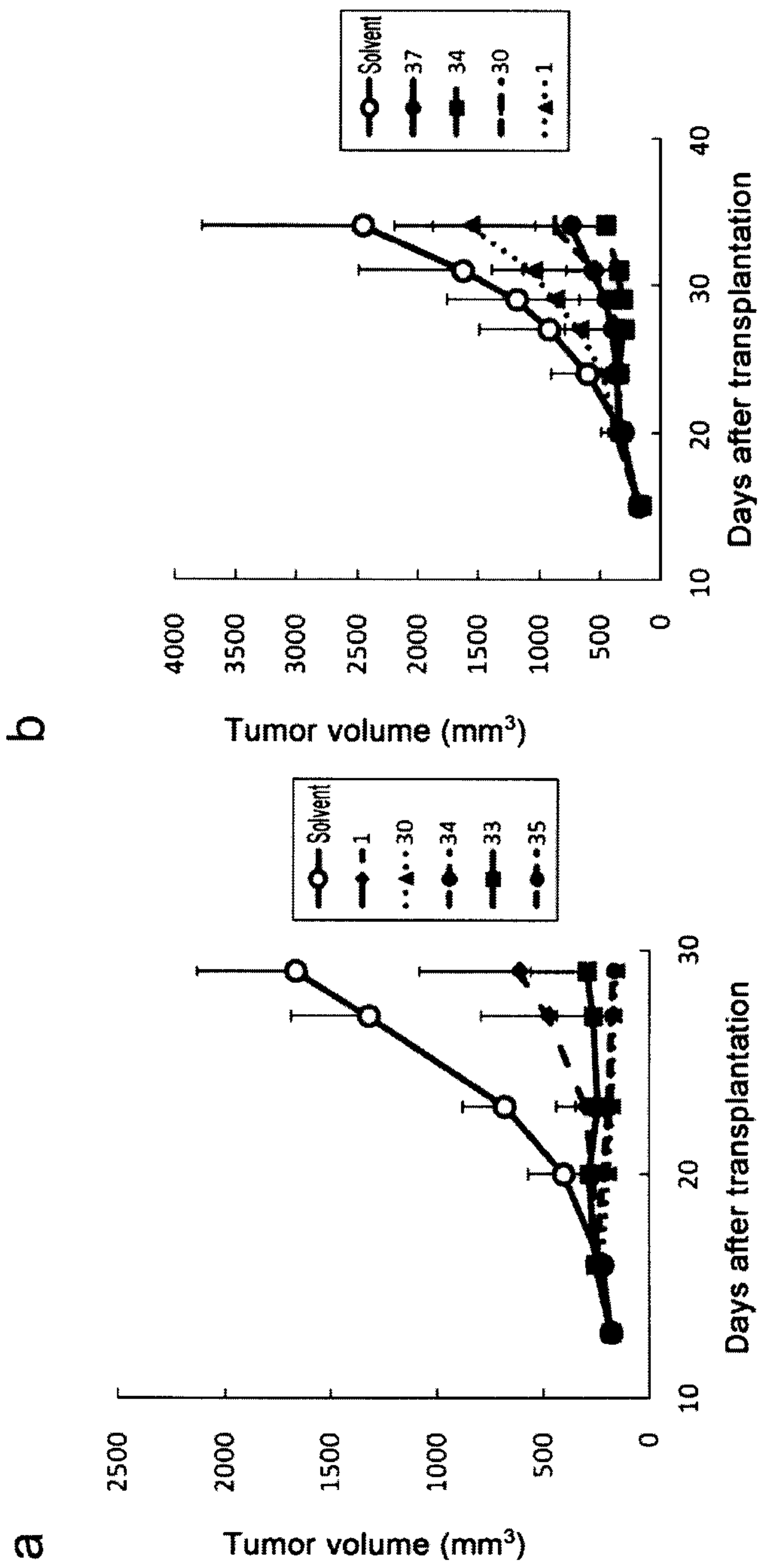


FIG. 22

[illegible]

FIG. 23

Antibody name	Chothia CDR H1																																							Kabat CDR H1			
	Contact CDR H1																																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	35a	35b	36	37	38	39	40	
CE115HA000	Q	V	Q	L	V	E	S	G	G	G	G	V	V	Q	P	G	R	S	L	R	L	S	C	A	A	S	G	F	T	F	S	N	A	W	M	H	-	-	W	V	R	Q	A
TR01H040	Q	V	Q	L	V	E	S	G	G	G	G	L	V	Q	P	G	R	S	L	R	L	S	C	A	A	S	G	F	T	F	S	N	A	W	M	H	-	-	W	V	R	Q	A
TR01H071	Q	V	Q	L	V	E	S	G	G	G	G	L	V	Q	P	G	R	S	L	R	L	S	C	A	A	S	G	F	T	F	S	N	A	W	M	H	-	-	W	V	R	Q	A
TR01H082	Q	V	Q	L	V	E	S	G	G	G	G	L	V	Q	P	G	R	S	L	R	L	S	C	A	A	S	G	F	T	F	S	N	A	W	M	H	-	-	W	V	R	Q	A
TR01H084	Q	V	Q	L	V	E	S	G	G	G	G	L	V	Q	P	G	R	S	L	R	L	S	C	A	A	S	G	F	T	F	S	N	A	W	M	H	-	-	W	V	R	Q	A
TR01H108	Q	V	Q	L	V	E	S	G	G	G	G	L	V	Q	P	G	R	S	L	R	L	S	C	A	A	S	G	F	T	F	S	N	A	W	M	H	-	-	W	V	R	Q	A
TR01H113	Q	V	Q	L	V	E	S	G	G	G	G	L	V	Q	P	G	R	S	L	R	L	S	C	A	A	S	G	F	T	F	S	N	A	W	M	H	-	-	W	V	R	Q	A
H0000	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	P	G	A	S	V	K	V	S	C	K	A	S	G	Y	T	F	T	D	Y	E	M	H	-	-	W	I	R	Q	P
H0610	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	P	G	A	S	V	K	V	S	C	K	A	S	G	Y	T	F	T	D	Y	E	M	H	-	-	W	I	R	Q	P
GCH054	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	P	G	A	S	V	K	V	S	C	K	A	S	G	Y	T	F	T	D	Y	E	M	H	-	-	W	I	R	Q	P
GCH085	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	P	G	A	S	V	K	V	S	C	K	A	S	G	Y	T	F	T	D	Y	E	M	H	-	-	W	I	R	Q	P
GCH094	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	P	G	A	S	V	K	V	S	C	K	A	S	G	Y	T	F	T	D	Y	E	M	H	-	-	W	V	R	Q	A

Antibody name	Chothia CDR H2																																							Kabat CDR H2			
	Contact CDR H2																																										
	41	42	43	44	45	46	47	48	49	50	51	52	52a	52b	52c	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	
CE115HA000	P	G	K	G	L	E	W	V	A	Q	I	K	A	K	S	N	N	Y	A	T	Y	Y	Y	A	E	S	V	K	G	R	F	T	I	S	R	D	D	S	K	N	S	L	Y
TR01H040	P	G	K	G	L	E	W	V	A	Q	I	K	D	K	S	Q	N	Y	A	T	Y	Y	Y	A	E	S	V	K	G	R	F	T	I	S	R	A	D	S	K	N	S	I	Y
TR01H071	P	G	K	G	L	E	W	V	A	Q	I	K	D	K	S	Q	N	Y	A	T	Y	Y	Y	A	E	S	V	K	G	R	F	T	I	S	R	A	D	S	K	N	S	I	Y
TR01H082	P	G	K	G	L	E	W	V	A	Q	I	K	D	K	S	Q	N	Y	A	T	Y	Y	Y	A	E	S	V	K	G	R	F	T	I	S	R	D	D	S	K	N	S	L	Y
TR01H084	P	G	K	G	L	E	W	V	A	Q	I	K	D	K	S	Q	N	Y	A	T	Y	Y	Y	A	E	S	V	K	G	R	F	T	I	S	R	A	D	S	K	N	S	I	Y
TR01H108	P	G	K	G	L	E	W	V	A	Q	I	K	D	K	S	Q	N	Y	A	T	Y	V	V	A	E	S	V	K	G	R	F	T	I	S	R	D	D	S	K	N	S	L	Y
TR01H113	P	G	K	G	L	E	W	V	A	Q	I	K	D	K	S	Q	N	Y	A	T	Y	V	V	A	E	S	V	K	G	R	F	T	I	S	R	A	D	S	K	N	S	I	Y
H0000	P	G	Q	G	L	E	W	I	G	A	I	D	P	-	-	K	T	G	D	T	A	Y	S	Q	K	F	K	G	R	V	T	L	T	A	D	K	S	T	S	T	A	Y	
H0610	P	G	Q	G	L	E	W	I	G	A	I	D	G	-	-	K	T	P	D	T	A	Y	S	Q	K	F	K	G	R	V	T	L	T	A	D	K	S	T	S	T	A	Y	
GCH054	P	G	E	G	L	E	W	I	G	A	I	D	G	-	-	E	T	P	D	T	A	Y	S	E	K	F	K	G	R	V	T	L	T	A	D	K	S	T	S	T	A	Y	
GCH085	P	G	E	G	L	E	W	I	G	A	I	D	G	-	-	E	T	P	D	T	A	Y	S	E	K	F	K	G	R	V	T	L	T	A	D	K	S	T	S	T	A	Y	
GCH094	P	G	E	G	L	E	W	M	G	A	I	D	G	-	-	E	T	P	D	T	A	Y	S	E	K	F	K	G	R	V	T	L	T	A	D	K	S	T	S	T	A	Y	

FIG. 24-1

[illegible]

Antibody name	108	109	110	111	112	113
CE115HA000	T	V	T	V	S	S
TR01H040	T	V	T	V	S	S
TR01H071	T	V	T	V	S	S
TR01H082	T	V	T	V	S	S
TR01H084	T	V	T	V	S	S
TR01H109	T	V	T	V	S	S
TR01H113	T	V	T	V	S	S
H0000	L	V	T	V	S	S
H0810	L	V	T	V	S	S
GC-H054	L	V	T	V	S	S
GC-H085	L	V	T	V	S	S
GC-H084	L	V	T	V	S	S

FIG. 24-2

Kabat CDR L1																																										
Chothia CDR L1																																										
Antibody name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	27a	27b	27c	27d	27e	27f	28	29	30	31	32	33	34	35	36
L0000	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	S	L	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0011	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	P	L	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0201	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	P	P	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0203	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	S	P	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0204	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	S	P	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0206	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	S	P	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0208	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	S	P	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0209	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	S	P	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0211	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	P	P	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0212	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	S	P	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0222	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	S	P	V	H	S	-	N	R	N	T	Y	L	H	W	Y
L0272	D	I	V	M	T	Q	S	P	L	S	L	P	V	T	P	G	E	P	A	S	I	S	C	R	S	S	Q	P	L	V	H	S	-	N	R	Q	T	Y	L	H	W	Y

Kabat CDR L2																									
Chothia CDR L2																									
Contact CDR L2																									

Antibody name	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78
L0000	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0011	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0201	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0203	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0204	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0206	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0208	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0209	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0211	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0212	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0222	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V
L0272	Q	Q	K	P	G	Q	A	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V

Kabat CDR L3																									
Chothia CDR L3																									
Contact CDR L3																									

Antibody name	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	95a	95b	95c	95d	95e	95f	96	97	98	99	100	101	102	103	104	105	106	107
L0000	E	A	E	D	V	G	V	Y	Y	C	G	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K
L0011	E	A	E	D	V	G	V	Y	Y	C	G	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K
L0201	E	A	E	D	V	G	V	Y	Y	C	G	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K
L0203	E	A	E	D	V	G	V	Y	Y	C	G	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K
L0204	E	A	E	D	V	G	V	Y	Y	C	G	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K
L0206	E	A	E	D	V	G	V	Y	Y	C	Y	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K
L0208	E	A	E	D	V	G	V	Y	Y	C	Y	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K
L0211	E	A	E	D	V	G	V	Y	Y	C	Y	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K
L0212	E	A	E	D	V	G	V	Y	Y	C	Y	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K
L0222	E	A	E	D	V	G	V	Y	Y	C	Y	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K
L0272	E	A	E	D	V	G	V	Y	Y	C	G	Q	G	T	Q	V	P	-	-	-	-	-	-	Y	T	F	G	Q	G	T	K	L	E	I	K

FIG. 25

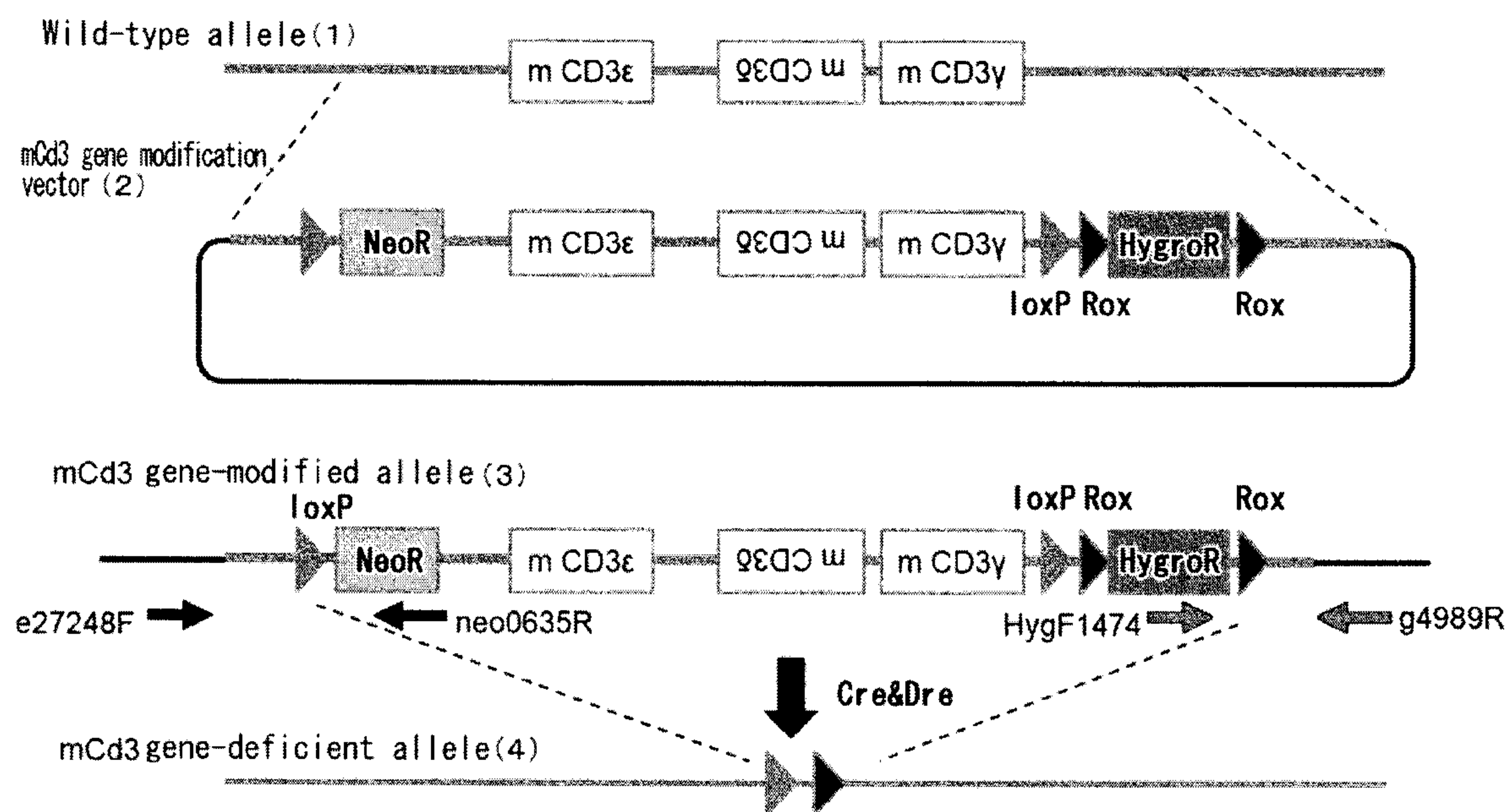


FIG. 26A

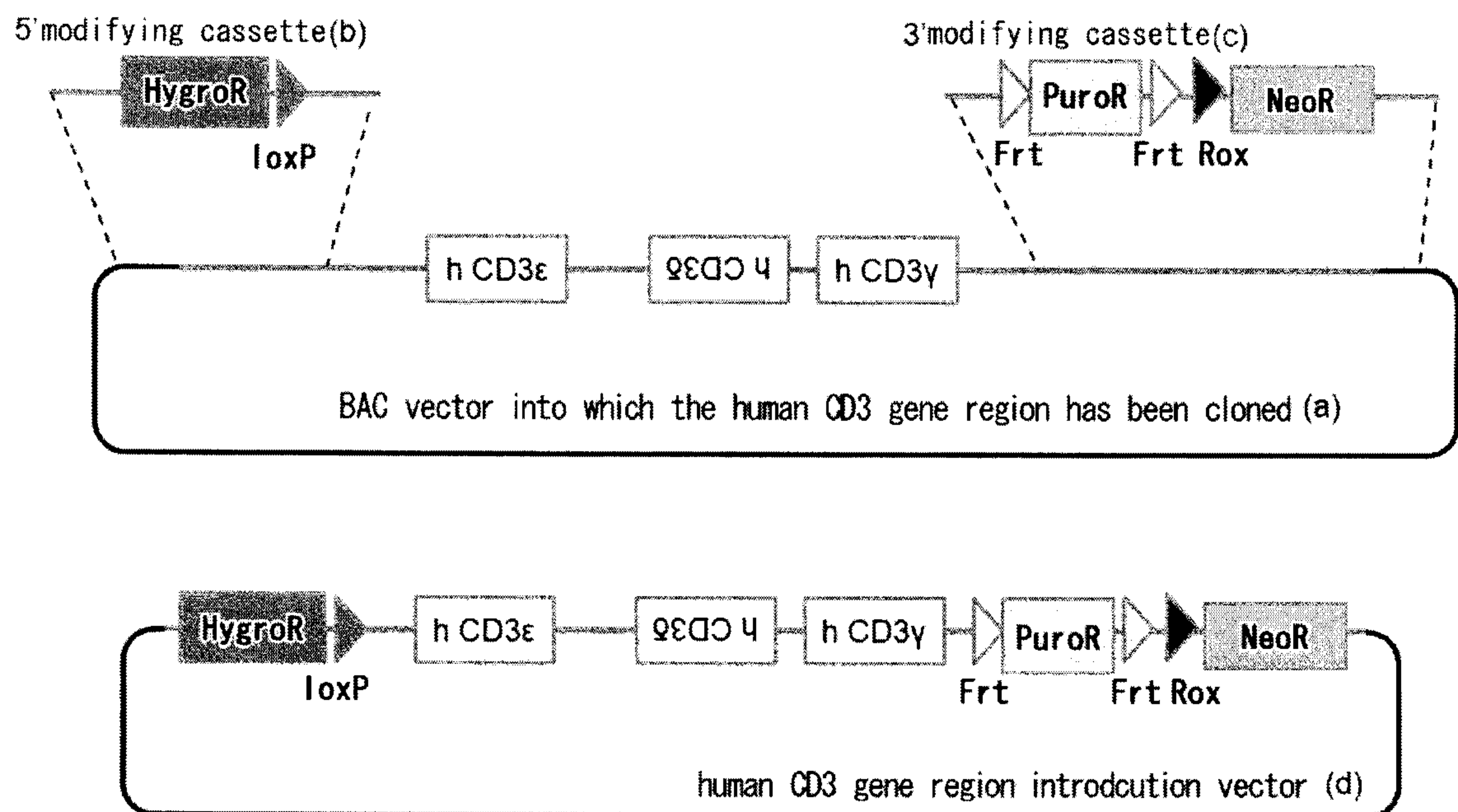


FIG. 26B

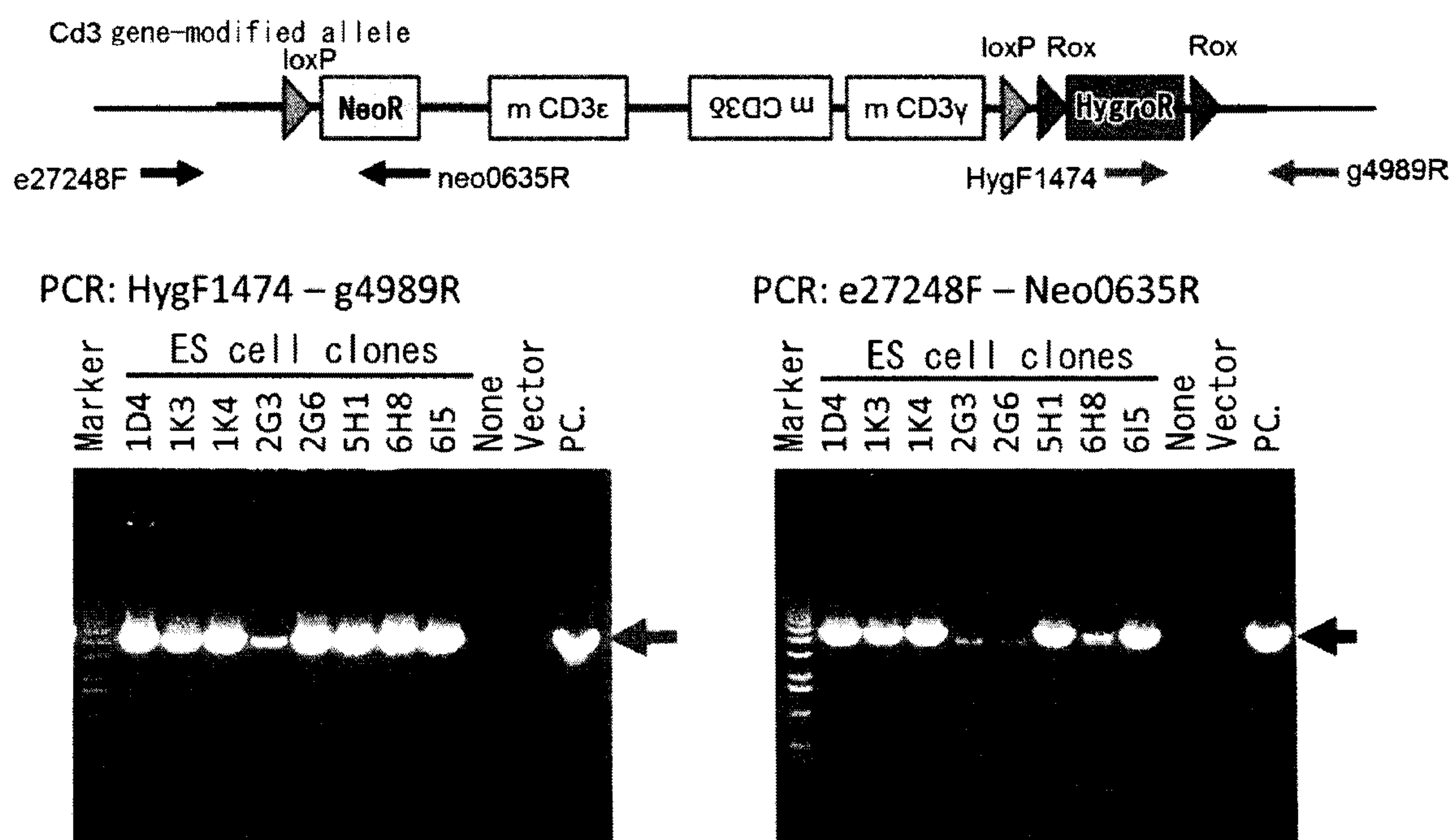


FIG. 27

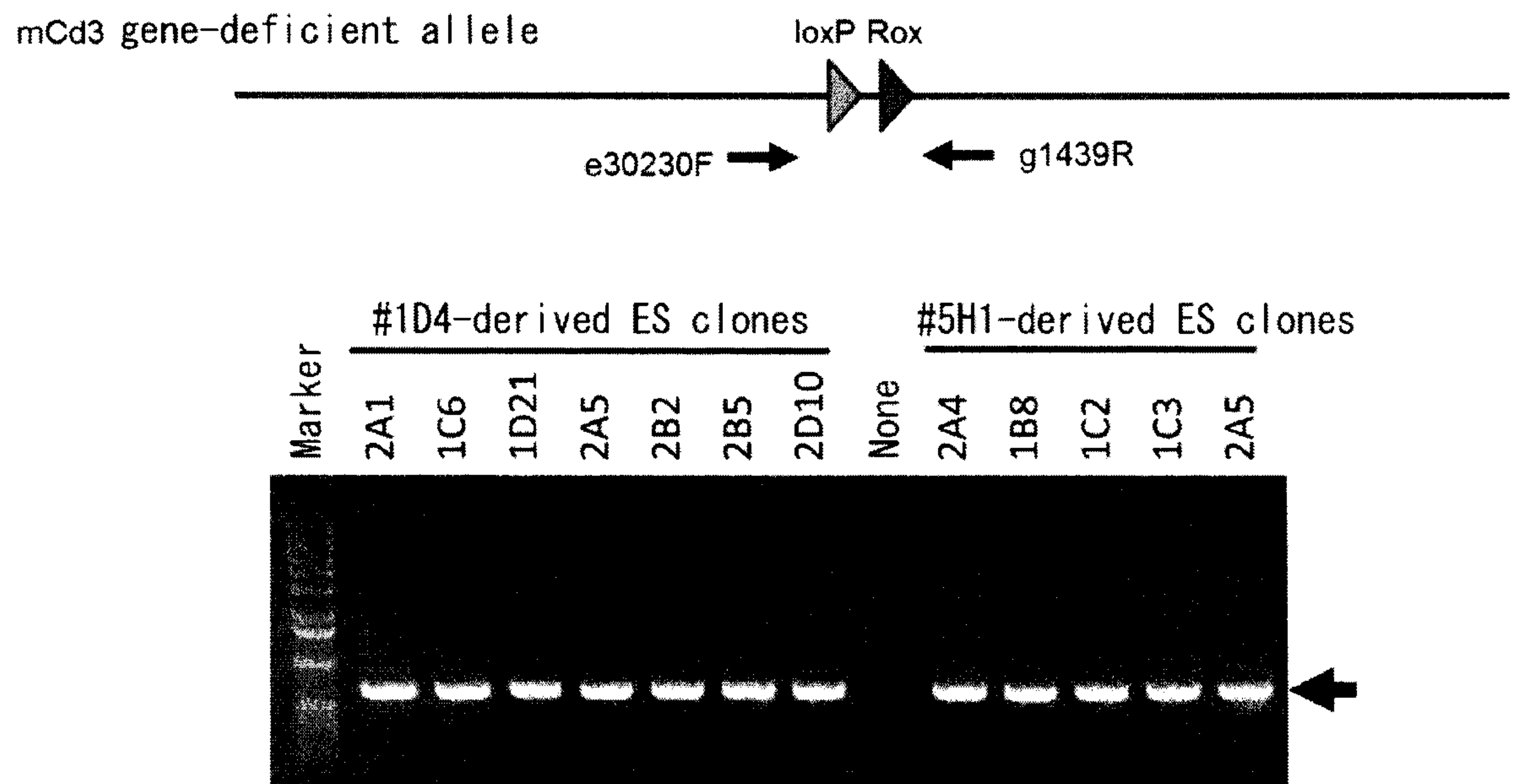


FIG. 28A

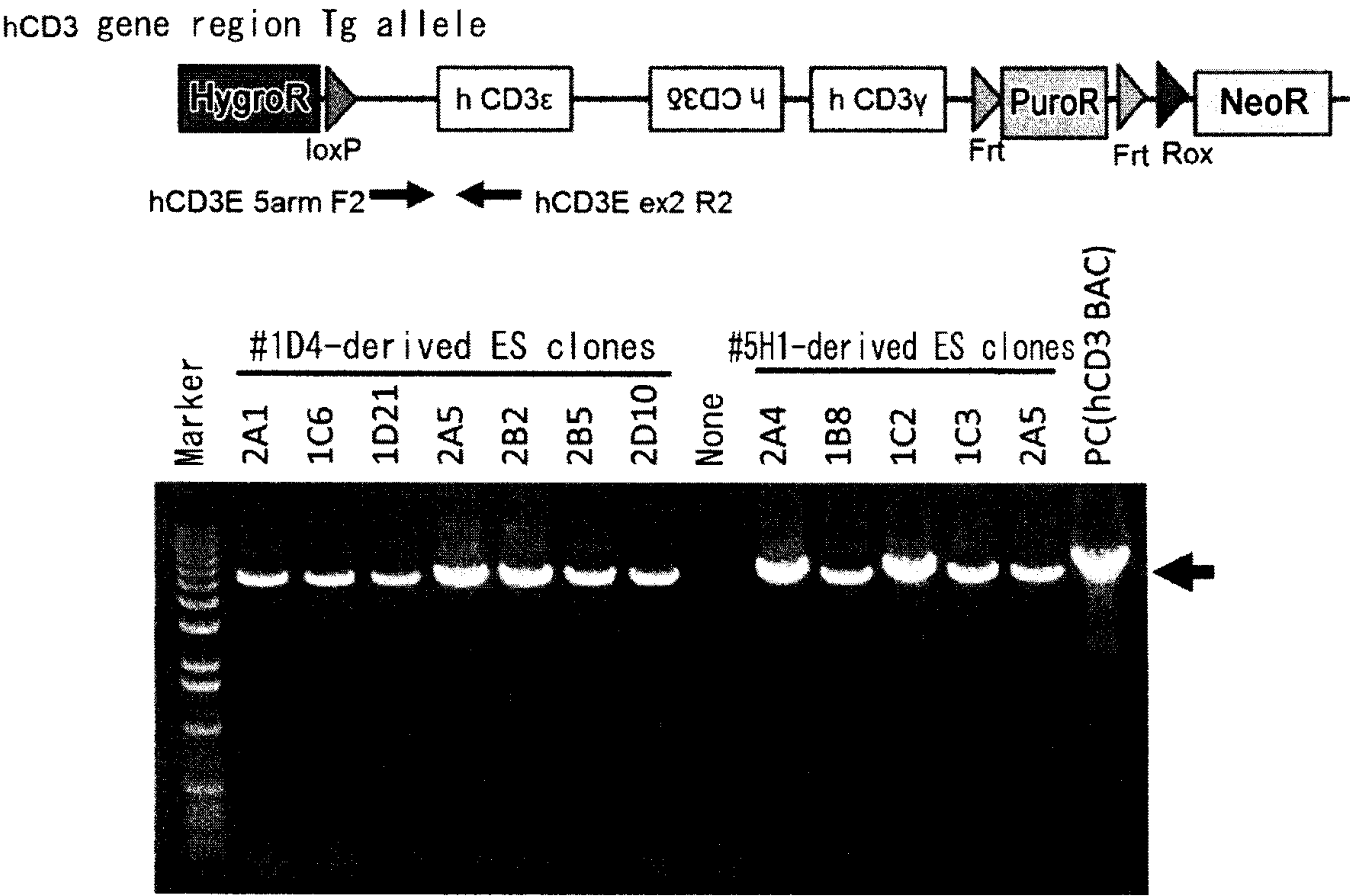


FIG. 28B

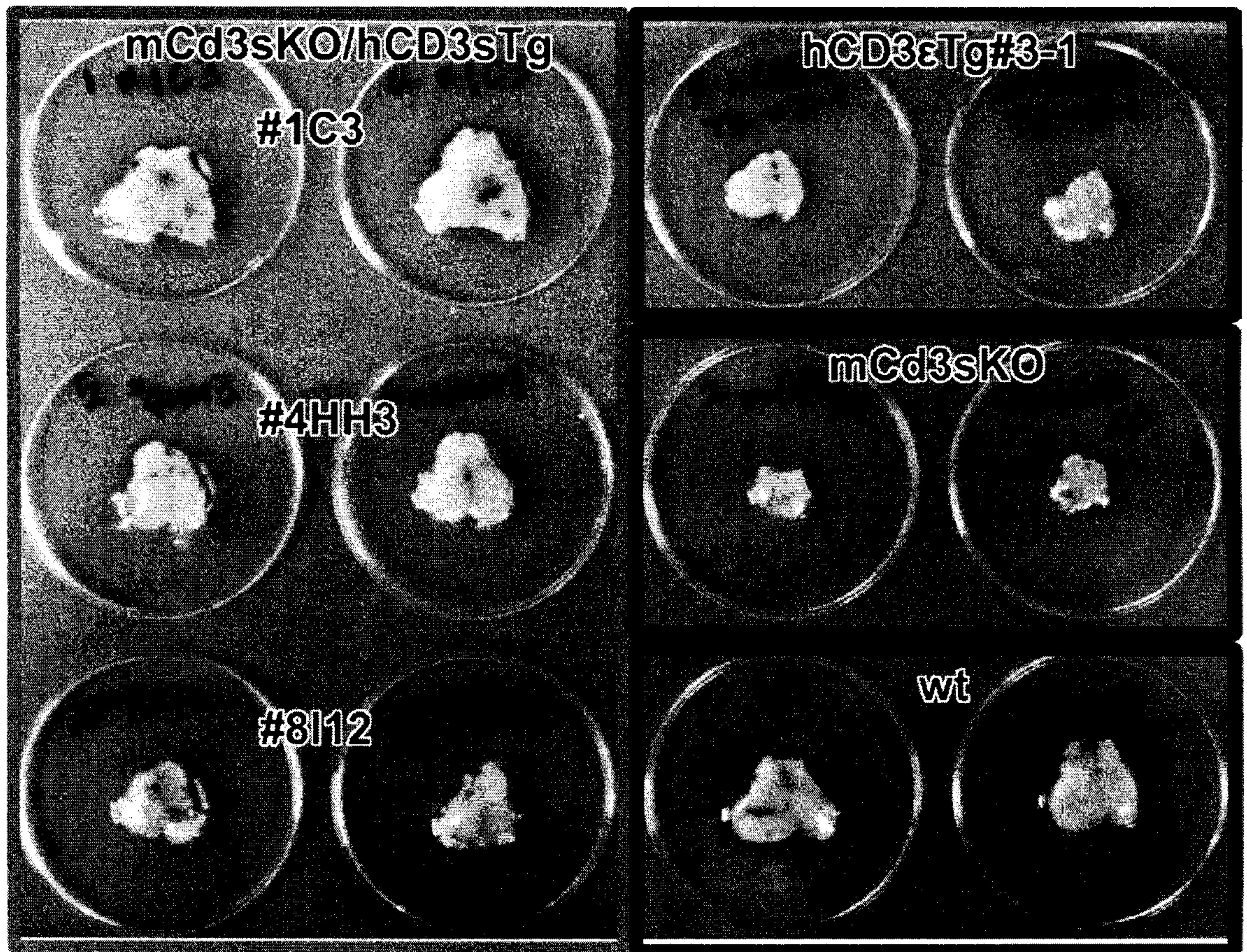


FIG. 29

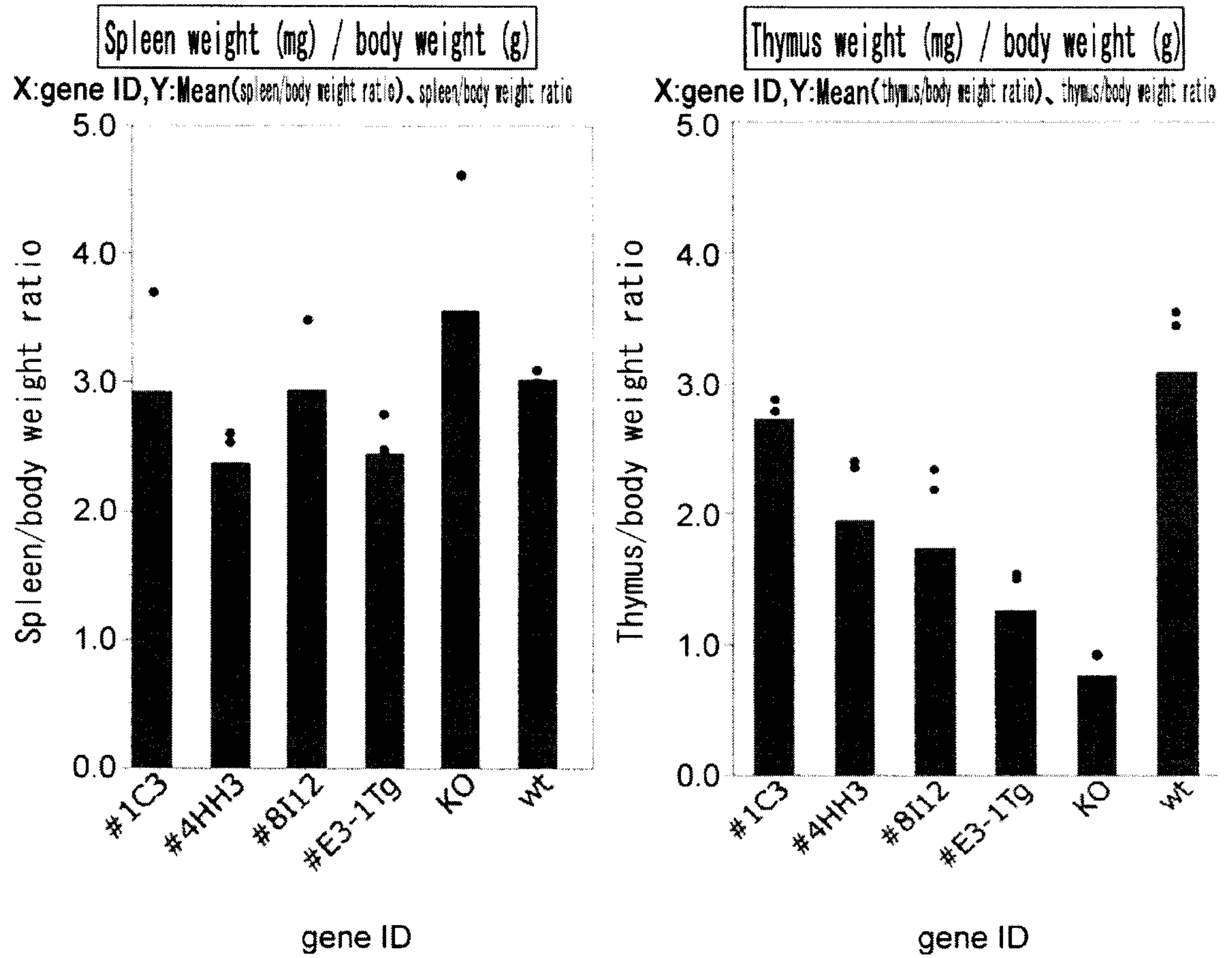


FIG. 30

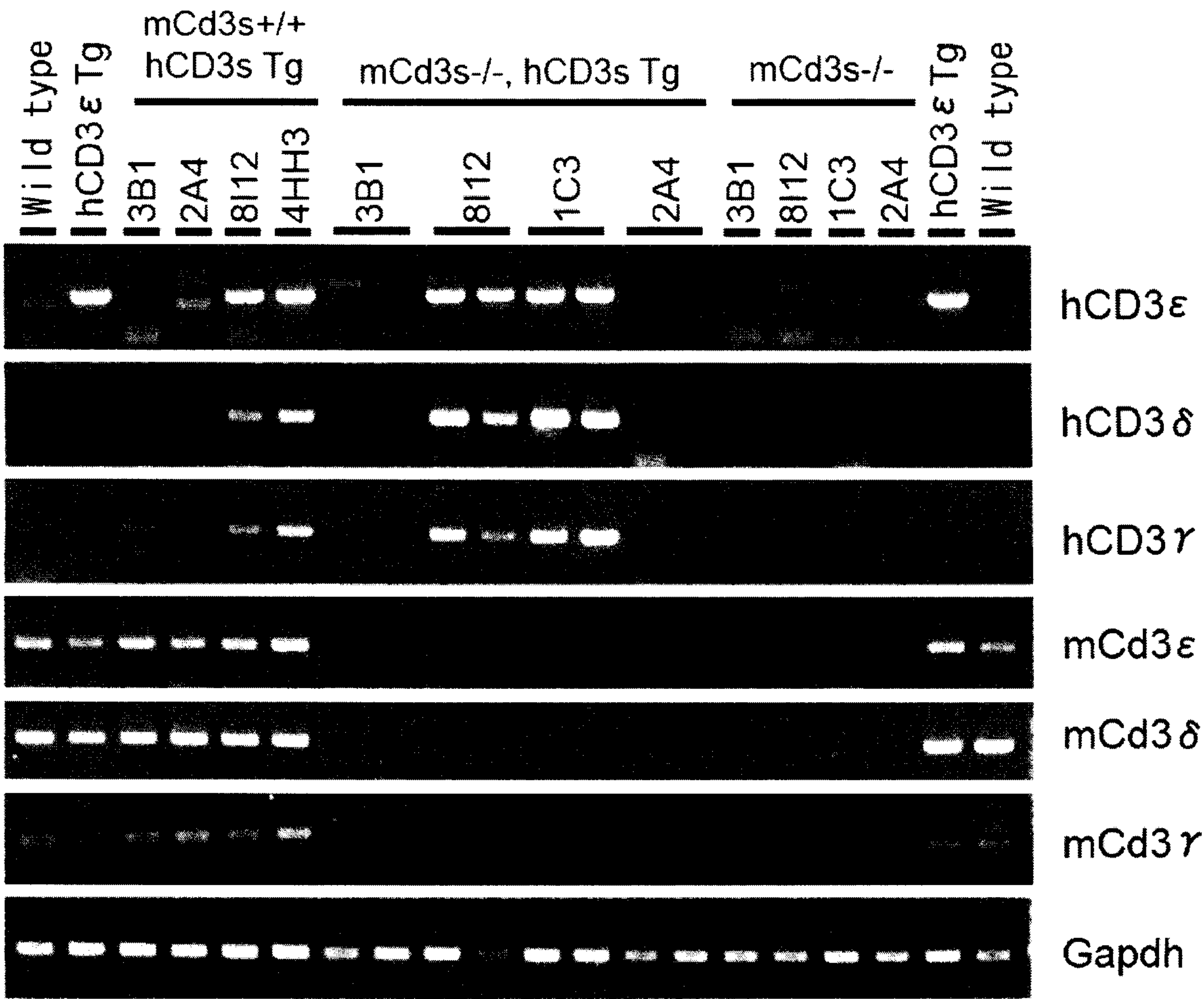


FIG. 31

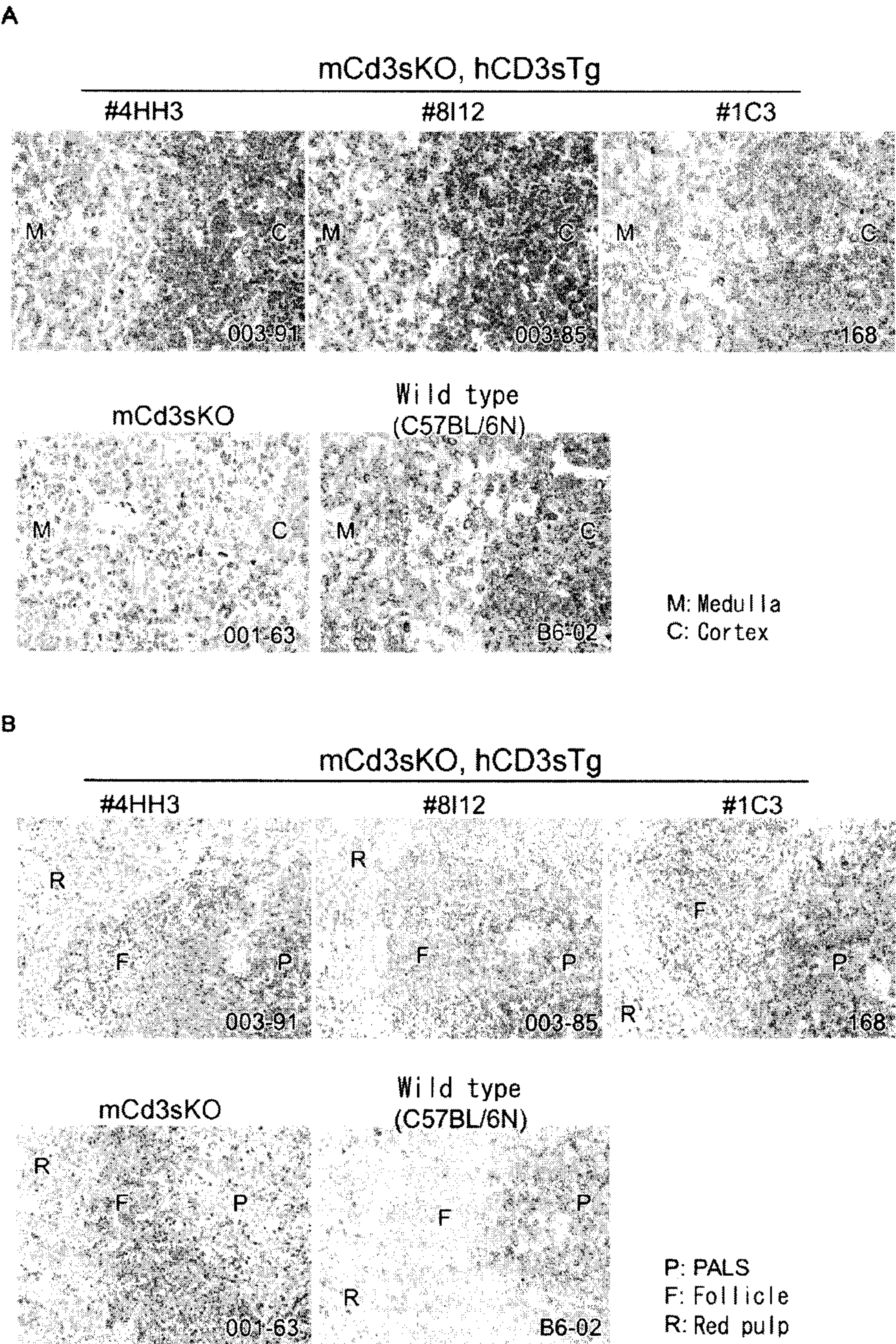


FIG. 32

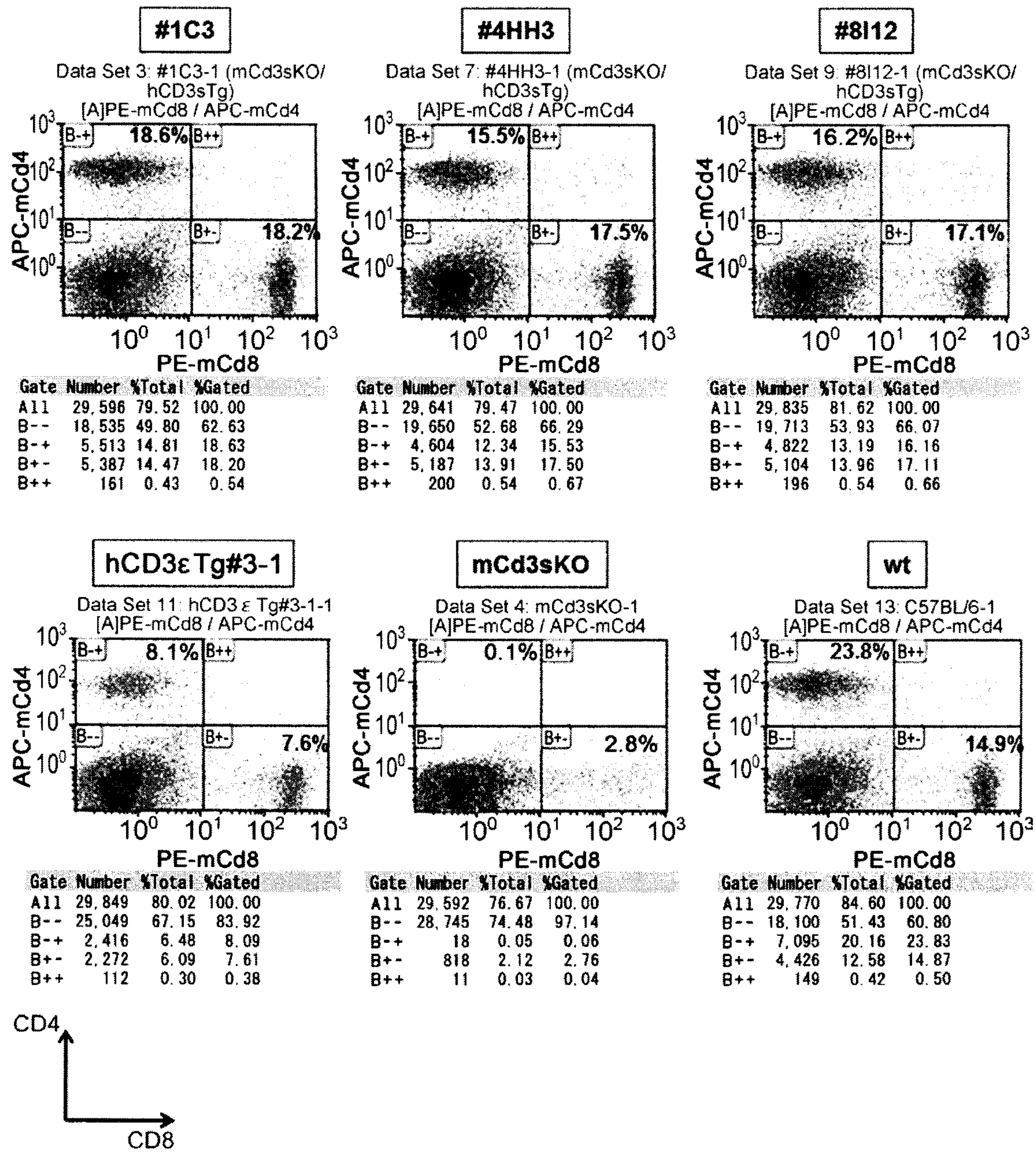


FIG. 33

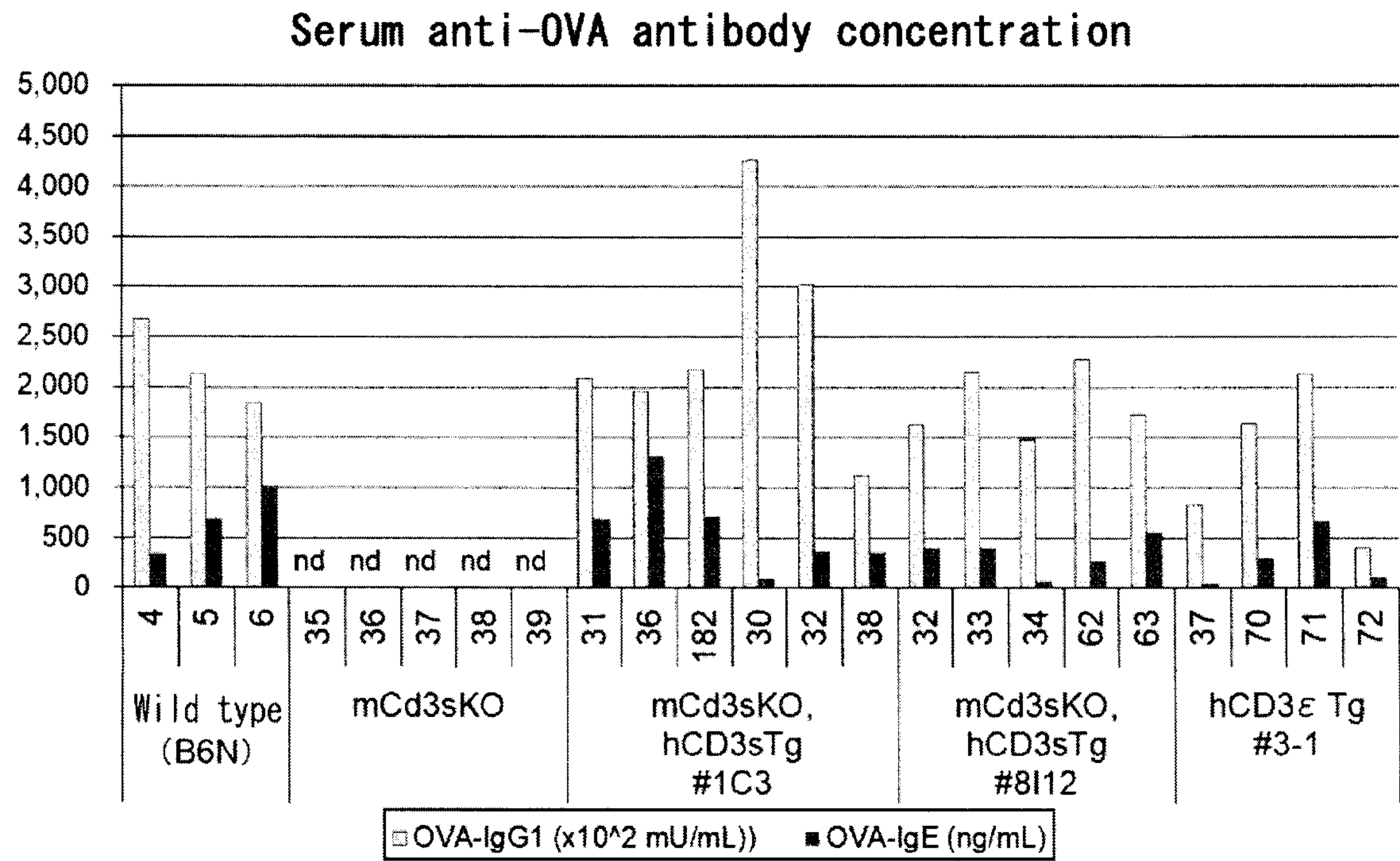


FIG. 34

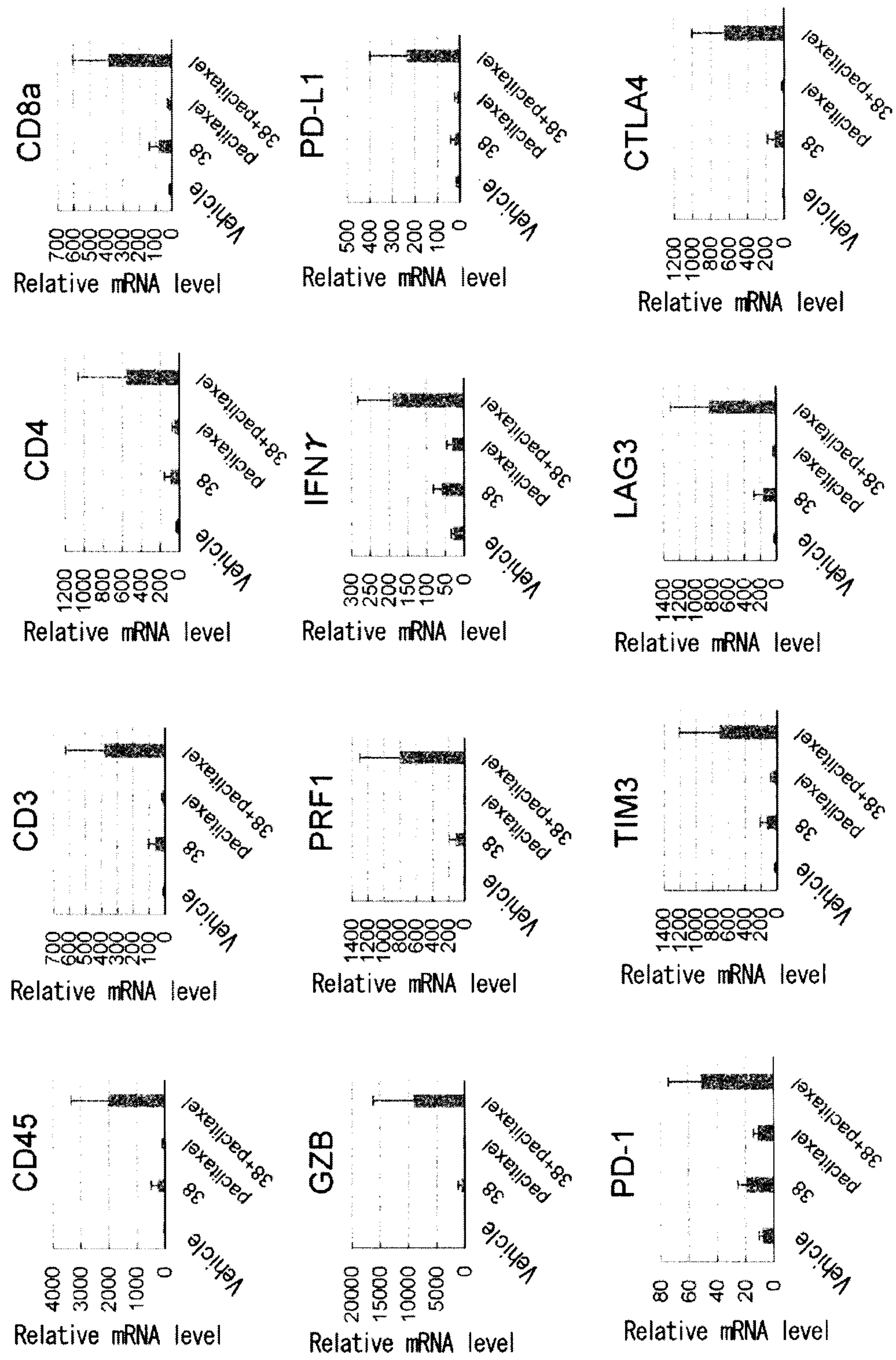


FIG. 35-1

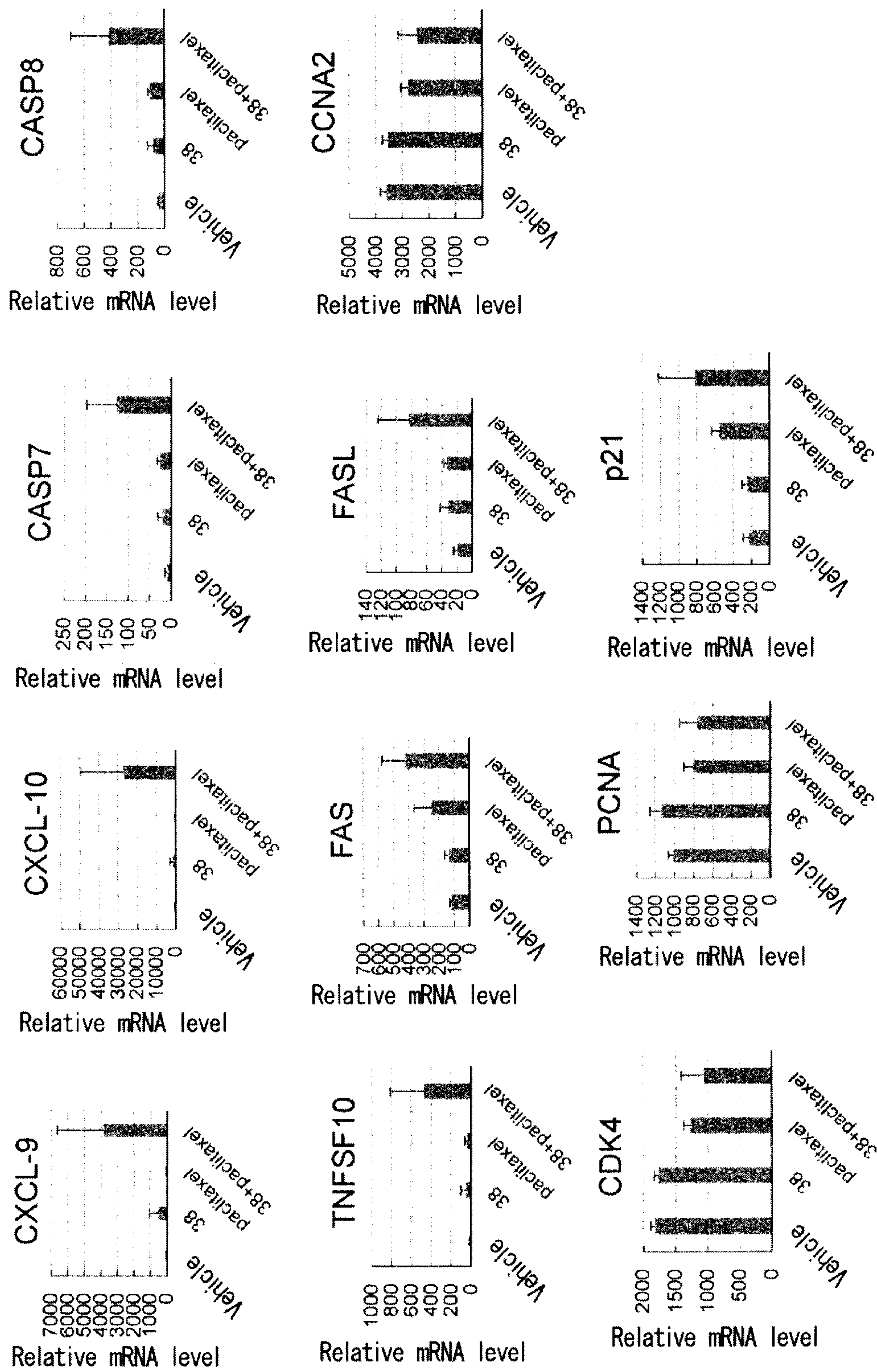


FIG. 35-2

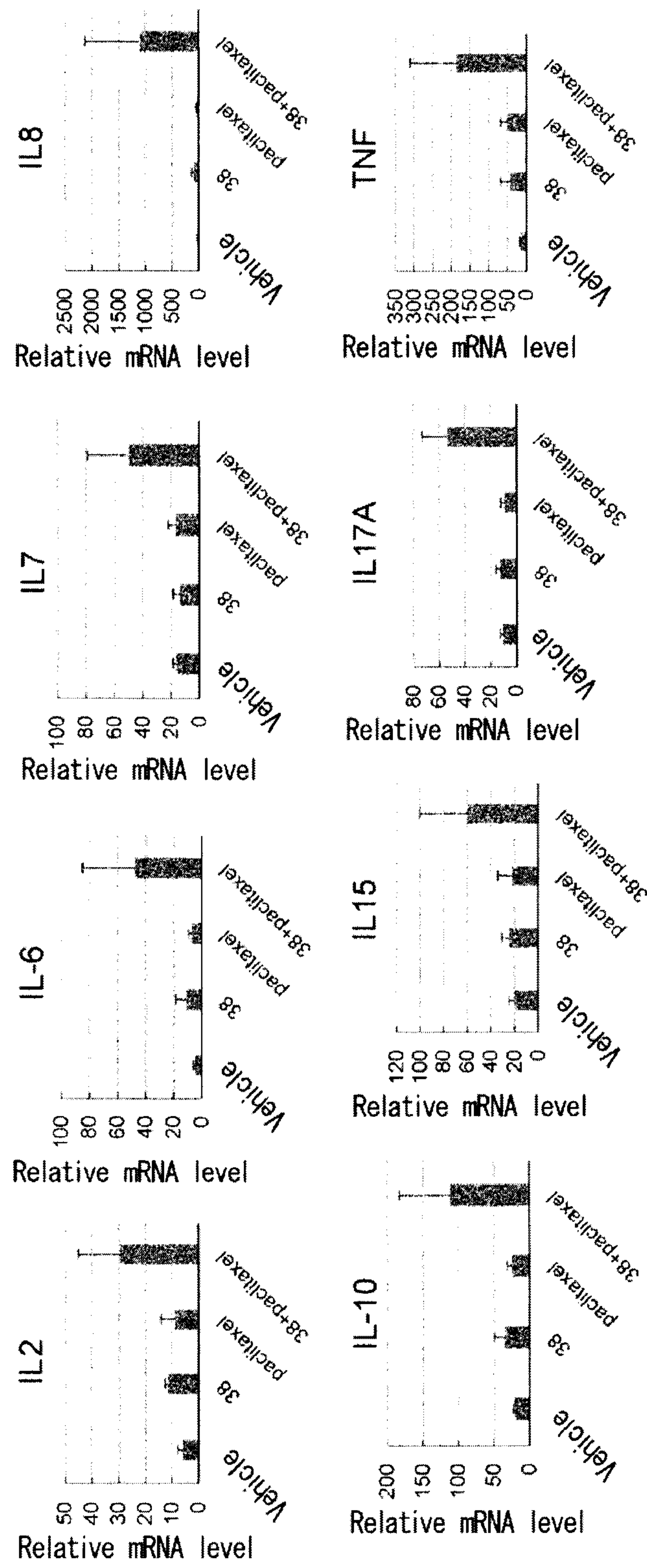


FIG. 35-3

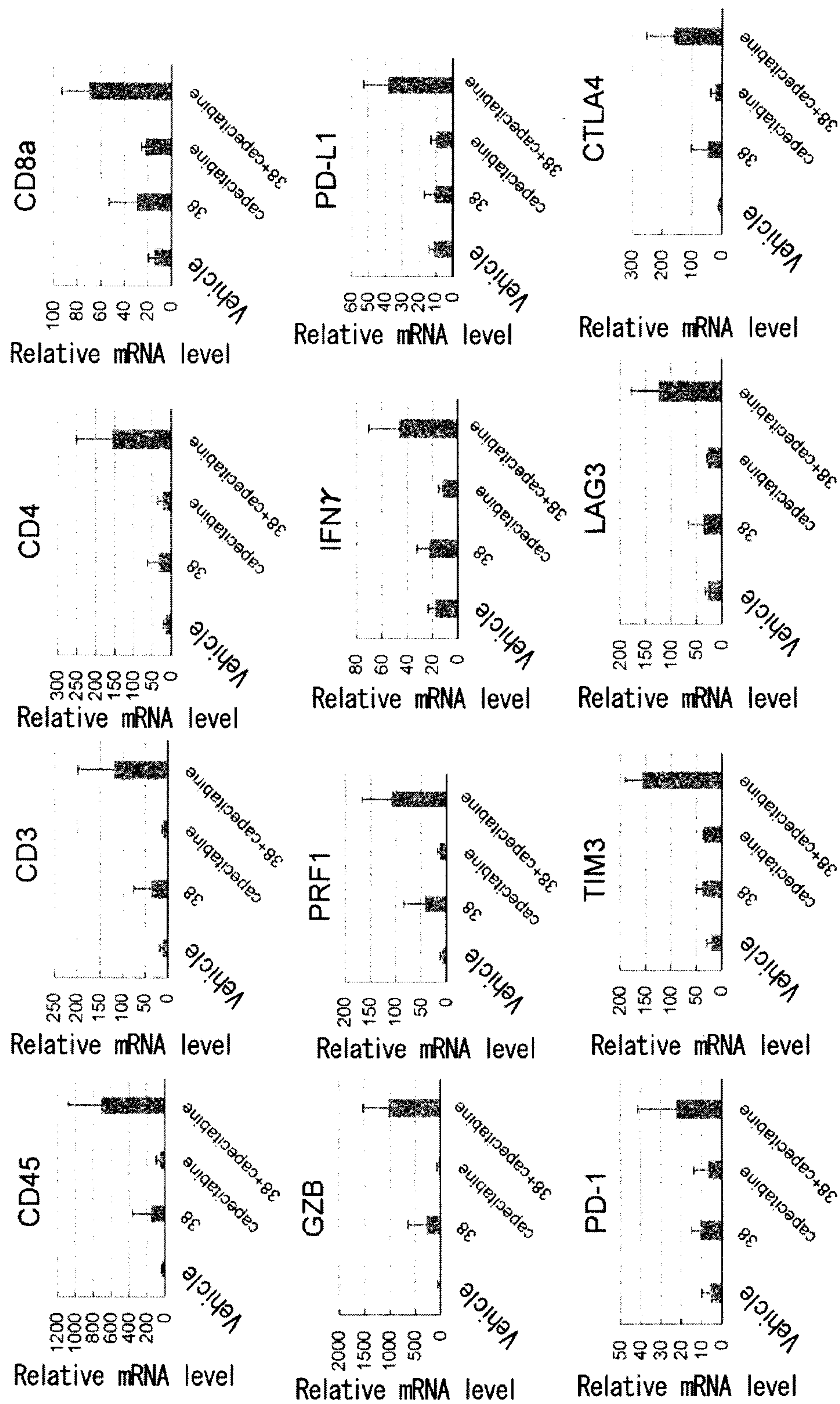


FIG. 35-4

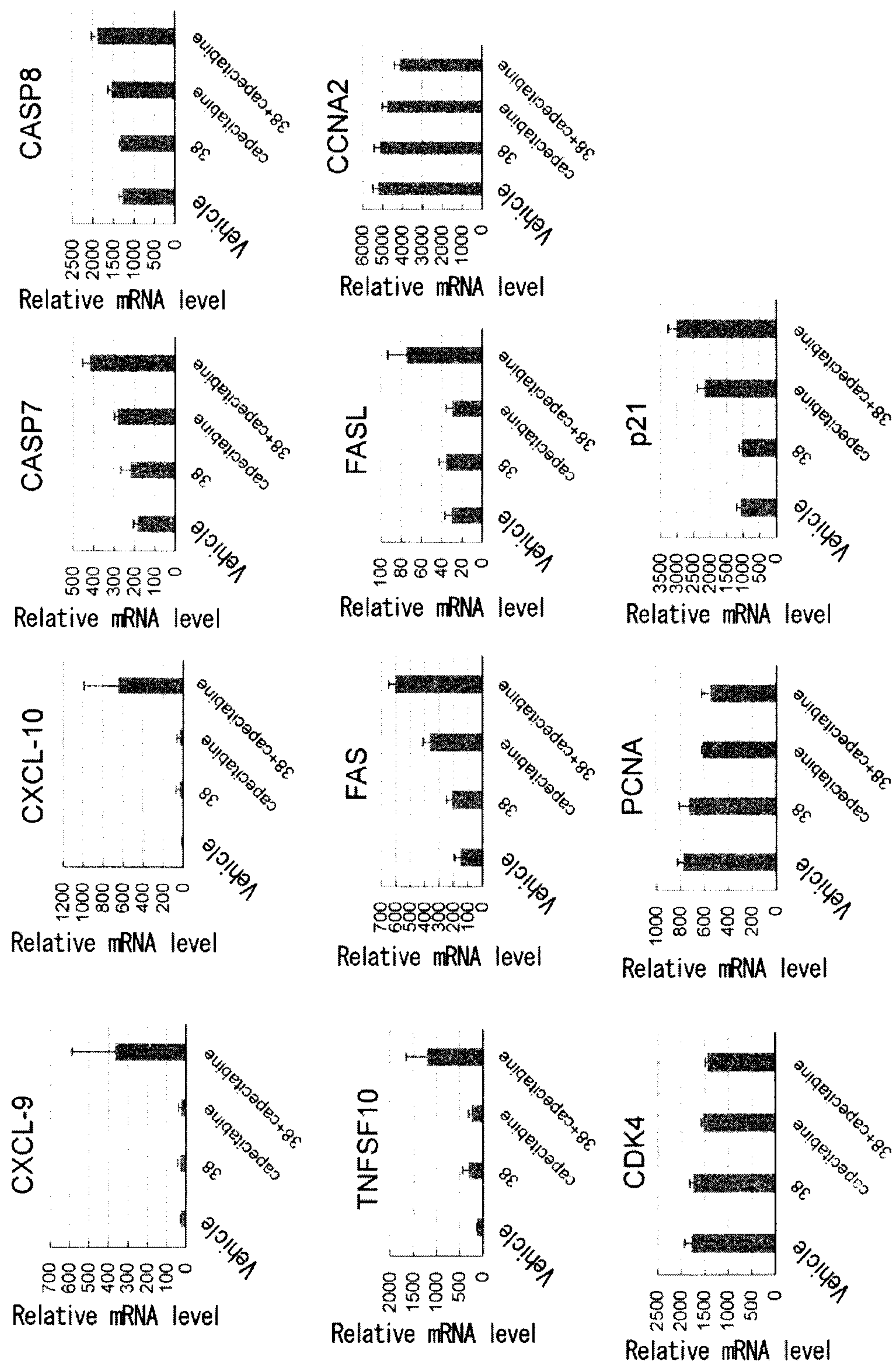


FIG. 35-5

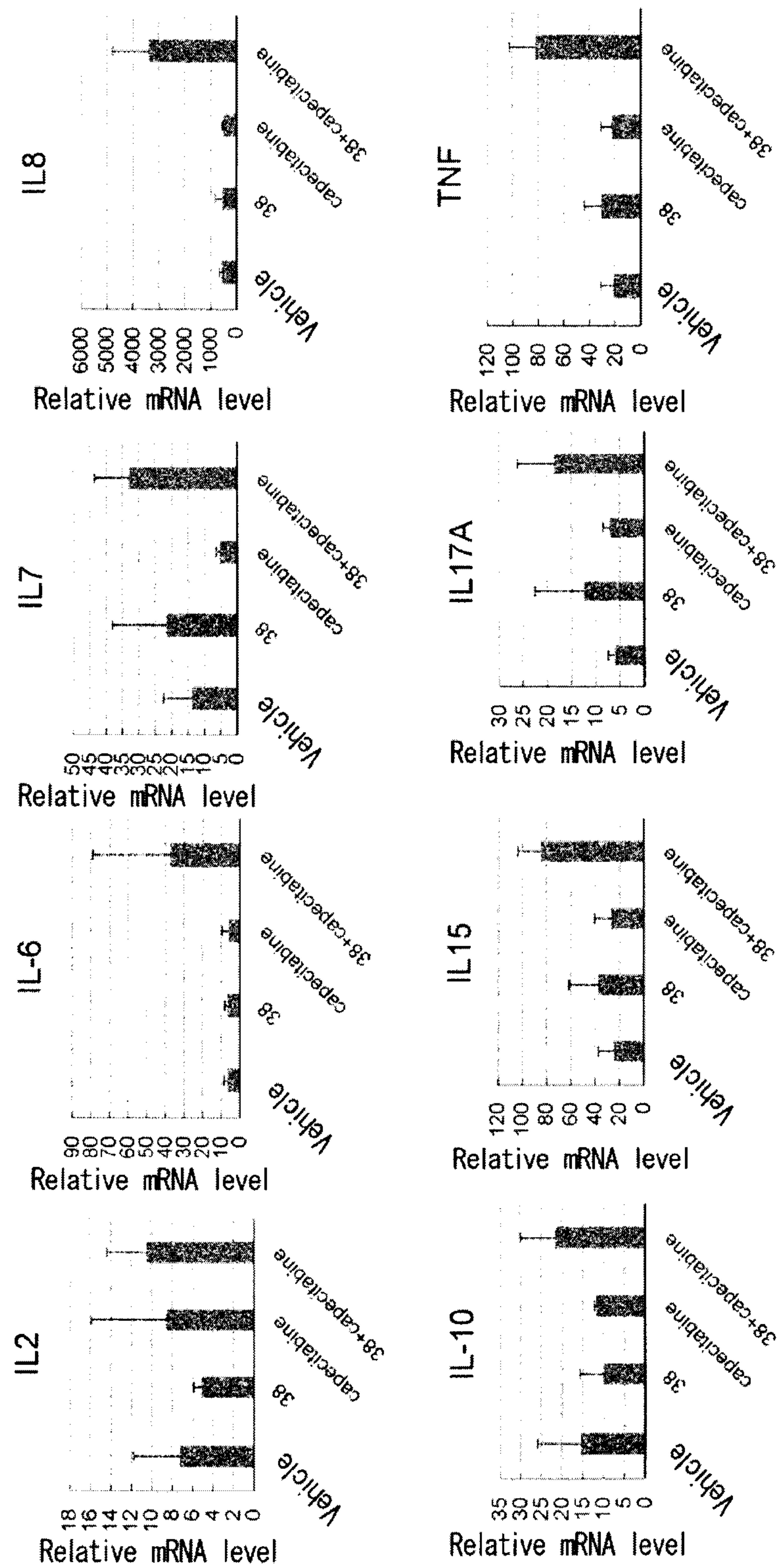


FIG. 35-6

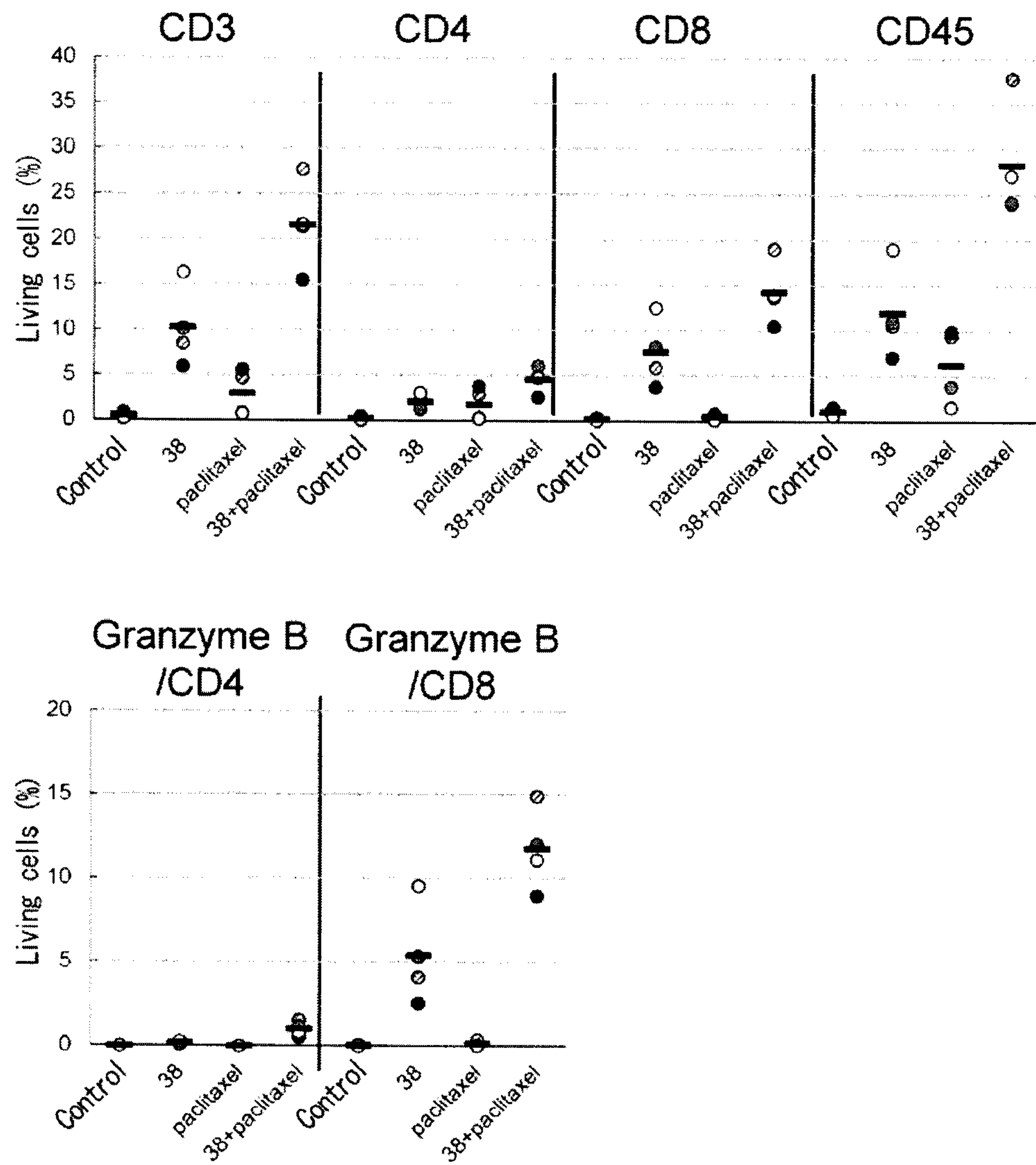


FIG. 36-1

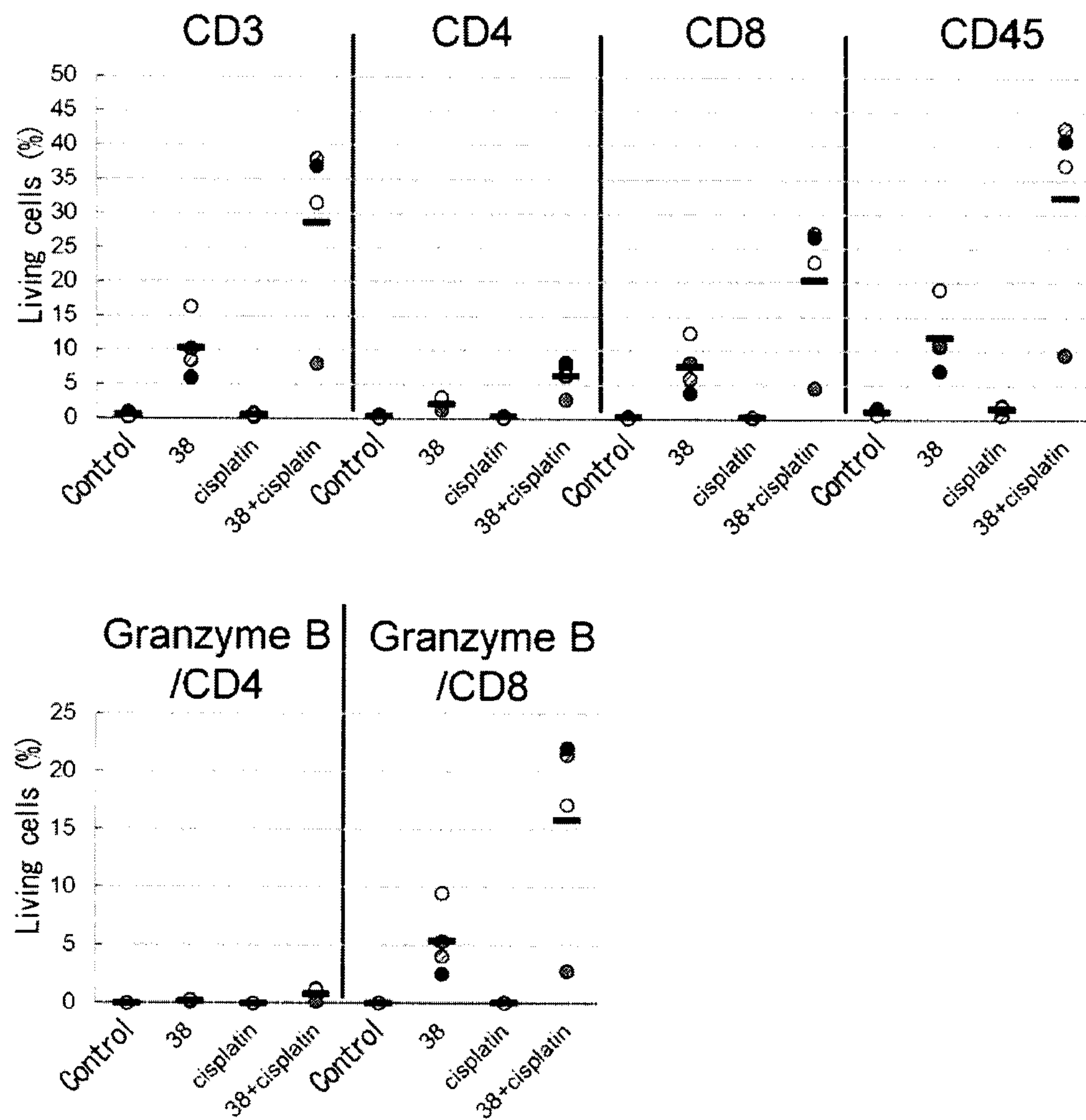


FIG. 36-2

[図8]

AA
NCI-H446 (肺)

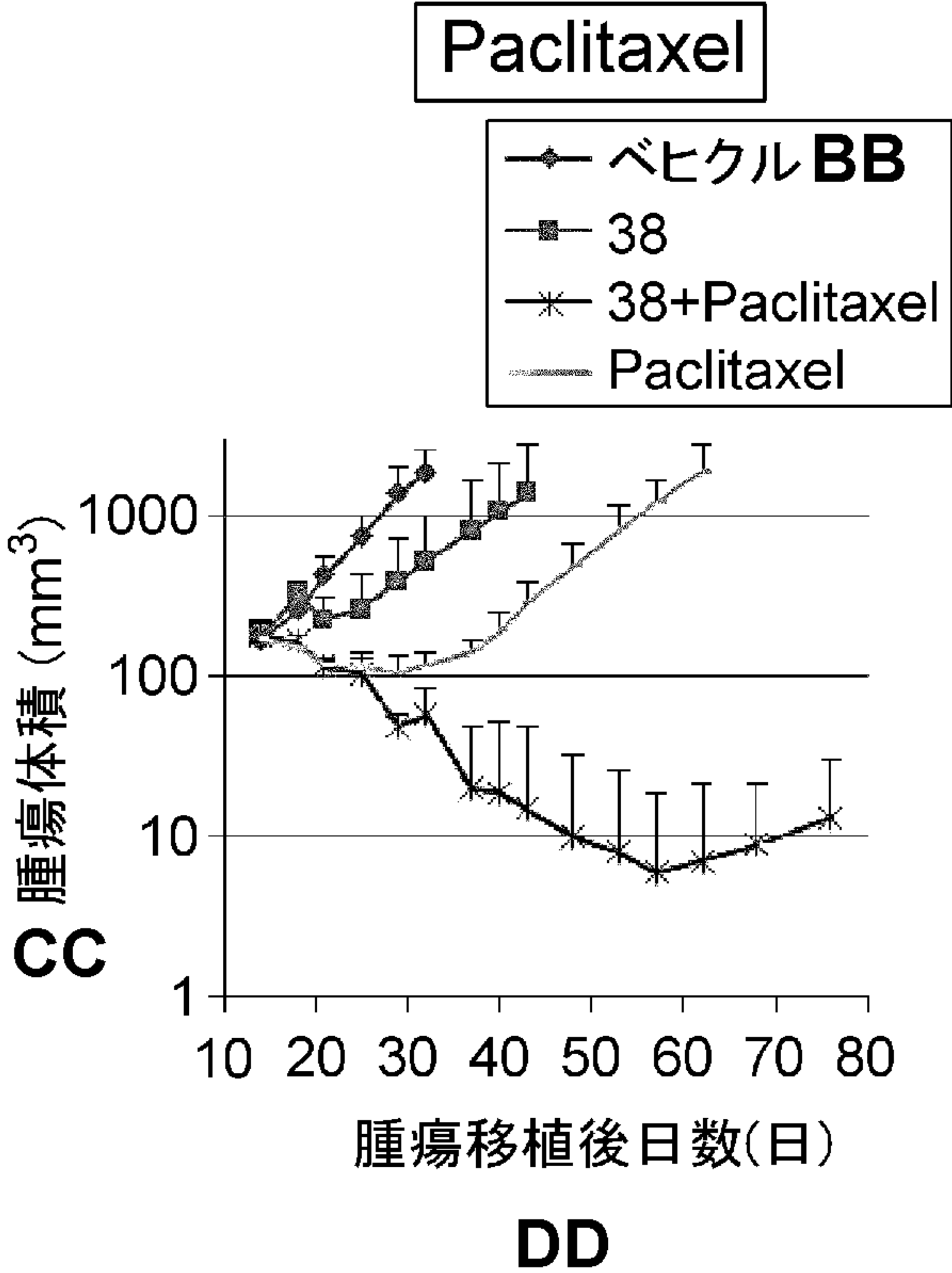
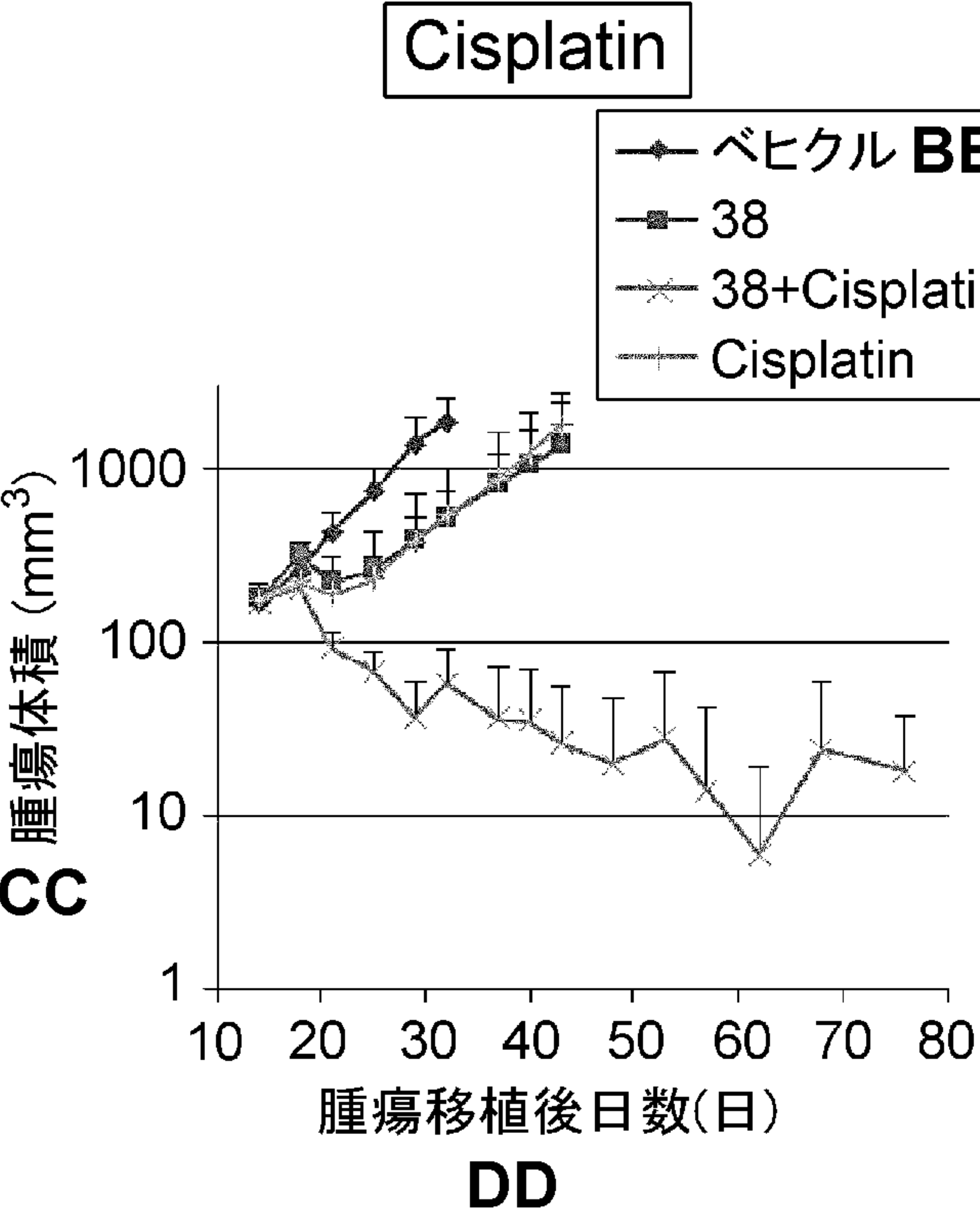


FIG. 8:

AA Lung

BB Vehicle

CC Tumor volume

DD Number of days after tumor transplantation (days)