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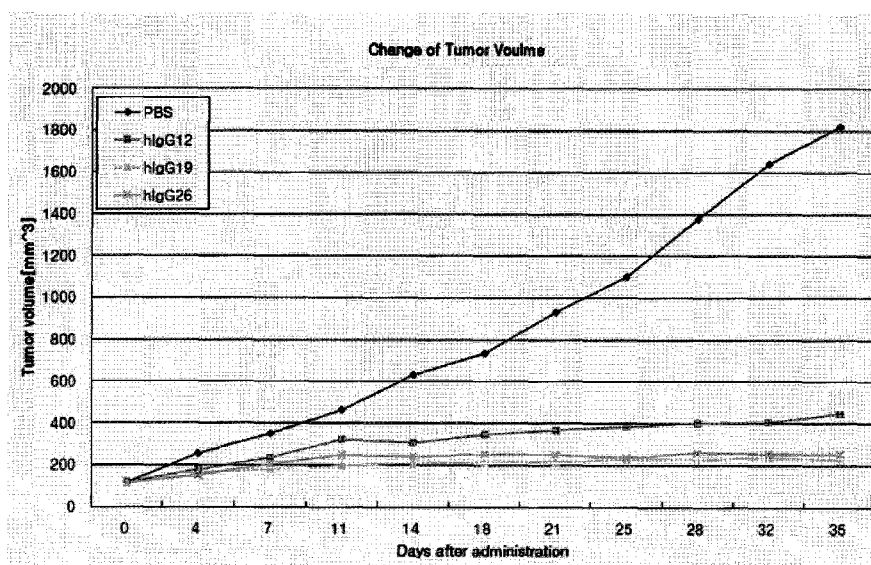
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(54) Title: HUMANIZED MONOCLONAL ANTIBODY HIGHLY BINDING TO EPIDERMAL GROWTH FACTOR RECEPTOR, EGF RECEPTOR



(57) Abstract: The present invention relates to a humanized antibody having excellent stability and high binding capacity to epidermal growth factor receptor (EGFR), more precisely a humanized antibody composed of immunized rabbit originated complementarity determining region (CDR) and human immunoglobulin originated framework region. The humanized antibody of the present invention has high binding capacity to EGF receptor and thus inhibits EGF binding and increases stability, resulting in tumor suppressing effect in vivo. In addition, the humanized antibody minimizes immune response so that it can be effectively used for the diagnosis and treatment of cancer over-expressing EGF receptor.

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【DESCRIPTION】**【Invention Title】**

**HUMANIZED MONOCLONAL ANTIBODY HIGHLY BINDING TO
EPIDERMAL GROWTH FACTOR RECEPTOR, EGF RECEPTOR**

5

【Technical Field】

The present invention relates to a humanized antibody having high binding specificity to EGF receptor and excellent stability, more precisely a humanized monoclonal antibody composed of complementarity determining region
10 derived from an immunized rabbit and framework region induced from a human immunoglobulin and an anticancer composition containing the same.

15 **【Background Art】**

Epidermal growth factor (EGF) is a polypeptide hormone stimulating mitosis of epidermal cells and epithelial cells. EGF acts by binding with high affinity to epidermal growth factor receptor (EGFR) on the cell
20 surface and stimulating the intrinsic protein-tyrosine kinase activity of the receptor.

EGF receptor is a transmembrane protein of approximately 170 kDa and is a genetic product of c-erb-B, a proto-oncogen. In particular, EGF receptor is a tumor-

related cell surface membrane protein, so that it has been widely used as a target of the development of a mouse monoclonal antibody (430) highly binding specifically to EGF receptor using an osteosarcoma cell line 791T as an antigen (Durrant, et al., Prenatal Diagnosis, 14, 131-140, 1994) or the development of a mouse monoclonal antibody (MAb 425) inhibiting the EGF binding using a human superficial cancer cell line A431 as an antigen (Rodeck, et al., Cancer Res., 47, 3692-3696, 1987).

The phage-antibody library provides an alternative technique substituting hybridoma technique in order to separate an antibody from an immunized animal. Particularly, the hybridoma technique is based on the immortalization of cells generating an antibody. On the contrary, the phage-antibody library technique is based on the immortalization of a gene encoding a target antibody (Winter, G. and Milstein, C., Nature, 349, 293-299, 1991). This phage-antibody technique is summarized as follows; heavy chain variable region and light chain variable region genes are amplified by PCR, the variable region (V) is forced to bind to be expressed as an antibody fragment on the surface of the phage particle, and then the phage-antibody binding to the corresponding antigen is screened by the phage-antibody library.

The hybridoma technique is especially useful to separate a mouse monoclonal antibody when a strong immune response is expected in the mouse spleen. For example, the mouse monoclonal antibody against EGF receptor can be
5 allegedly separated from the mouse spleen immunized with A431, a human tumor cell line, by the intra-abdominal injection (Murthy, et al., Arch. Biochem. Biophys., 252, 549-560, 1987).

In the meantime, the phage-antibody technique can use
10 any antibody-expressing cell as a starting material, unlike the conventional hybridoma technique, and more over facilitates fast screening of numbers of different antibodies. Thus, according to this method, a gene encoding the variable region (V) of the corresponding
15 antibody is already cloned, so that the gene can be used right away for other genetic engineering purposes.

It is one of the new attempts to prepare a monoclonal antibody that a gene corresponding to each variable region of heavy chain (V_H) and light chain (V_L) is amplified by PCR
20 and then expressed via phage exhibition library as a recombinant gene. More specifically, the antibody variable region gene is cloned first and then fused to a small envelope protein as a single-chain variable fragment (scFv). The phage particle expresses the antibody fragment on its

surface, leading to the selection by panning using the interaction with an antibody. The advantage of this method is producing a novel pair that has specificity and affinity, based on which the pair can be distinguished easily, and
5 this selection was not easy with the random recombination of the variable region (V) gene and producing an immunized antibody to a mouse or human originated antigen.

The conventional method to produce a monoclonal antibody to EGF receptor using B cells, such as *in vitro*
10 immunization and hybridoma technique, results in low affinity and cross-reactivity. To overcome the above problem, the present inventors hire a recombination method using PCR cloning after *in vivo* immunization.

The repeated administration of the mouse monoclonal
15 antibody to human induces immune response with side effects and results in reduced therapeutic effects. That is, the effect of an antibody against an antigen decreases by human anti-mouse antibody (HAMA) response. To minimize the HAMA response, a chimeric antibody has been developed in which
20 mouse variable region (V) is bound to human constant region (C), and more precisely a humanized antibody has been developed by transplanting some amino acid residues of human framework region (FR) and mouse complementarity determining region (CDR) to a human antibody in order to

increase antigen binding capacity (Lobuglio, et al., Proc. Nat. Acad. Sci. USA, 86, 4220-4224, 1989). In fact there have been reports saying that repeated administration of such humanized antibodies reduces human immune response (Brown, et al., Proc. Natl. Acad. Sci. USA, 88, 2663-2667, 1991). However, there is still a possibility for the humanized antibody to cause immune response by its amino acid residues derived from a mouse. Therefore, it is still requested to develop an advanced humanized antibody with antigen binding capacity and less mouse originated amino acid residues.

To bring a proper effect of a recombinant antibody protein *in vivo*, stability of the protein is a key factor. Structural stability and thermo-stability increase half-life *in vivo*, leading to the increase of a therapeutic effect. It has also been reported that thermo-stability of an antibody plays an important role in targeting of a tumor region (Willuda J, Honegger A, Waibel R, Schubiger PA, Stahel R, Zangemeister-Wittke U, Pluckthun A. Cancer Res. 1999, 59(22): 5758-5767).

In the meantime, stability of an antibody affects aggregation. In the production of biopharmaceuticals using cell culture system, the aggregation of proteins during cell culture has been a problem. Protein aggregates are

generally formed by more than one protein molecule, which contains partially or completely denatured molecules. Aggregates cannot be eliminated by filtering or other pre-purification processes because of its homology with biopharmaceuticals.

Protein aggregation is a huge problem in producing biopharmaceuticals. The primary aggregate ratio in the bulk of biopharmaceuticals has to be less than 0.1% and high concentration biopharmaceuticals having at least 100 mg/ml has to be formulated with the acceptable low aggregate content. The conventional method to eliminate protein aggregates in the bulk solution is gel filtration, which is to separate aggregates by using the difference in size between aggregates and target monomer proteins. However, this method is not easy to apply to the mass-production of biopharmaceuticals in industry and requires a high cost. Therefore, it is important to select a very stable protein as a lead compound, which does not form protein aggregates during cell culture process for the production efficiency and cost reduction.

If a biological drug has an unstable structure or it is apt to generate protein aggregates, it might cause immunogenicity more easily than monomer proteins after being administered in human or the half-life of the drug in

blood will be shorten because of the decreased stability in blood. In addition, targeting efficiency will be significantly reduced because aggregates might be eliminated in the liver or spleen non-specifically. Thus,
5 to secure high stability of biopharmaceuticals and targeting efficiency thereof, the development of a novel protein having a stable structure is very important.

【Disclosure】

10 **【Technical Problem】**

It is an object of the present invention to select a humanized antibody having high binding capacity to EGF receptor and high stability and further to provide a novel antibody having excellent *in vitro/in vivo* anticancer
15 effect owing to improved stability and high binding capacity to EGF receptor, and an anticancer composition containing the same.

【Technical Solution】

20 The present inventors selected a novel humanized antibody having high stability and high binding capacity to EGF receptor using the recombination technique and then completed this invention by confirming its excellent *in vivo/in vitro* anticancer effect.

To achieve the above object, the present invention provides an EGF receptor specific antibody which comprises a recombinant heavy chain comprising human originated heavy chain constant region (C_H) linked to the heavy chain variable region (V_H) containing rabbit originated heavy chain complementarity determining region (HCDR) and a recombinant light chain comprising human originated light chain constant region (C_L) linked to the light chain variable region (V_L) containing rabbit originated light chain complementarity determining region (LCDR).

The present invention also provides a gene encoding the heavy chain variable region of the EGF receptor specific antibody.

The present invention further provides a gene encoding the light chain variable region specific to the EGF receptor.

The present invention also provides an expression vector containing the gene encoding the heavy chain of the antibody and an expression vector containing the gene encoding the light chain of the antibody.

The present invention also provides a transformant prepared by transfecting host cells with the expression vectors.

The present invention also provides a preparation method of an EGF receptor specific antibody comprising the step of culturing the transformant.

5 The present invention also provides a pharmaceutical composition containing the antibody of the invention as an active ingredient for the treatment of malignant tumor over-expressing EGF receptor.

10 The present invention also provides a treatment method for malignant tumor over-expressing EGF receptor containing the step of administering the effective dose of the pharmaceutical composition of the invention to a subject.

15 Hereinafter, the present invention is described in detail.

20 The present inventors injected EGF-EGF receptor mixture or epithelial cancer cells to a rabbit to induce immune response. Antibody production therein was confirmed and then total RNA was separated from the bone marrow and spleen of the rabbit. The separated RNA was reverse-transcribed into cDNA. Fab fragments of rabbit variable regions heavy chain (V_H) and light chain (V_L) and human constant region heavy chain (C_{H1}) and light chain (C_L) were amplified by using the above cDNA as a template with a

primer set described in the report of Carlos, F. The amplified each rabbit variable region fragments (V_H , V_L) and human constant region fragments (C_{H1} , C_L) were amplified to convert them into 'rabbit V_H -human C_{H1} ' and 'rabbit V_L -human C_L ' using overlapping primers. The amplified two fragments were amplified again with those overlapping primers to prepare chimeric Fab having binding capacity to EGF receptor (chimeric antibody binding fragment). The both ends of the chimeric Fab DNA fragment of the library were digested with a restriction enzyme, which was then inserted into pComb3X (Genbank No. AF268281; Rader, C. and Barbas, C.F. III. (in) PHAGE DISPLAY, A LABORATORY MANUAL. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2000) to construct chimeric Fab library.

The present inventors transfected the host cell (ex: ER2738) with the prepared chimeric Fab library to infect helper phage, resulting in the preparation of chimeric Fab phage library. Competitive cell panning was performed using the above library to select antibody clones having high binding capacity to EGF receptor. Competitive cell panning was performed as follows; cancer cells, membrane fragments of normal fibroblasts, an EGF receptor antibody and the phage library were added and cultured. Then, centrifugation was performed to obtain phages, to which the

host cells and helper phages were added again. After repeating this process, phages binding to EGF receptor were selected and amplified. At last, FACS was performed using anti-Fab FITC antibody to select a phage clone having high binding capacity to EGF receptor. Sequence analysis was performed and as a result it was confirmed that the clone was novel CDR containing clone which was distinguished from the conventional EGF receptor antibody (see Fig. 1).

To produce the selected chimeric Fab phage clone as a whole IgG form, the present inventors amplified the heavy chain and light chain of the chimeric Fab, to which a signal sequence and a restriction enzyme sequence were inserted. The amplified products were respectively inserted into pCDH-101 vector expressing human heavy chain cDNA and pCDK-101 vector expressing human light chain (k chain) cDNA (see Fig. 2).

The above two vectors each expressing the heavy chain and light chain respectively were mixed and this vector mixture was inserted into host cells (ex: 293T/72 cells) to produce a transformant. A whole IgG form generated in the transformant was separated by column. The purified antibody was tested for EGF receptor binding capacity by BIAcore assay. As a result, dissociation constant K_D value was in the range of $6.7E-10M \sim 2.8E-11M$, suggesting that

the antibody had high affinity (see Table 2). As a result, the binding capacity of CRH44, CRH46, CRH47, CRH49, CRH411 and CRH412 was high, compared with that of CRH4 antibody (see Fig. 4).

5 The present inventors also investigated the *in vitro* effect of the antibody of the invention on tumor growth using A431 cells. As a result, tumor cell growth inhibitory effect (under 40% of relative growth) was confirmed at the antibody concentration of 1 $\mu\text{g/ml}$,
10 regardless of the kind of antibodies above (see Fig. 5). The antibody of the invention was also treated to *in vivo* tumor models. As a result, tumor cell growth inhibitory effect of CRH4 was greater than that of CRH412 (see Fig. 6), but the effect was not necessarily antibody-dose dependent.

15 Tumor suppressing effect of CRH412 was significantly low in spite of its high affinity ($K_D = 3.09\text{E-}11\text{M}$). This was considered to be attributed to the low stability of CRH412, indicating that the tumor targeting was interrupted.

20 Thus, to overcome the low stability problem of CRH412 antibody, the present inventors transplanted a sequence containing CDR of CRH412 antibody into hu4D5-framework having comparatively high stability. As shown in Table 4, some amino acids were modified. Then, a novel Fab library transplanted with the CDR was synthesized and CRH412

derivative Fab antibody phage clone binding to EGF receptor was selected by FACS. The DNA sequence of the selected EGF receptor binding clone was analyzed. The selected clones were produced in a whole IgG form. The EGF receptor binding capacity of the novel CRH412 modified antibody was investigated. As a result, the EGF receptor binding capacity of the modified antibodies, except hIgG2 antibody, was equal to that of CRH412 antibody (see Table 6).

The present inventors further investigated whether the stability of the novel modified antibodies was improved or not. First, aggregate contents of the novel modified antibodies were investigated. As a result, aggregate content of CRH412 antibody was 7%, while aggregate contents of hIgG2, hIgG12, hIgG18, hIgG26 and hIgG32 were all reduced to 1 - 2% (see Fig. 7).

Second, thermo-stability of the novel modified antibodies was investigated. As a result, CRH412 variants exhibited significantly high thermo-stability, compared with a control group, commercial anti-EGFR antibody C255 (Erbitux) (see Fig. 8).

The present inventors injected CRH412 and its variants, hIgG12, hIgG18, hIgG19, hIgG26 and hIgG 32, into mouse tumor models. As a result, the half-life of CRH412 antibody was short in blood and non-specific absorption in

the liver and spleen was high. In the meantime, CRH412 variants with improved stability exhibited excellent tumor targeting capacity. Non-specific absorption in the liver and spleen was reduced and the half-life in blood was increased (see Fig. 9). The present inventors injected hIgG12, hIgG19 and hIgG26, among the CRH412 variants, into mouse tumor models. As a result, they all exhibited excellent tumor suppressing effect (see Fig. 10).

As confirmed in the above results, the present inventors selected a novel antibody having high affinity and new CDR distinguished from the conventional EGF receptor binding antibodies. And then the inventors prepared a novel antibody with improved stability and thereby excellent *in vivo* functions and effect.

The present invention provides an EGF receptor specific antibody which comprises a recombinant heavy chain comprising human originated heavy chain constant region (C_H) linked to the heavy chain variable region (V_H) containing rabbit originated heavy chain complementarity determining region (HCDR) and a recombinant light chain comprising human originated light chain constant region (C_L) linked to the light chain variable region (V_L)

containing rabbit originated light chain complementarity determining region (LCDR).

The antibody herein is preferably a humanized antibody or a chimeric antibody or its fragment.

5 The heavy chain complementarity determining region (CDR) preferably includes CDR-H1 represented by SEQ. ID. NO: 19, CDR-H2 represented by SEQ. ID. NO: 20 and CDR-H3 represented by SEQ. ID. NO: 21 or NO: 22.

10 The light chain complementarity determining region preferably includes CDR-L1 represented by one of SEQ. ID. NO: 23 - NO: 26, CDR-L2 represented by one of SEQ. ID. NO: 27 - NO: 29 and CDR-L3 represented by one of SEQ. ID. NO: 30 - NO: 32.

15 The heavy chain variable region herein is preferably selected from the group consisting of the sequences represented by SEQ. ID. NO: 33 - NO: 56, and the light chain variable region herein is preferably selected from the group consisting of sequences represented by SEQ. ID. NO: 58 - NO: 84.

20 The variable region herein indicates a part of an antibody that has an antigen specific binding capacity and various mutations in its sequence, which includes CDR1, CDR2 and CDR3. The complementarity determining region is a ring shaped region involved in recognition of an antigen

and the specificity of an antibody against an antigen depends on the sequence of this region. There are framework regions between CDRs which play a role in supporting the CDR rings. In the present invention, some parts of CDR and the amino acids of the framework were modified with human immunoglobulin originated sequences to prepare a humanized antibody having high stability without damaging its EGF receptor binding capacity.

At this time the fact that the variable region containing CDR can be easily modified without losing its binding capacity to EGF receptor is well understood by those skilled in the art. The modified sequence competes with CRH412 to bind EGF receptor and it can be included in the present invention as long as it has higher stability than CRH412. The stability can be measured by various ways such as binding capacity before and after heat treatment, aggregate content and tumor tissue targeting, etc.

Various modifications can happen in an antibody. Thus, the 'antibody' of the invention includes not only a whole antibody but also a functional fragment of an antibody. The whole antibody is composed of two full length light chains and two full length heavy chains and each light chain is linked to each heavy chain by disulfide bond. The functional fragment of an antibody indicates a fragment

that has an antigen-binding capacity, which is exemplified by (i) a Fab fragment composed of light chain variable region (VL), heavy chain variable region (VH), light chain constant region (CL) and the first constant region of heavy chain (CH1); (ii) a Fd fragment composed of VH and CH1 domains; (iii) a Fv fragment composed of VL and VH domains; (iv) a dAb fragment composed of VH domain (Ward, E. S. et al., Nature 341: 544-546, 1989); (v) an isolated CDR region; (vi) a F(ab')₂ fragment composed of two linked Fab fragments; (vii) a single chain Fv molecule (ScFv) in which VH domain and VL domain are bound by a peptide linker to form an antigen binding site; (viii) a bi-specific single chain Fv dimer (PCT/US92/09965) and (ix) a multivalent or multi-specific fragment diabody (9W094/13804) prepared by gene fusion. In the present invention the antibody is preferably a whole antibody or a Fab fragment.

The humanized antibody or chimeric antibody provided by the invention can be linked to any constant region by the recombination technique. The heavy chain variable region has γ , μ , α , δ and ϵ types and the light chain variable region has κ and λ types. IgG1 type antibody is preferably used in the invention.

The present invention also provides a gene encoding the heavy chain variable region of the EGF receptor specific antibody.

5 The gene preferably comprises a nucleic acid sequence encoding the heavy chain variable region containing CDR-H1 represented by SEQ. ID. NO: 19, CDR-H2 represented by SEQ. ID. NO: 20 and CDR-H3 represented by SEQ. ID. NO: 21 or NO: 22. A nucleic acid sequence encoding the heavy chain variable region represented by one of SEQ. ID. NO: 33 - NO: 10 56 is more preferred. In a preferred embodiment of the present invention, the invention provides a nucleic acid sequence encoding the heavy chain variable region H19 of the antibody hIgG19. The nucleic acid sequences encoding the various heavy chain variable regions can be easily 15 prepared by substituting codons encoding a couple of amino acids based on the sequence represented by SEQ. ID. NO: 85, according to the conventional method well-known to those skilled in the art.

20 The present invention further provides a gene encoding the light chain variable region specific to EGF receptor.

The gene preferably comprises a nucleic acid sequence encoding the light chain variable region containing CDR-L1

represented by one of SEQ. ID. NO: 23 - NO: 26, CDR-L2 represented by one of SEQ. ID. NO: 27 - NO: 29 and CDR-L3 represented by one of SEQ. ID. NO: 30 - NO: 32. It is more preferably a nucleic acid sequence encoding the light chain variable region represented by one of SEQ. ID. NO: 57 - NO: 84. In a preferred embodiment of the present invention, the invention provides a nucleic acid sequence encoding the light chain variable region L19 of the antibody hIgG19. The nucleic acid sequences encoding the various light chain variable regions can be easily prepared by substituting codons encoding a couple of amino acids based on the sequence represented by SEQ. ID. NO: 86, according to the conventional method well-known to those skilled in the art.

The present invention also provides an expression vector containing the gene encoding the heavy chain variable region of the antibody.

It is preferred to use pCDH-101 of Fig. 2 as a mother vector for the expression vector, but not always limited thereto.

The present invention also provides an expression vector containing the gene encoding the light chain variable region of the antibody.

It is preferred to use pCDK-101 of Fig. 2 as a mother vector for the expression vector, but not always limited thereto.

5 The present invention also provides a transformant prepared by transfecting host cells with the expression vectors containing the heavy chain region and the light chain region genes.

10 The host cells herein can be prokaryotic cells such as *E. coli* or *Bacillus subtilis* or eukaryotic cells such as yeast like *Saccharomyces cerevisiae*, insect cells, plant cells or animal cells. The host cells can be selected from the group consisting of mouse myeloma cells, CHO cells, monkey kidney cells (COS1, COS7, etc), NSO cells, SP2/O
15 cells, W138 cells, BHK cells, Namalwa cells, BALL-1 cells, JBL cells and 293 cells, but not always limited thereto.

The introduction of the expression vector to the host cell is performed by any conventional method known to those skilled in the art.

20

The present invention also provides a preparation method of an EGF receptor specific antibody comprising the steps of (1) culturing the transformant; and (2) purifying an antibody from the culture solution.

The culture medium can be any of those appropriate for the transformant which has been well-known to those skilled in the art. The antibody purification can be performed by one of the conventional methods known to those skilled in the art.

Particularly, the preparation method of an EGF receptor specific antibody is as follow:

1) inducing immune response by injecting EGF receptor into a mammal;

2) constructing a gene library with animal tissues obtained from the immune response induced animal;

3) constructing a phage library by inserting the gene into an expression vector, then transfecting host cells with the vector and then infecting the cells with helper phage;

4) selecting a phage expressing an anti-EGF receptor antibody having high binding capacity to EGF receptor by bio-panning with the phage library;

5) constructing an expression vector for chimeric antibody by separating DNA encoding the antibody from the phage and then inserting the region encoding the variable region of the DNA operably into a vector containing the gene encoding human antibody constant region;

6) preparing a transformant by transfecting host cells with the expression vector constructed in step (5) and culturing the transformant; and

7) purifying the antibody from the culture solution.

5 The host cells herein can be prokaryotic cells such as *E. coli* or *Bacillus subtilis* or eukaryotic cells such as yeast like *Saccharomyces cerevisiae*, insect cells, plant cells or animal cells. The host cells can be selected from the group consisting of mouse myeloma cells, CHO cells,
10 monkey kidney cells (COS1, COS7, etc), NSO cells, SP2/O cells, W138 cells, BHK cells, Namalwa cells, BALL-1 cells, JBL cells and 293 cells, but not always limited thereto. In step 2), the tissue is preferably bone marrow or spleen, but not always limited thereto.

15

 The present invention also provides a pharmaceutical composition containing the antibody of the invention as an active ingredient for the treatment of malignant tumor over-expressing EGF receptor.

20

 The present inventors investigated the tumor cell growth inhibitory effect of the whole IgG chimeric antibody of the invention in human tumor models using the method described in Ishiyama's paper (Ishiyama, et al., Biol, Pharmacol, Bull., 19, 158-1520, 1996). Particularly, 7

different IgG chimeric antibodies were treated to the tumor models. As a result, the tumor cell growth inhibitory effect was observed in A431 cells cultured in a serum-free medium at the antibody concentration of 1 μ g/ml (relative growth : less than 40%) (Fig. 5).

To evaluate the anticancer activity of the chimeric antibody of the invention not only *in vitro* but also *in vivo*, the chimeric antibody was treated to the nude mouse tumor model and the tumor inhibitory effect was measured. As a result, the selected chimeric antibody suppressed the growth of A431 epidermoid carcinoma cell line, compared with the negative control.

The present inventors treated CRH412 antibody and its variants with improved stability, hIgG12, hIgG18, hIgG19, hIgG26 and hIgG32 to mouse tumor models. As a result, CRH412 antibody exhibited short half-life in blood and high level of non-specific absorption in the liver and spleen. On the other hand, the CRH412 variants with improved stability exhibited excellent tumor targeting and decreased non-specific absorption in the liver and spleen and increased half-life in blood (see Fig. 9). The present inventors also confirmed that hIG12, hIgG19 and hIgG26 among the CRG412 variants showed excellent tumor suppressing activity in mouse tumor models (see Fig. 10).

According to the Dr. Ullrich's report, EGFR is over expressed in many different cancers including breast cancer, colon cancer, lung cancer and head/neck cancer (Ullrich et al., Endocrine-related cancer, 8, 11-31, 2001). Therefore, the pharmaceutical composition of the present invention can be effectively used for the treatment of such cancers overexpressing EGFR shown in Table 1.

【Table 1】

Type of tumours	Receptor	Overexpression (%)	Reference
Mammary	EGFR	14-91	Klijn et al. (1992); Beckmann et al. (1996); Walker & Dearing (1999)
	HER2	21	Paik et al. (1990)
Bladder	EGFR	31-48	Salomon et al. (1995); Chow et al. (1997)
	HER2	36	Sauter et al. (1993)
Colon	EGFR	25-77	Salomon et al. (1995); Messa et al. (1998)
	HER2	50	Caruso & Vallentini (1996)
Glioma	EGFR	40-50	Ekstrand et al. (1991); Salomon et al. (1995); Rieske et al. (1998)
Non-small-Cell-Lung	EGFR	40-80	Salomon et al. (1995); Fujino et al. (1996); Rusch et al. (1997); Fontanini et al. (1998)
Pancreatic	EGFR	30-50	Salomon et al. (1995); Uegaki et al. (1997)
Ovarian	EGFR	35-70	Salomon et al. (1995); Bartlett et al. (1996); Fischer-Colbrie et al. (1997)
	HER2	32	Berchuck et al. (1990)
Gastric	HER2	26	Lemoine et al. (1991)
Lung	HER2	28	Tateishi et al. (1991)
Salivary	HER2	32	Stenman et al. (1991)
Head and Neck	EGFR	80-100	Salomon et al. (1995); Grandis et al. (1996)

The effective dose of the pharmaceutical composition of the invention is preferably the concentration of serum

of the antibody enabling EGF receptor saturation. For example, more than 0.1 nM is considered to be enough. The composition includes the chimeric antibody, the humanized antibody or the transformant as an active ingredient. The pharmaceutical composition of the present invention can additionally include one or more active ingredients having the same or similar functions to the active ingredient of the invention. The composition of the present invention can also include, in addition to the above-mentioned effective ingredients, one or more pharmaceutically acceptable carriers for the administration. The pharmaceutically acceptable carrier can be selected or be prepared by mixing more than one ingredients selected from the group consisting of saline, sterilized water, Ringer's solution, buffered saline, dextrose solution, maltodextrose solution, glycerol and ethanol. Other general additives such as anti-oxidative agent, buffer solution, bacteriostatic agent, etc, can be added. In order to prepare injectable solutions, pills, capsules, granules or tablets, diluents, dispersing agents, surfactants, binders and lubricants can be additionally added. The composition of the present invention can further be prepared in suitable forms for each disease or according to ingredients

by following the method represented in Remington's Pharmaceutical Science, Mack Publishing Company, Easton PA.

The pharmaceutical composition of the present invention can be administered orally or parenterally (for example, intravenous, hypodermic, local or peritoneal injection). But, intravenous injection is preferred. To treat a solid tumor, local administration would be preferred because it is important for the antibody to reach the solid tumor fast and easy. The effective dosage of the composition can be determined according to weight, age, gender, health condition, diet, administration frequency, administration method, excretion and severity of a disease. One time dose of the chimeric antibody, the humanized antibody or the transformant is approximately 5 - 500 mg/m², which can be administered every day basis or every week basis. In the case of the antibody fragment, the administration has to be more frequent in order to keep serum to maintain EGF receptor level as saturation.

The composition of the present invention can be administered singly or treated along with surgical operation, hormone therapy, chemotherapy and biological reaction regulator, to prevent and treat malignant tumor.

The malignant tumor over-expressing EGF receptor that can be treated with the pharmaceutical composition of the

invention is preferably selected from the group consisting of breast cancer, bladder cancer, colon cancer, glioma, non-small-cell lung cancer, pancreatic cancer, ovarian cancer, stomach cancer, lung cancer, salivary cancer and head/neck cancer.

The present invention also provides a treatment method for malignant tumor over-expressing EGF receptor containing the step of administering the effective dose of the pharmaceutical composition of the invention to a subject.

The malignant tumor over-expressing EGF receptor above is preferably selected from the group consisting of breast cancer, bladder cancer, colon cancer, glioma, non-small-cell lung cancer, pancreatic cancer, ovarian cancer, stomach cancer, lung cancer, salivary cancer and head/neck cancer.

The effective dose can be determined by a doctor in charge.

【Description of Drawings】

The application of the preferred embodiments of the present invention is best understood with reference to the accompanying drawings, wherein:

Fig. 1 is a diagram illustrating the amino acid sequences of heavy chains and light chains (κ chains) of 7 antibodies binding to EGF receptor.

5 Fig. 2 is a diagram illustrating the antibody expression vector for the preparation of a whole IgG.

Fig. 3 is a diagram illustrating the result of SDS-PAGE with antibodies binding to the EGF receptor expressed, separated and purified from CHO cells.

10 Fig. 4 is a graph illustrating the EGF binding capacity of antibodies, measured by ELISA.

Fig. 5 is a graph illustrating the *in vitro* inhibition of cell growth of a human epidermoid carcinoma cell line A431 by the selected antibody.

15 Fig. 6 is a graph illustrating the tumor sizes (mm^3) in mouse models transplanted with tumor cells to examine the effect of the antibody.

Fig. 7 is a diagram illustrating the result of the investigation of aggregate content, measured by SEC-HPLC.

20 Fig. 8-1 is a diagram illustrating the maintenance of the binding capacity to EGF receptor after heat treatment, measured by ELISA.

Fig. 8-2 is a diagram illustrating the changes of aggregate content after heat-treatment, measured by SEC-HPLC.

Fig. 9 is a diagram illustrating the *in vivo* distribution of the antibody in a mouse tumor model.

Fig. 10 is a graph illustrating the tumor sizes in mouse tumor models to investigate the effect of the antibody.

10 **【Mode for Invention】**

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: Construction of chimeric Fab library

20 To construct EGF receptor specific chimeric Fab (chimeric antibody binding fragment) library, a recombinant EGF and EGF receptor protein mixture or epidermoid carcinoma cells (A431; ATCC, CRL-1555) were mixed with Ribi adjuvant (Sigma Co.) at the ratio of 1:1. This mixture was

injected into a rabbit at three week intervals to induce immune response. Blood samples were taken from the immunized rabbit (immunized by four times of injection) and antibody formation was confirmed. Total RNA was extracted from the bone marrow and spleen of the rabbit, which was reverse-transcribed into cDNA using Superscript II kit (Invitrogen).

The rabbit variable region heavy chain (V_H) and light chain (V_L) were amplified using the above cDNA as a template with the primer set described in the paper of Carlos, F (Phage Display: A Laboratory Manual, Carlos, F. et al., 2001 by Cold Spring Harbor Laboratory Press, Volume 1). The Fab fragments of the human constant region heavy chain (C_{H1}) and light chain (C_L) were also amplified with the same primer set. The amplified rabbit variable region fragments (V_H , V_L) and human constant region fragments (C_{H1} , C_L) were amplified to convert them into 'rabbit V_H -human C_{H1} ' and 'rabbit V_L -human C_L ' using overlapping primers. The amplified two fragments were amplified again with those overlapping primers to prepare chimeric Fab DNA.

The both ends of the chimeric Fab DNA fragment obtained from the above PCR were digested with a restriction enzyme, which was inserted into the expression vector pComb3X (Genebank No. AF268281; Rader, C. and

Barbas, C.F. III. (in) PHAGE DISPLAY, A LABORATORY MANUAL. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2000), resulting in the construction of chimeric Fab library.

5

Example 2: Construction of chimeric Fab phage library

The chimeric Fab library constructed in the above example was introduced into ER2738 cells (New England Biolabs), followed by culture for 2 hours. The cells were
10 infected with helper phage, followed by further culture for 16 - 18 hours. The cultured cells were centrifuged. Plasmid DNA was separated from the cells collected from the centrifugation. Polyethylene glycerol (PEG) was added to the supernatant, which proceeded to centrifugation to
15 obtain a pellet. The pellet was suspended in reaction buffer supplemented with 1% BSA and 0.02% sodium azide. The phage was recovered to prepare chimeric Fab phage library.

20 Example 3: Amplification of EGFR binding chimeric Fab antibody phage pool

To select antibody clones with high affinity to EGF receptor, competitive cell panning was performed using the chimeric Fab phage library prepared in example 1.

Particularly, A431 (ATCC, CRL-1555) epidermoid carcinoma cells ($1 \times 10^6/\text{ml}$) were suspended in reaction buffer supplemented with 1% BSA and 0.02% sodium azide, followed by culture at 4°C for one hour. WI38 VA-13 (ATCC, CCL-75.1) normal fibroblasts were added thereto, followed by further culture. Membrane fragments ($100 \mu\text{g}/100 \mu\text{l}$) isolated by ultracentrifugation, 255 monoclonal antibody ($50 \mu\text{g}/10 \mu\text{l}$) (ATCC HTB-8508 hybridoma was cultured and purified by Protein-A column to produce the monoclonal antibody) and the phage library ($4 \times 10^{12}/\text{ml}$) were mixed, followed by culture at 4°C for one hour.

The reaction mixture was centrifuged to obtain a pellet, which was washed four times with reaction buffer supplemented with 0.1% Tween 20. 1 ml of 100 mM TEA was added thereto, followed by culture at 37°C for 10 minutes to elute the phage. The eluted phage solution was neutralized with 1 M Tris (pH 7.4). 1 ml of the neutralized solution was mixed with 10 ml of ER2738 cell solution ($\text{OD}_{600} \leq 1$), followed by culture for one hour. Helper phage was added to the culture solution, followed by further culture for 16 - 19 hours.

Upon completion of the culture, supernatant was obtained by centrifugation. PEG was added to the supernatant, followed by centrifugation again. The phage

was precipitated by the same manner as described in example 1, which was collected and used for panning. The cell panning was repeated 5 times and finally a phage pool was obtained.

5

Example 4: Selection of the chimeric Fab phage highly binding to EGFR

A431 cells were treated with dissociation buffer (GIBCO) and then detached from the plate. After washing
10 three times with PBS, the cells were suspended in FACS (1% BSA, 0.02% sodium azide, PBS) at the concentration of 5×10^5 cell/ml, which were distributed in a 96-well v bottom plate (Corning, #3897) by 200 μ l/well. The plate was incubated at 4°C for one hour, followed by centrifugation to remove
15 supernatant.

The phage pool prepared in example 3 was distributed in the plate, followed by culture at 37°C for overnight to obtain colonies. The obtained colonies were inoculated in SB medium (Beckton Dickenson), which was then infected with
20 helper phage by the same manner as described in example 3. The resultant phage was added to the plate by 100 μ l/well, followed by culture at 4°C for one hour. After washing with FACS solution twice, 50 μ l of anti-Fab FITC antibody (Jackson ImmnoResearch: FITC-conjugated affiniPure Goat

anti-Human IgG, F(ab)₂ fragment specific) was added to the phage, followed by further culture at 4°C for one hour. The culture solution was washed with FACS solution three times and then washed once again with reaction buffer, followed
5 by FACS to select phage clones binding to A431 cells. Phagemid was obtained from the 12 phage clones binding to A431. DNA sequences of the heavy chain variable region and light chain variable region of the phagemid were analyzed using primers represented by SEQ. ID. NO: 1 and NO: 2. The
10 identified sequences are shown in Fig. 1, which were confirmed to be the clones with high homology with only 2 - 3 amino acid difference.

Example 5: Construction of an expression vector for whole
15 IgG production from chimeric phage antibody and the
expression thereof in 293T/17 cells

To construct an expression vector for the expression of a whole IgG, Apal site of pCND4 3.1 vector (Invitrogen) was removed by Klenow polymerase, to which human heavy
20 chain cDNA (Genebank No. BC 089417) and light chain cDNA (Genebank No. BC 067092) were inserted respectively, resulting in the construction of the expression vector pCDH-101 expressing human heavy chain and the expression vector pCDK-101 expressing human light chain. The vector

maps are shown in Fig. 2.

To prepare the chimeric Fab clones selected in Example 4 as a whole IgG form, the heavy chain variable region of the chimeric Fab was amplified, to which a signal sequence was inserted. Restriction enzyme recognition sites (*Eco* RI and *Apa* I) were also inserted in both ends.

Particularly, the first PCR was performed using the phagemid DNA obtained from the chimeric Fab phage clone as a template with primers C4H_Fw1 (SEQ. ID. NO: 3) and C4H_Rv1 (SEQ. ID. NO: 4), the second PCR was performed using the first PCR product as a template with primers C4HK_Fw2 (SEQ. ID. NO: 5) and C4H_Rv2 (SEQ. ID. NO: 6), and the third PCR was performed using the second PCR product as a template with primers C4H_Fw3 (SEQ. ID. NO: 7) and C4H_Rv2 (SEQ. ID. NO: 8). All the PCRs were performed using DNA polymerase (Taq polymerase, Takara) as follows; predenaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, polymerization at 72°C for 1 minute, 30 cycles from denaturation to polymerization, and final extension at 72°C for 10 minutes.

The variable region of the pCDH-101 vector expressing human heavy chain cDNA was digested with *Eco* RI and *Apa* I, to which the third PCR product digested with *Eco* RI and *Apa*

I was cloned. The resulted expression vectors were named as pCDH-4, pCDH-44, pCDH-46, pCDH-47, pCHD-49, pCDH-H411 and pCDH-412.

5 The light chain (κ chain) variable region of the chimeric Fab phage was amplified, to which a signal sequence was inserted and so were *Hind* III and *Bsiw* I restriction enzyme sites in both ends. Particularly, the first PCR was performed using the phagemid DNA obtained from the chimeric Fab phage clone as a template with
10 primers C4K_Fw1 (SEQ. ID. NO: 8) and C4K_Rv1 (SEQ. ID. NO: 9), the second PCR was performed using the first PCR product as a template with primers C4HK_Fw2 (SEQ. ID. NO: 5) and C4K_Rv1 (SEQ. ID. NO: 9), and the third PCR was performed using the second PCR product as a template with
15 primers C4K_Fw3 (SEQ. ID. NO: 10) and C4K_Rv1 (SEQ. ID. NO: 9).

The variable region of the pCDK-101 expressing chimeric light chain (κ chain) cDNA was digested with *Hind* III and *Bsiw* I, to which the third PCR product digested
20 with *Hind* III and *Bsiw* I was inserted. The resultant expression vectors were named as pCDK-4, pCDK-44, pCDK-46, pCDK-47 pCDK-49, pCDK-411 and pCDK-412.

The complementarity determining region (CDR) was determined by Kabat numbering

(<http://www.bioinf.org.uk/abs/#cdrdef>).

At last, the present inventors prepared an antibody as a whole IgG form by introducing the expression vectors produced above into animal cells. Particularly, the heavy chain and light chain expression vectors pCDH-X and pCDK-X were mixed at the ratio of 3: 7 (to form a whole antibody with a set of one heavy chain and one light chain that have same number), which was introduced into 293T/17 cells (ATCC) by transfection using calcium phosphate (Girard, et al., Cytotechnology 35, 175-180, 2001).

The transformant was cultured in HYQ SFM4CH0 medium (Hyclone) for one week and the antibody was purified using protein-A column (Amersham). The purified antibody was analyzed by using NuPAGE 4-12% Bis-Tris Gels 1.0 (Invitrogen) under the unreduced condition to obtain IgG with at least 95% of purity (Figure 3).

Example 6: Binding capacity

<6-1> BIAcore analysis

The affinity constant of the purified antibody to EGF receptor was determined by BIAcore analysis. BIAcore 3000 was used for the analysis. Recombinant human EGF receptor (R&D systems, Cat.1095-ER) was diluted in 10 mM sodium acetate (pH 5.0) at the concentration of 30 μ g/ml and

linked to CM5 sensor chip by amine coupling method. HBS-EP solution (10 mM HEPES, 150 mM NaCl, 3 mM EDTA & 0,005% SP20, pH7.4) was used as running buffer. As a result, dissociation constant K_D was $6.7E-10M \sim 2.8E-11M$, suggesting that the antibody of the invention has high binding capacity (Table 2).

【Table 2】

10 Measurement of Affinity of 7 antibodies by BIAcore

	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
CRH4	5.33E+05	3.57E-04	6.70E-10
CRH44	1.81E+05	3.09E-04	1.70E-10
CRH46	3.38E+05	1.01E-05	2.90E-11
CRH47	1.87E+05	1.85E-05	1.00E-10
CRH49	3.26E+05	9.45E-06	2.80E-11
CRH411	1.89E+05	1.30E-05	6.90E-11
CRH412	4.85E+05	1.89E-05	3.90E-11

* CRH : Chimeric Rabbit Human

<6-2> ELISA

15 The binding capacity of the IgG antibody purified in Example 5 to the recombinant human EGF receptor (R&D systems) was measured *in vitro* by ELISA. Particularly, the EGF receptor was loaded in each well of a 96-well plate at the concentrations of 300 ng/ml and 1 μ g/ml, followed by
20 coating at 4°C for 16 hours or at 37°C for 3 hours. Then,

the plate was washed three times with TBST supplemented with 0.05% Tween 20.

Blocking was performed with TBST supplemented with 2% BSA at 37°C for 2 hours, followed by washing three times
5 with 0.05%-TBST. The IgG antibody was serially diluted, 5-fold each time (1526.5 ng/ml - 0.1 ng/ml), which was distributed in the 96-well plate, followed by ~~conjugation~~ incubation at 37°C for 1 hour.

The plate was washed three times with 0.05%-TBST, to
10 which HRP labeled goat anti-human IgG (Fc, Sigma) secondary antibody diluted 3000 fold in 2% BSA-TBST was added, followed by incubation at 37°C for one hour. After washing three times with 0.05%-TBST, color development was induced at 37°C for 7 minutes with OPD (OPD (O-Phenylenediamine
15 dihydrochloride, Sigma). The reaction was terminated by 2.5 N sulfuric acid and OD₄₉₀ was measured.

As a result, all the antibodies selected above exhibited binding capacity specific to human EGF receptor. Considering comparative binding capacity, CRH44, CRH46,
20 CRH47, CRH49, CRH411 and CRH412 showed higher binding capacity than that of CRH4 antibody (Fig. 4).

Example 7: Tumor growth inhibition effect *in vitro*

Inhibitory effect of the chimeric antibody of the

invention on the human tumor cell growth was investigated by the method described in the paper of Ishiyama, et al (Ishiyama, et al., Biol. Pharmacol. Bull., 19, 1518-1520, 1996). Particularly, A431 cells were cultured in a serum free mixed medium (DMEM:F12 = 1:1). The cells (2×10^3) were diluted in 200 μ l medium, which were inoculated in a 96-well plate, to which the chimeric antibody diluted in 100 μ l medium was added, followed by culture at 37°C for 3 - 5 days. A control was prepared without adding the antibody of the present invention but cultured under the same condition.

After eliminating the medium, the cells were fixed with 0.25% glutaraldehyde, washed with 0.9% NaCl solution and dried at room temperature. The cells were stained with 0.4% crystal violet, washed with water and dried at room temperature. 100 μ l of methanol was added thereto, followed by mixing at room temperature for 5 minutes. Then, OD₅₉₅ was measured. The relative cell growth was calculated by comparing the cell growth of the experimental group with that of the control group added with a medium without antibodies, which was presented as %.

As a result, A431 cells cultured in the serum free medium exhibited the relative growth of less than 40% at the antibody concentration of 1 μ g/ml, suggesting that the

tumor cell growth inhibitory effect of the antibody was confirmed (Fig. 5).

Example 8: *In vivo* anticancer effect in mouse tumor models

To evaluate the *in vivo* anticancer effect of the chimeric antibody of the invention, balb/c nude mouse tumor models were used. Particularly, an epidermoid carcinoma cell line (A431; ATCC, CRL-1555) was used as a cell line for tumor transplantation. The A431 cells (5×10^6) in 100 μ l buffer were transplanted into the abdominal region of balb/c nude mice by hypodermic injection. When the tumor size reached 100 mm³, the mice were grouped randomly and then administered with the antibody through intra-abdominal cavity at different concentrations and different frequencies. Then, the tumor growth was observed over time and concentrations.

【Table 3】

Cell Line	Group	Antibodies	Dose	Cycle
A431	I	Negative control PBS	0 mg	q3d, 5weeks
	II	CRH4	0.1 mg	
	III	CRH4	0.5 mg	
A431	I	Negative control PBS	0 mg	q3d, 5weeks
	II	CRH4	0.5 mg	
	III	CRH412	0.5 mg	

As shown in Fig. 6A, the tumor growth inhibitory effect of CRH4 antibody in A431 cells was observed even at low concentration of 0.1 mg, which was not much different from the effect at the concentration of 0.5 mg. The tumor growth inhibitory effect continued approximately 7 days until the tumor was grown to 500 mm³.

As shown in Fig. 6B, the tumor growth inhibitory effects of CRH4 and CRH412 antibodies (each administered by 0.5 mg) were compared. The tumor growth inhibition by CRH4 antibody was significant. Unlike the negative control, the tumor growth inhibitory effect continued approximately 21 days until the tumor was grown to 500 mm³. On the contrary, the tumor growth inhibition by CRH412 (0.5 mg) was not significant, which was similar with that in the negative control.

Example 9: Construction of an antibody library with improved stability

In spite of its high affinity ($K_D = 3.90E-11$ M), the chimeric antibody CRH412 did not exhibit tumor inhibitory effect in xenograft model transplanted with A431 epidermoid carcinoma cells. This was presumably because of low tumor targeting resulted from the low stability of the CRH412 antibody (Willuda J, Honegger A, Waibel R, Schubiger PA,

Stahel R, Zangemeister-Wittke U, Pluckthun A. Cancer Res. 1999, 59(22):5758-5767).

Thus, to overcome the problem of the low stability of the CRH412 antibody, a sequence containing CDR (complementary determining region) of the CRH412 antibody was transplanted into hu4D5-framework (referred as "4D5" hereinafter; Willuda J, Honegger A, Waibel R, Schubiger PA, Stahel R, Zangemeister-Wittke U, Pluckthun A. Cancer Res. 1999, 59(22):5758-5767) which has been known to have high stability. At this time, some amino acids of CDR and framework were modified, aiming at: 1) degeneracy was given in order for the CDR sequence to contain all the sequences of CRH4, CRH411 and CRH412, 2) degeneracy was given in order for the CDR adjacent framework region to contain the CRH412 framework sequence as it was or to contain the sequence of Fig. 3, and 3) degeneracy was given to eliminate negative charge or to introduce positive charge to increase isoelectric point value of a humanized antibody. Specifically, the amino acid modifications were made as shown in Table 4. Numbering of the variable region of an antibody was performed by Kabat numbering (Kabat et al., "Sequences of Proteins of Immunological Interest", Bethesda, MD: National Institute of Health pp. 14-32(1983)).

【Table 4】

	Kabat No.	Hu- Library	Locatio n	Reason
VH Domain	2	V/Q	FR-H1	Combination of 4D5/CRH4 sequence
	24	A/V	FR-H1	Combination of 4D5/CRH4 sequence
	28,29,30	S L S	FR-H1	Three amino acids adjacent to CDR-H1 (28-30) are changed into the sequence of CRH4
	47	W/Y	FR-H2	Three amino acids adjacent to CDR-H2 (47-49) are changed into the combination of 4D5/CRH4 sequence
	48	V/I	FR-H2	
	49	A/G	FR-H2	
	71	A/K/R	FR-H3	Amino acid 71 is adjacent to FR-H3 loop (72-76) and changed into the combination of 4D5/CRH4/other human antibodies sequence
	72~76	DNSKNT	FR-H3	Four amino acids of FR-H3 loop(72-76) are changed into other human antibody sequence and thereby increased into 6 amino acids
	77	A/V/L	FR-H3	Amino acid 77 is adjacent to FR-H3 loop(72-76) and changed into the combination of 4D5/CRH4/other human antibodies sequence
	93	A	FR-H3	Three amino acids adjacent to CDR-H3(92-94) are changed into CRH4 sequence
	101	N/D	CDR-H3	Combination of CRH4/CRH411 sequence for removing negatively charged amino acid aspartate
VL	1,2	DI/EL	FR-L1	Combination of 4D5/CRH4 sequence (block

Domain				change)
	24	Q/R	CDR-L1	Combination of 4D5/CRH4 sequence for introduction of positively charged amino acid arginine
	27	E/Q	CDR-L1	Combination of 4D5/CRH4 sequence for removing negatively charged amino acid glutamate
	31	D/N	CDR-L1	Combination of CRH4/CRH411 sequence for removing negatively charged amino acid asparagine
	54	L/R	CDR-L2	Amino acid were varied for introduction of positively charged amino acid arginine
	55	E/Q	CDR-L2	Amino acid were varied for removing negatively charged amino acid glutamate
	66	R/G	FR-L3	Combination of 4D5/CRH4 sequence for introduction of positively charged amino acid arginine
	92	D/N	CDR-L3	Amino acid were varied for removing negatively charged amino acid aspartate
	96	F/L	CDR-L3	Combination of CRH4/CRH12 sequence

To synthesize a novel Fab library transplanted with CDR, each light chain and heavy chain variable region (VK, VH) was synthesized by PCR using oligo nucleotide. The synthesis was performed by the method described in the paper of Gao, et al (Gao X, Yo P, Keith A, Ragan TJ, Harris TK. Nucleic Acids Res. 2003; 31(22):e143.), and the synthetic oligo nucleotide was designed by DNAWorks 3.1

program (<http://helixweb.nih.gov/dnaworks/>). The synthesized VK sequence and VH sequence were inserted into the vector pComb3X (GeneBank No. AF268281) for phage display by the same manner as described in Example 1. At last, a Fab phage library was constructed by the same manner as described in Example 2.

Example 10: Selection of CRH412 derivative Fab antibody phage clone binding to EGF receptor

Epoxy bead panning was performed to selectively amplify the EGFR binding antibody pool using the novel CRH412 Fab phage library constructed in Example 9.

Particularly, the phage library (4×10^{12} /ml) was mixed with the epoxy bead (1×10^7 , Dynal) coated with BSA, followed by reaction at 4°C for one hour. In the meantime, the epoxy bead coated with the recombinant EGFR extra cellular domain (EGFR ECD) was suspended in reaction buffer supplemented with 1% BSA and 0.02% sodium azide, followed by reaction at 4°C for one hour. The EGFR ECD coated bead and the phage library were recovered and mixed, followed by culture at 4°C for 2 hours.

The bead was collected from the reaction mixture using a magnet and washed four times with reaction buffer supplemented with 0.1% Tween 20, to which dilution buffer

(0.1 M citrate, pH 3.1) was added. After culturing at room temperature for 10 minutes, the phage was eluted. The phage eluate was neutralized with 1 M Tris (pH 8.0), from which 1 ml was taken and mixed with 20 ml of XL1-blue cells (OD₆₀₀: ≤ 1), followed by culture for one hour. Helper phage was added thereto, followed by further culture for 16 - 19 hours. Upon completion of the culture, centrifugation was performed to obtain supernatant. After adding PEG, centrifugation was performed again to precipitate the phage. The precipitated phage was collected and used for panning. The above panning was repeated three times and at last the phage pool was obtained.

FACS analysis was performed to select an individual antibody clone binding specifically to EGF receptor from the Fab phage pool. Particularly, the culture medium (RPMI, 10% FBS) was eliminated from the plate where A431 cells were cultured. The cells were detached from the plate by treating dissociation buffer (GIBCO), washed with PBS three times, suspended in FACS solution (1% BSA, 0.02% sodium azide, PBS) at the concentration of 5×10^5 cell/ml, distributed in a 96-well V-bottom plate (Corning, #3897) at the concentration of 200 μ l/well and cultured at 4°C for one hour. Centrifugation was performed to eliminate supernatant. In the meantime, the phage pool obtained in

the above was distributed on the plate, followed by culture at 37°C for one day and then colonies were collected. The colonies were inoculated to SB medium (Beckton Dickenson), which was infected with helper phage by the same manner as described in Example 2. The obtained phage was added to the plate by 100 μ l/well, followed by culture at 4°C for one hour. After washing with FACS solution twice, 50 μ l of anti-Fab FITC antibody (FITC-conjugated anti-Human IgG, F(ab')₂ fragment specific, Jackson ImmnoRese) was added to the phage, followed by further culture at 4°C for one hour. The culture solution was washed with FACS solution three times and then washed once again with reaction buffer, followed by FACS to select phage clones binding to A431 cells expressing EGF receptor.

DNA sequence of the selected clones binding to A431 EGFR was analyzed and as a result the antibody clones having the amino acid sequences shown in Fig. 7 were obtained.

Example 11: Construction of an expression vector for the preparation of a whole IgG from a humanized phage antibody and the expression thereof in 293T/17 cells

CRH412 originated Fab phage clone selected in Example 10 was prepared as a whole IgG form by the same method as

described in Example 5.

The heavy chain variable region and constant region of the Fab phagemid DNA were amplified, to which a signal sequence was inserted and so were restriction enzyme sites (Eco RI and Age I) into the both ends. Particularly, the first PCR was performed by using the phagemid DNA obtained from the Fab phage clone of Example 10 as a template with primers hH_Fw and Heavy_CH1_Rev. Amplification of the signal sequence was performed by using the pCHD-101 vector DNA as a template with primers CMV_Fw and C4_Ov_Rv, and the second PCR was performed by using the first PCR product as a template with primers CMV_Fw and Heavy_CH1-Rev. All the PCRs were performed using DNA polymerase (High fidelity Taq polymerase, Roche) as follows; predenaturation at 95°C for 5 minutes, denaturation at 95°C for 45 seconds, annealing at 56°C for 45 seconds, polymerization at 72°C for 2 minutes, 15 cycles from denaturation to polymerization, and final extension at 72°C for 10 minutes. The variable region of the vector pCDH-101 expressing human heavy chain cDNA was digested with Eco RI and Age I, to which the PCR product digested with Eco RI and Age I was cloned.

The light chain (κ chain) variable region of the Fab phagemid DNA was amplified, to which a signal sequence was

inserted and so were restriction enzyme sites (*Hind* III and *Bsi* WI) into the both ends. Particularly, the first PCR was performed by using the phagemid DNA obtained from the Fab phage clone of Example 10 as a template with primers hL_Fw1/2 and hL_Rv1/2. Amplification of the signal sequence was performed by using the pCHD-101 vector DNA as a template with primers CMV_Fw and C4_Ov_Rv, and the second PCR was performed by using the first PCR product as a template with primers CMV_Fw and hL_Rv1/2. The variable region of the vector pCDK-101 expressing human light chain (κ chain) cDNA was digested with *Hind* III and *Bsi* WI, to which the PCR product digested with *Hind* III and *Bsi* WI was inserted.

【Table 5】

hH_FW	5'- gctacaggtgtccactccgaagtgcagctggtgga gt-3'	SEQ. ID. NO: 11
Heavy_CH1_Rev	5'-ccacgctgctgagggagtagagtc-3'	SEQ. ID. NO: 12
CMV_Fw1	5'-cgctattaccatggtgatgc- 3'	SEQ. ID. NO: 13
C4_Ov_Rv	5'-ggagtggacacctgtagc-3'	SEQ. ID. NO: 14
hL_FW1	5'- gctacaggtgtccactccgaactgcagatgaccca gtc-3'	SEQ. ID. NO: 15
hL_FW2	5'- gctacaggtgtccactccgatatccagatgaccca gtc-3'	SEQ. ID. NO: 16
hL_Rv 1	5'-cgccgtacgtttgatctccaccttagttcc- 3'	SEQ. ID. NO: 17

hL_Rv 2	5'-cgccgtacgtttgatttccaccttagttccc- 3'	SEQ. ID. NO: 18
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Finally, the IgG expression vector prepared above was inserted into animal cells by the same manner as described in Example 5 and IgG was purified therefrom.

5

Example 12: EGF receptor binding capacity of the novel CRH412 modified antibody

The EGF receptor binding capacity of the antibody was measured by BIAcore according to the method described in Example 6. In Table 6, hIgG2 is the antibody composed of the heavy chain variable region H2 and the light chain variable region L2 as shown in Fig. 7, and other antibodies were composed of the heavy chain and the light chain having same numbers by the same manner. The novel CRH412 modified antibodies were confirmed to have the same level of EGF receptor binding capacity with CRH412, except hIgG2 (Table 6).

【Table 6】

20

	ka (M ⁻¹ s ⁻¹)	kd (s ⁻¹)	KD (M)
hIgG2	6.49E+04	4.40E-04	6.78E-09
hIgG36	1.08E+05	6.19E-05	5.73E-10
hIgG19	1.27E+05	3.86E-06	5.81E-11
hIgG18	9.06E+04	2.26E-05	2.49E-10

hIgG26	1.28E+05	1.48E-05	1.16E-10
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Example 13: Improvement of the stability of the antibody

<13-1> Analysis of the aggregate content of the antibody

CRH412 antibody and its variants were purified by
 5 protein-A column and the size of the purified antibody was
 measured by Size Exclusion Chromatography (SEC) HPLC.
 Particularly, two TSK-GELG3000SWXL columns were connected
 in series, over which the elution buffer (150 mM NaCl, 200
 mM KPO₄, pH 6.9) was flowing and OD was measured at 280 nm
 10 and at 220 nm.

In case of the CRH412 antibody, the aggregate content
 after protein-A column purification was approximately 7%.
 In the meantime, the aggregate contents of hIgG2, hIgG12,
 hIgG18, hIgG19, hIgG26 and hIgG32 were all as low as 1 - 2%
 15 of total protein, suggesting that they were all very stable
 antibodies. Fig. 7 shows the result of aggregation of
 hIgG19, indicating that the aggregation aggregate content
 thereof was as low as 0.65%.

20 <13-2> Analysis of the thermo-stability of the antibody

To investigate the thermo-stability of the antibody,
 100 μ l of each CRH412 and CRH412 modified antibodies (1.56
 μ g/ml) respectively was loaded in a microtube, followed by

heat treatment at 80°C for 10 minutes. After the heat-treatment, centrifugation was performed at 13,000 rpm for 10 minutes. The obtained supernatant was filtered with a 0.22 µm filter and the filtrate was diluted serially in PBS + 0.1% BSA solution, 5 fold each time, followed by EGFR binding ELISA. Particularly, ELISA was performed as follows; each well of a 96-well plate was coated with 300 ng/ml of EGF receptor at 4°C for 16 hours, and then washed three times with TBST (Tris-Buffered Saline Tween 20) supplemented with 0.05% Tween 20.

Blocking was performed with TBST supplemented with 2% BSA at 37°C for 2 hours, followed by washing three times with 0.05%-TBST. IgG antibody was diluted serially in 2% BSA-TBST, 5 fold each time (1526.5 ng/ml - 0.1 ng/ml), and the solution was distributed in a 96-well plate, followed by conjugation at 37°C for 1 hour.

The plate was washed three times with 0.05%-TBST, followed by conjugation with HRP (Horse radish peroxidase) labeled goat anti-human IgG (goat anti-human Fc, Sigma) secondary antibody diluted 3000-fold in 2% BSA-TBST at 37°C for 1 hour. After washing three times again with 0.05%-TBST, color development was induced with OPD (O-Phenylenediamine dihydrochloride, Sigma) at 37°C for 7 minutes. The reaction was terminated by 2.5 N sulfuric

acid and then OD₄₉₀ was measured.

As shown in Fig. 8-1, compared with the control (commercial anti-EGFR antibody C225, Erbitux), the CRH412 variants were confirmed to have significantly enhanced thermo-stability. In Fig. 8-1, the C225-control indicates the binding capacity of C225 before the heat-treatment and others indicate the clone specific binding capacity of C225, CRH412, hIgG12, hIgG18, hIgG19, hIgG26 and hIgG32 after the heat-treatment. EGF receptor binding capacities of C225 and CRH412 were significantly reduced to 0% after the heat treatment. On the other hand, EGF receptor specific binding capacities of the CRH412 variants were not reduced after the heat-treatment and maintained the EGF receptor binding capacity level to the equal level of C225 not treated with heat.

Another experiment to verify the thermo-stability of the antibody was performed as follows; 50 μ l of each C225 (Erbitux), CRH412 and CRH412 modified antibodies were loaded in a microtube respectively at the concentration of 2 mg/ml, followed by heat-treatment at 65°C for 10 minutes. After the heat-treatment, 40 μ g of each antibody proceeded to SEC (size exclusion chromatography) by the same manner as described in Example <13-1>. In case of the C225 antibody, the aggregate content after the heat treatment

(65°C, 10 minutes) was approximately 12%. In the meantime, the aggregate contents of hIgG2, hIgG12, hIgG18, hIgG19, hIgG26 and hIgG32 were all less than 1% of total protein, suggesting that they were all very stable antibodies.

5 Compared with the commercial anti-EGFR antibody, C225 (Erbix), CRH412 variants were confirmed to have excellent thermo-stability. Fig. 8-2 illustrates the result of comparison of the thermo-stability between hIgG26 and C225.

10 Example 14: Improvement of *in vivo* effect

<14-1> Tumor targeting analysis

To investigate *in vivo* dynamics and tumor targeting in mouse tumor models, CRH412 antibody and hIgG12, hIgG18, hIgG19, hIgG26 and hIgG32 antibodies were labeled with DOTA-linker and then labeled again with ¹¹¹Indium. The labeled antibodies were intravenously-injected into the tail vein of the xenograft nude mouse transplanted with A431 cells. After 24 hours and 48 hours from the injection, the test animals were sacrificed and each organ was extracted and radioactivity was measured. Compensating the natural decay of radioactivity at each check point, the *in vivo* distribution was presented by %ID (Injected Dose) per gram tissue.

15
20

First, the labeling was performed with DOTA-linker as

follows. 460 μl (460 μg , 666.7 nmol) of **p**-SCN-Bn-DOTA[2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclo dodecane-1,4,7,10-tetraacetic acid] at 1 mg/ml concentration (in 0.1M Sodium bicarbonate, pH 8.7) was added to 1 mg/200 μl of each
5 CRH412 (experimental antibody) and C225 (control antibody), followed by reaction at 37°C for 60 minutes in the dark. PD-10 column was loaded with 1% BSA and 1 ml of 20 mM cold DOTA and then fully washed with DDW (deionized distilled water). The DOTA binding antibody was loaded into the PD-
10 10 column, followed by fractionation by 0.5 ml using 0.9% NaCl (in DDW, ultra pure reagent). The DOTA binding antibody was aliquoted by 50 μg (approximately 1 mg/2 ml), which was stored at -70°C.

¹¹¹Indium labeling was performed as follows. 50 μg of
15 the DOTA binding antibody was mixed with 200 μl of 0.1 M sodium acetate (pH 5.4, reaction solution), to which 500 μCi of ¹¹¹Indium was added, followed by reaction for one hour at room temperature. PD-10 column was loaded with 1 ml of 1% BSA, followed by washing with injectable saline.
20 The ¹¹¹In-DOTA-antibody was loaded into the PD-10 column, followed by fractionation by 0.5 ml using 0.9% NaCl (in DDW, ultra pure reagent). Radioactivity of the fraction was measured.

A431 xenograft model was generated as follows. A431

cells (5×10^6) were added into 100 μl of buffer, which was transplanted into the abdominal region of balb/c nude mice by hypodermic injection. When the tumor size reached 100 mm^3 , the mice were grouped randomly. 8 of each group were
5 injected intravenously with 5 μCi of the ^{111}In labeled antibody into the tail vein. After 24 and 48 hours from the injection, 4 of each group were sacrificed and the *in vivo* distribution of the antibody was examined. The organs such as blood, liver, spleen, kidney, muscle, skin, bone,
10 lung, heart, stomach, brain, tumor, intestine, and tail were extracted and the radioactivity therein was measured. Triplicate standard of the ^{111}In labeled antibody injected was prepared to compensate the natural decay of the radioactivity measured at each sampling point. The *in vivo*
15 distribution was presented by mean $\pm$ standard deviation, indicating %ID (Injected Dose) per gram tissue.

The *in vivo* distributions of CRH412 and its modified antibodies with improved stability after Indium-111 labeling were shown in Fig. 9. The CRH412 antibody showed
20 very weak tumor targeting of 2 ± 1 %ID/g and 2 ± 1 %ID/g respectively at 24th and 48th hour from the labeling. The CRH412 also exhibited short half-life in blood and high non-specific absorption in the liver and spleen. These results indicate that the CRH412 antibody had weak tumor

targeting in the xenograft model transplanted with A431 epidermoid carcinoma cells in spite of its high affinity ($K_D = 3.90E-11$ M). In the meantime, the modified antibodies with improved stability exhibited excellent tumor targeting. And also, those variants exhibited very low non-specific absorption in the liver and spleen and increased half-life in blood.

<14-2> In vivo tumor inhibitory effect

The *in vivo* tumor inhibitory effect of the CRH412 modified antibodies with improved stability (hIgG12, hIgG19 and hIgG26) on mouse tumor models was investigated by the same manner as described in Example 8. As shown in Fig. 10, the antibody variants having CDR of the CRH412 with improved stability had excellent tumor inhibitory effect. As shown in Fig. 6 and Fig. 10, the *in vivo* tumor inhibitory effect can be improved by increasing the stability of an antibody.

【Industrial Applicability】

As explained hereinbefore, the anti-EGF receptor antibody of the present invention has high binding capacity to EGF receptor, so that it can inhibit EGF binding and has excellent tumor suppressing effect and tumor targeting with

high stability. And also, the anti-EGF receptor antibody can minimize immune response against the antibody by containing human immunoglobulin constant regions, so that it can be effectively used for the diagnosis and treatment
5 of cancer expressing EGF receptor.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis
10 for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

15

【CLAIMS】**【Claim 1】**

An EGF receptor specific antibody which comprises a recombinant heavy chain comprising human originated heavy chain constant region (C_H) linked to the heavy chain variable region (V_H) containing rabbit originated heavy chain complementarity determining region (HCDR) and a recombinant light chain comprising human originated light chain constant region (C_L) linked to the light chain variable region (V_L) containing rabbit originated light chain complementarity determining region (LCDR).

【Claim 2】

The EGF receptor specific antibody according to claim 1, wherein the antibody is a humanized antibody, a chimeric antibody or its fragment.

【Claim 3】

The EGF receptor specific antibody according to claim 1, wherein the heavy chain complementarity determining region contains CDR-H1 represented by SEQ. ID. NO: 19, CDR-H2 represented by SEQ. ID. NO: 20 and CDR-H3 represented by SEQ. ID. NO: 21 or NO: 22.

【Claim 4】

The EGF receptor specific antibody according to claim 1, wherein the light chain complementarity determining region preferably includes CDR-L1 represented by one of SEQ. ID. NO: 23 - NO: 26, CDR-L2 represented by one of SEQ. ID. NO: 27 - NO: 29 and CDR-L3 represented by one of SEQ. ID. NO: 30 - NO: 32.

【Claim 5】

The EGF receptor specific antibody according to claim 1, wherein the heavy chain variable region is selected from the group consisting of sequences represented by SEQ. ID. NO: 33 - NO: 56.

【Claim 6】

The EGF receptor specific antibody according to claim 1, wherein the light chain variable region is selected from the group consisting of sequences represented by SEQ. ID. NO: 58 - NO: 84.

【Claim 7】

A gene encoding one of the recombinant heavy chain variable regions of the EGF receptor specific antibodies of

claim 1 - claim 6.

【Claim 8】

5 A gene encoding one of the recombinant light chain
variable regions of the EGF receptor specific antibodies of
claim 1 - claim 6.

【Claim 9】

10 An expression vector containing the gene of claim 7.

【Claim 10】

An expression vector containing the gene of claim 8.

【Claim 11】

15 A transformant generated by introducing the
expression vectors of claim 9 and claim 10 at the same time
into host cells.

【Claim 12】

20 A preparation method of an EGF receptor specific
antibody comprising the following steps:

- 1) culturing the transformant of claim 11; and
- 2) purifying the antibody of claim 1 from the culture
solution.

【Claim 13】

A pharmaceutical composition containing the antibody
of claim 1 as an active ingredient for the treatment of
5 malignant tumor over-expressing EGF receptor.

【Claim 14】

The pharmaceutical composition according to claim 13,
wherein the malignant tumor over-expressing EGF receptor is
10 selected from the group consisting of breast cancer,
bladder cancer, colon cancer, glioma, non-small-cell lung
cancer, pancreatic cancer, ovarian cancer, stomach cancer,
lung cancer, salivary cancer and head/neck cancer.

15 【Claim 15】

The pharmaceutical composition according to claim 13,
wherein the pharmaceutical composition is co-administered
with chemotherapeutic agents.

20 【Claim 16】

A treatment method for malignant tumor over-
expressing EGF receptor containing the step of
administering the effective dose of the pharmaceutical
composition of claim 13 to a subject.

【Claim 17】

The treatment method for malignant tumor over-expressing EGF receptor according to claim 16, wherein the
5 malignant tumor over-expressing EGF receptor is selected from the group consisting of breast cancer, bladder cancer, colon cancer, glioma, non-small-cell lung cancer, pancreatic cancer, ovarian cancer, stomach cancer, lung cancer, salivary cancer and head/neck cancer.

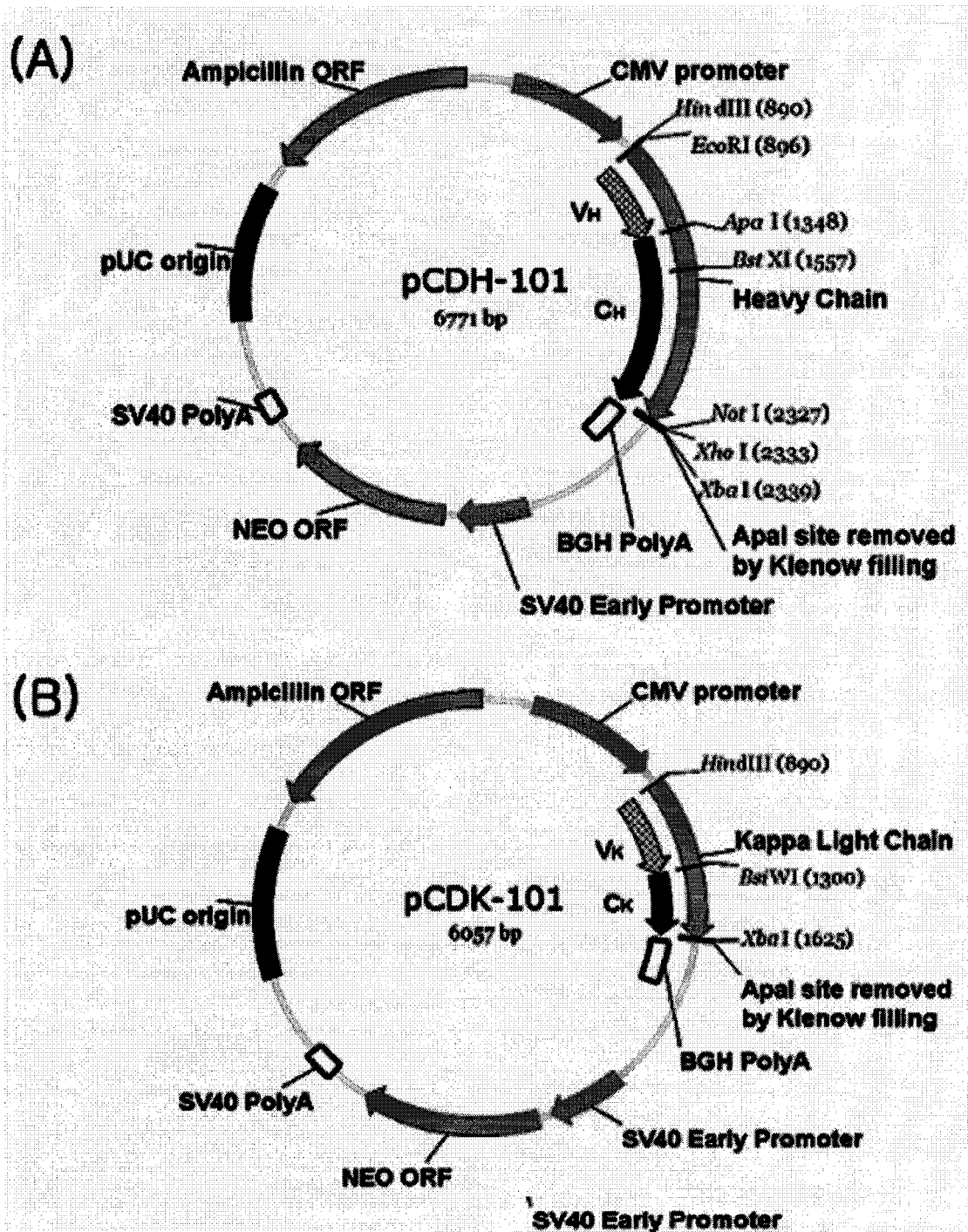
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Figures
Fig. 1

Heavy Chains		CDR1	CDR2	
		*****	*****	
CRH4	QSLEESGGRLVTPGTPLTLTCTVSGFSLSSYTMSWVRQPPGKGLQYIGIIDSSSTDYAS			60
CRH44	-----IG-----			60
CRH46	-----G-----			60
CRH47	-----G-----			60
CRH49	-----			60
CRH411	-----			60
CRH412	-----Q-----			60
		CDR2	CDR3	
		****	*****	
CRH4	WAKGRFTISKTSITVDLKLTSPTTEDTATYFCARVGDYYDSGYTWWYAFNLWGPGTLVT			120
CRH44	-----			120
CRH46	-----			120
CRH47	-----			120
CRH49	-----D--A-----			120
CRH411	-----D-----			120
CRH412	-----			120
Kappa Chains		CDR1	CDR2	
		*****	*****	
CRH4	ELVMTQTPSSVSAAVGGTVTINCQASESISNYLAWYQQKPGQPPKLLIYGASNLESGVPS			60
CRH44	-----			60
CRH46	-----D-----			60
CRH47	-----ND-----			60
CRH49	-----			60
CRH411	-----D-----			60
CRH412	-----D-----			60
		CDR3		

CRH4	RFRGSGSGTEFTLTISAMKAEDAATYYCQSVSDYSAGLTFGPGTKVEIK			108
CRH44	-----F-----			108
CRH46	-----E-----			108
CRH47	-----			108
CRH49	-----D-----			108
CRH411	-----			108
CRH412	-----F-----			108

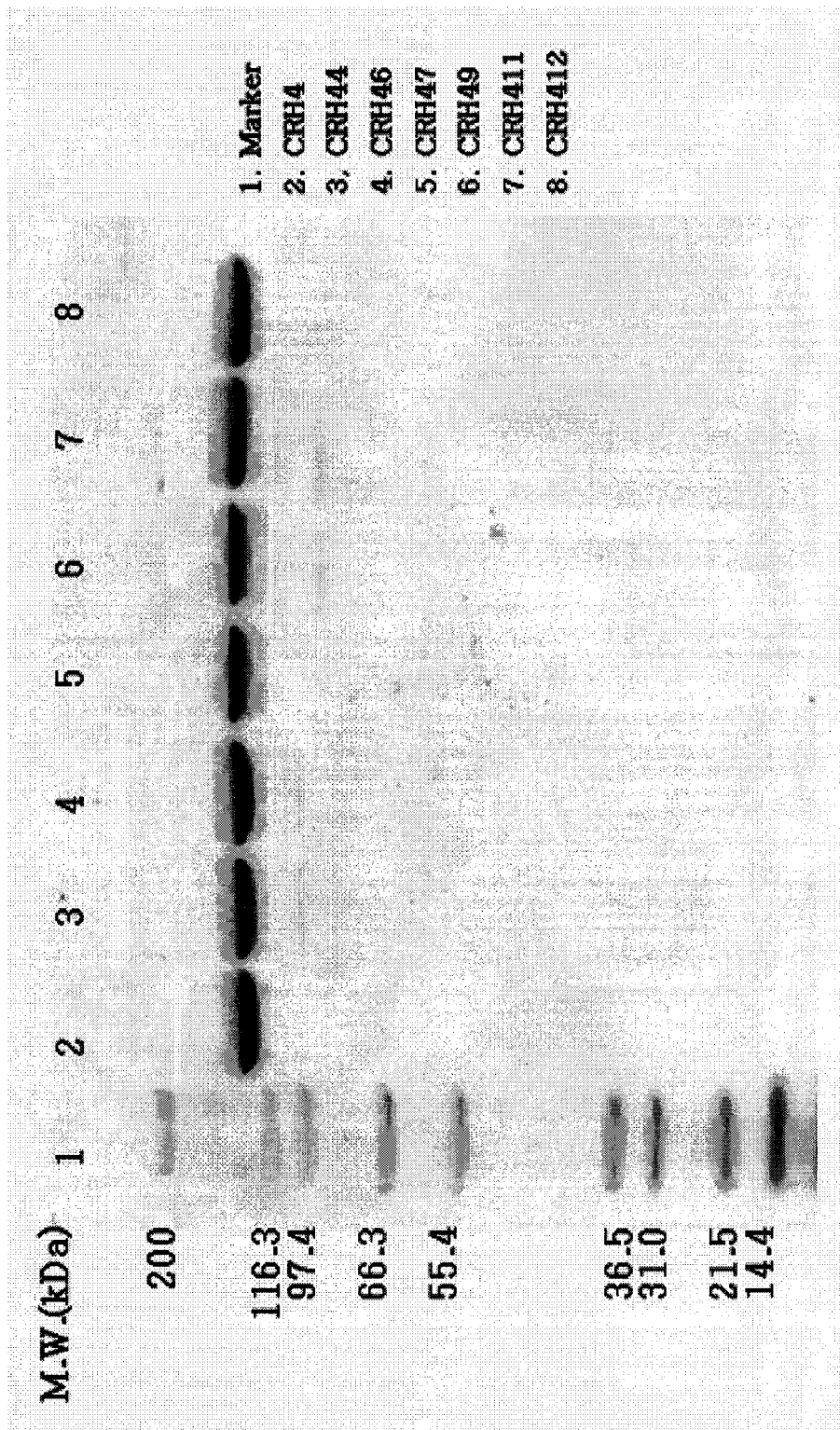
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Figures
Fig. 2



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Figures

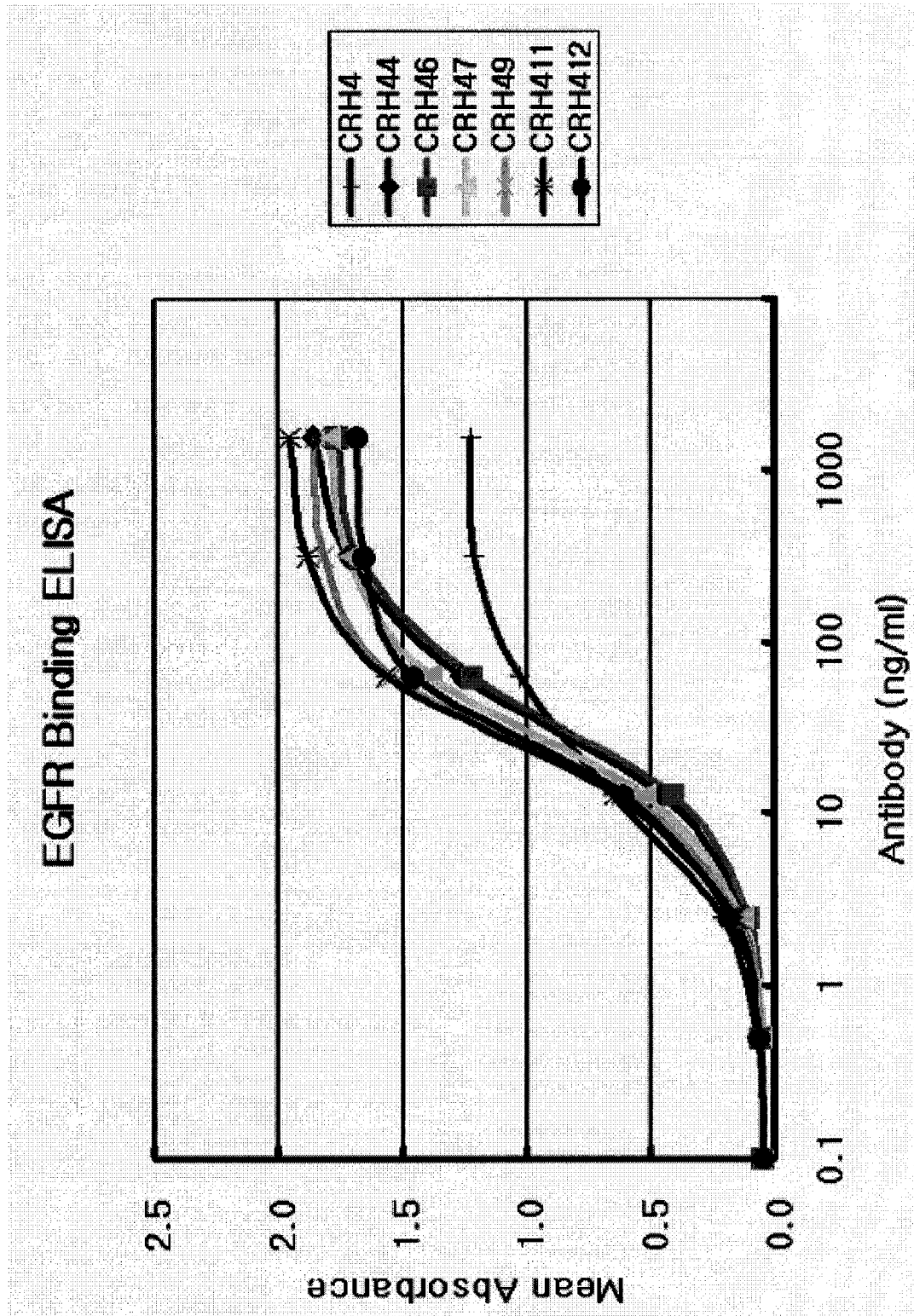
Fig. 3



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Figures

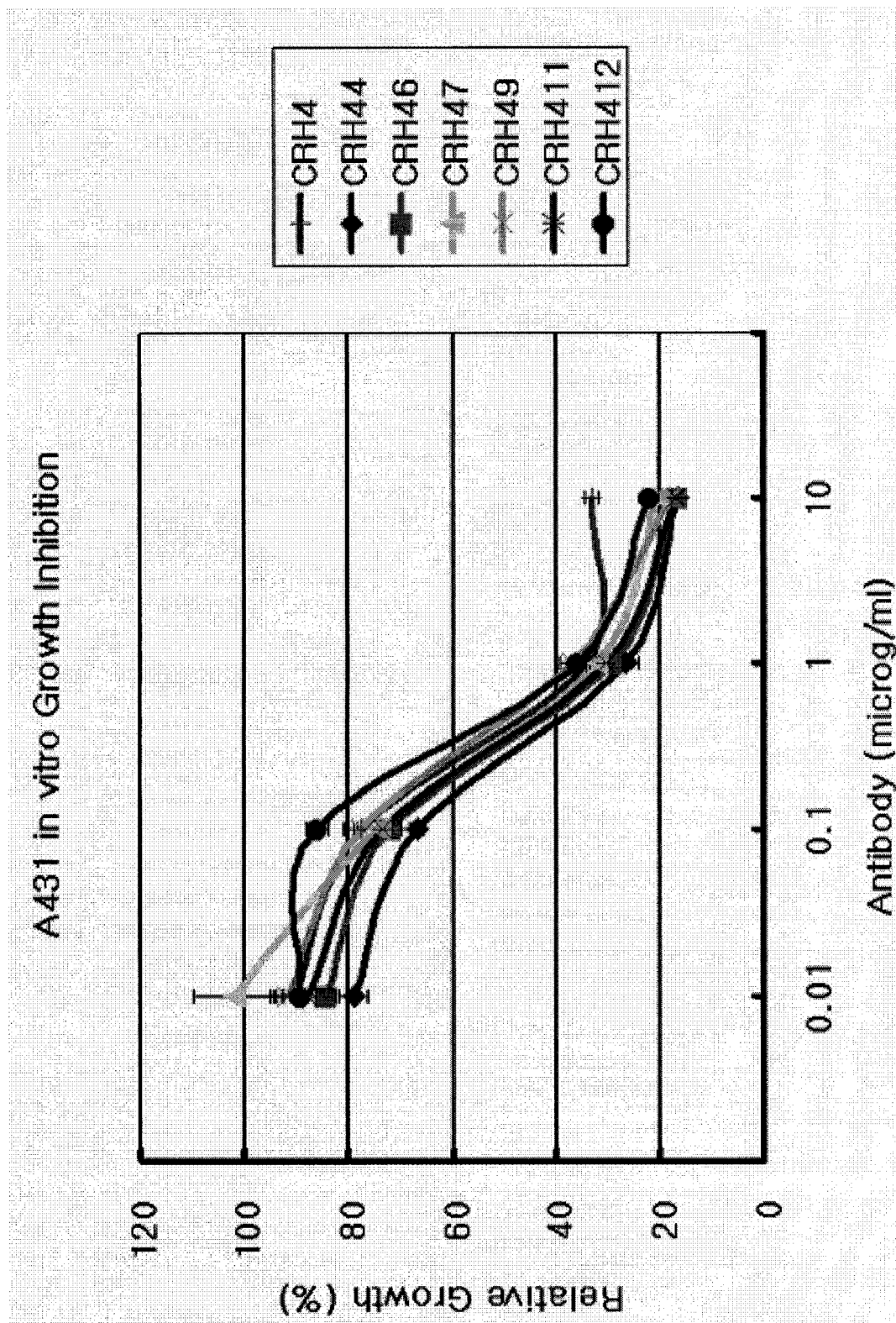
Fig. 4



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Figures

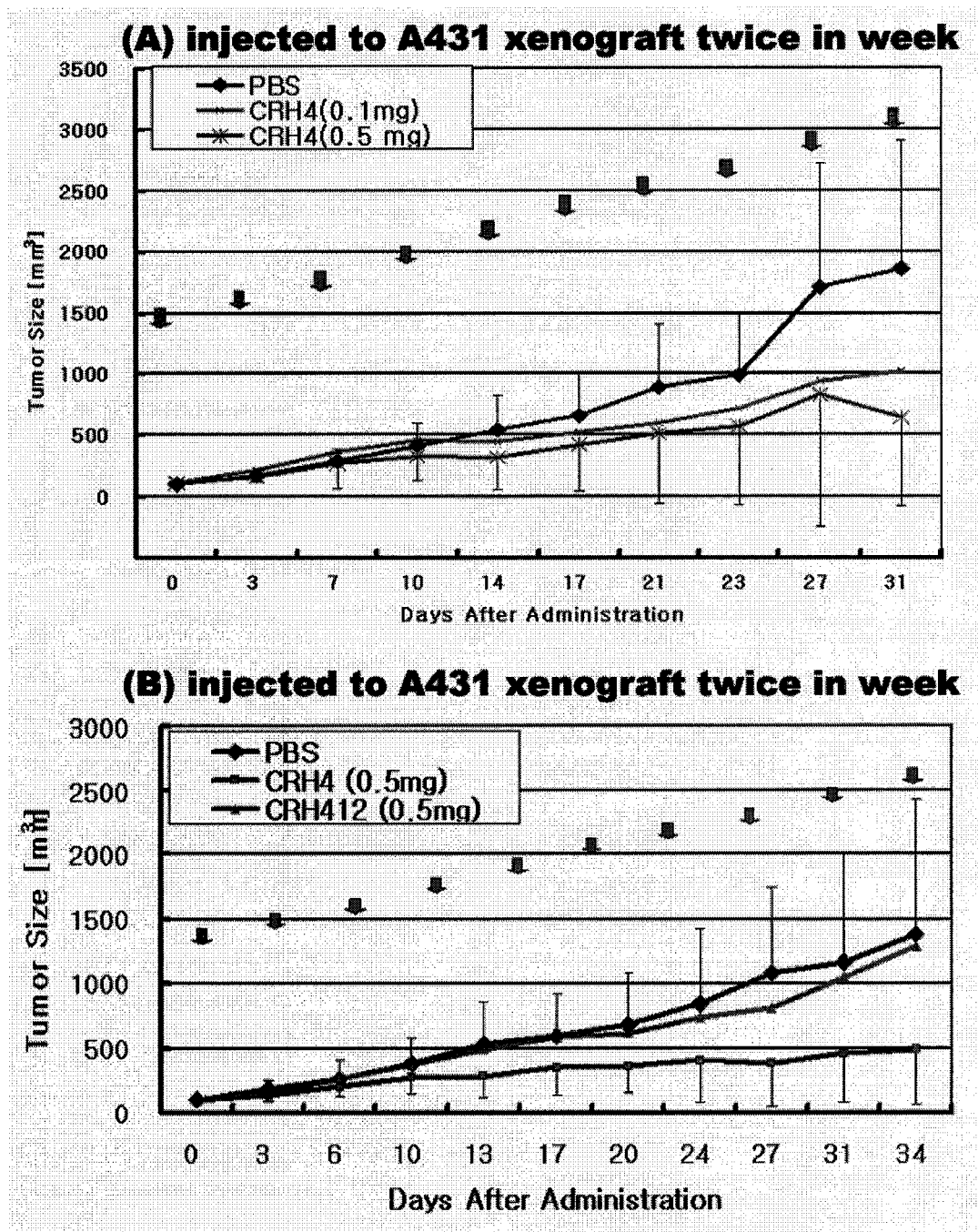
Fig. 5



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Figures

Fig. 6



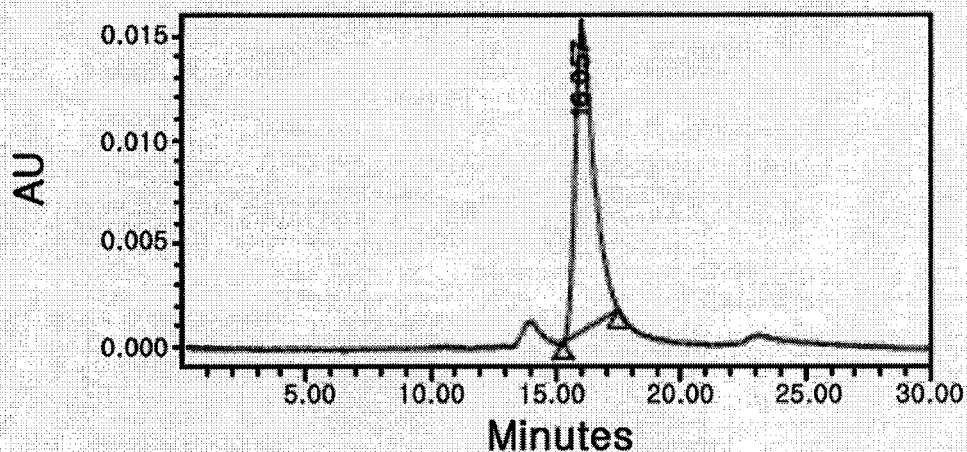
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Figures

Fig. 7

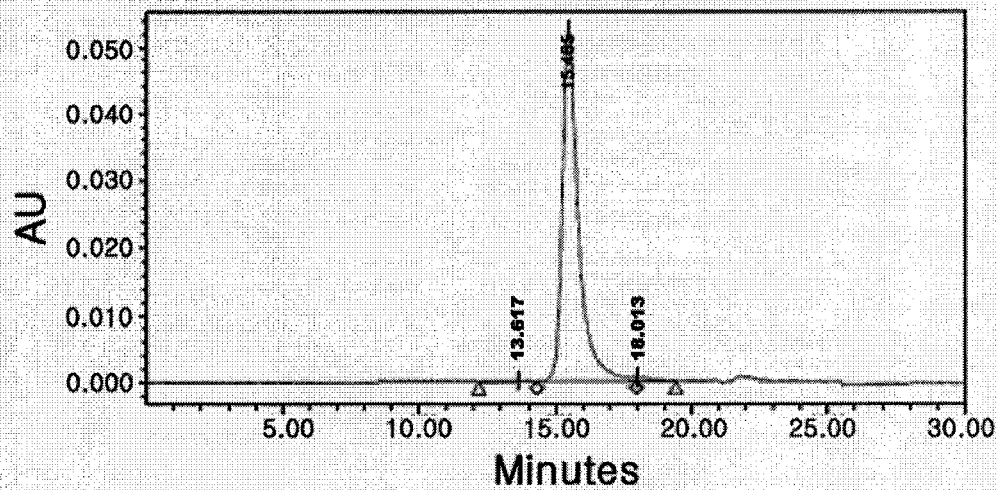
A. result of the investigation of CRH412 antibody by SEC-HPLC

Auto-Scaled Chromatogram



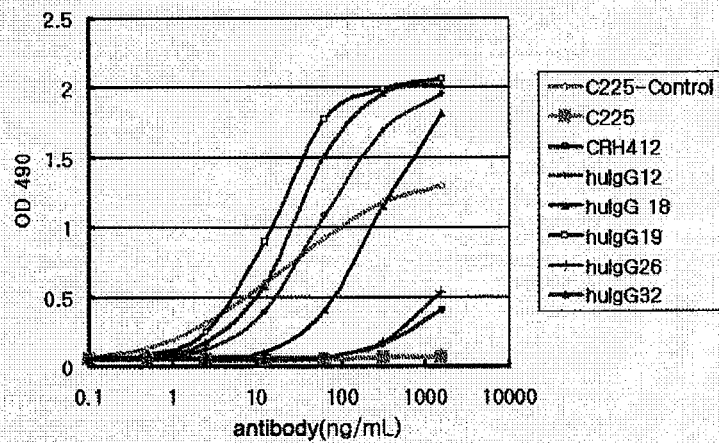
B. result of the investigation of CRH412 modified antibody by SEC-HPLC

Auto-Scaled Chromatogram

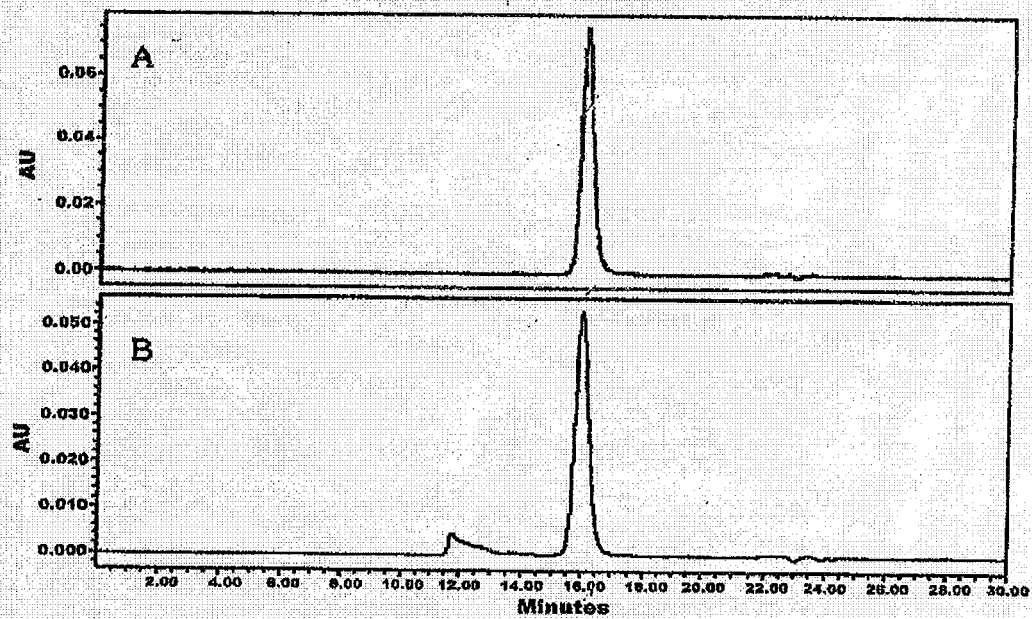


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Figures
Fig. 8

(1)



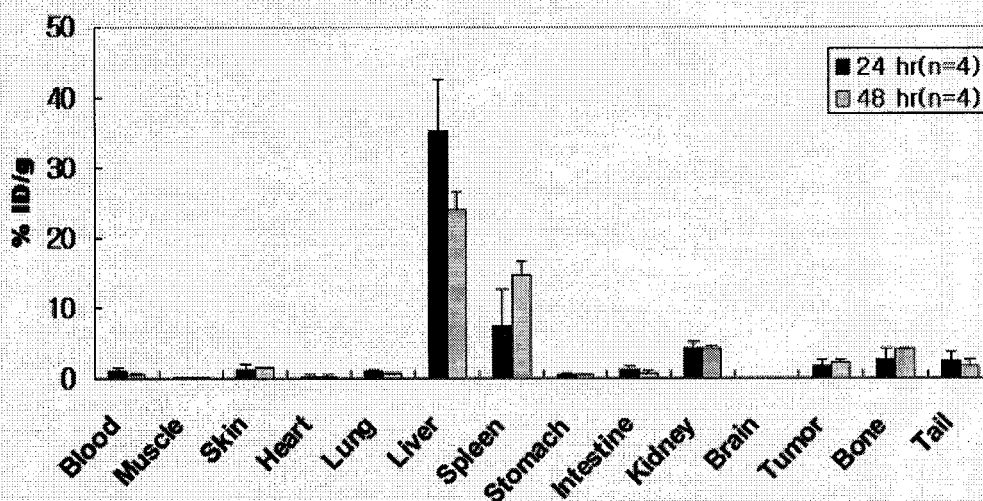
(2)



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Figures
Fig. 9

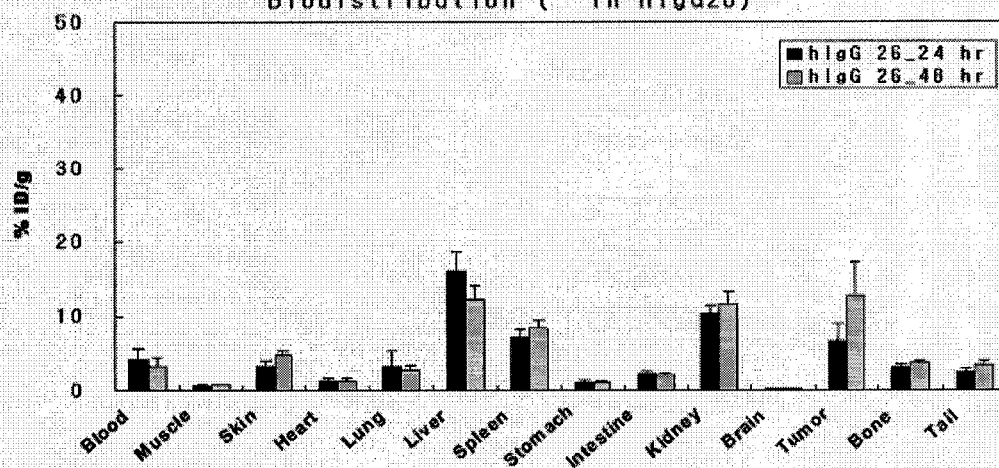
A. in vivo distribution of the CRH412 antibody

In-111-DOTA-CRH412

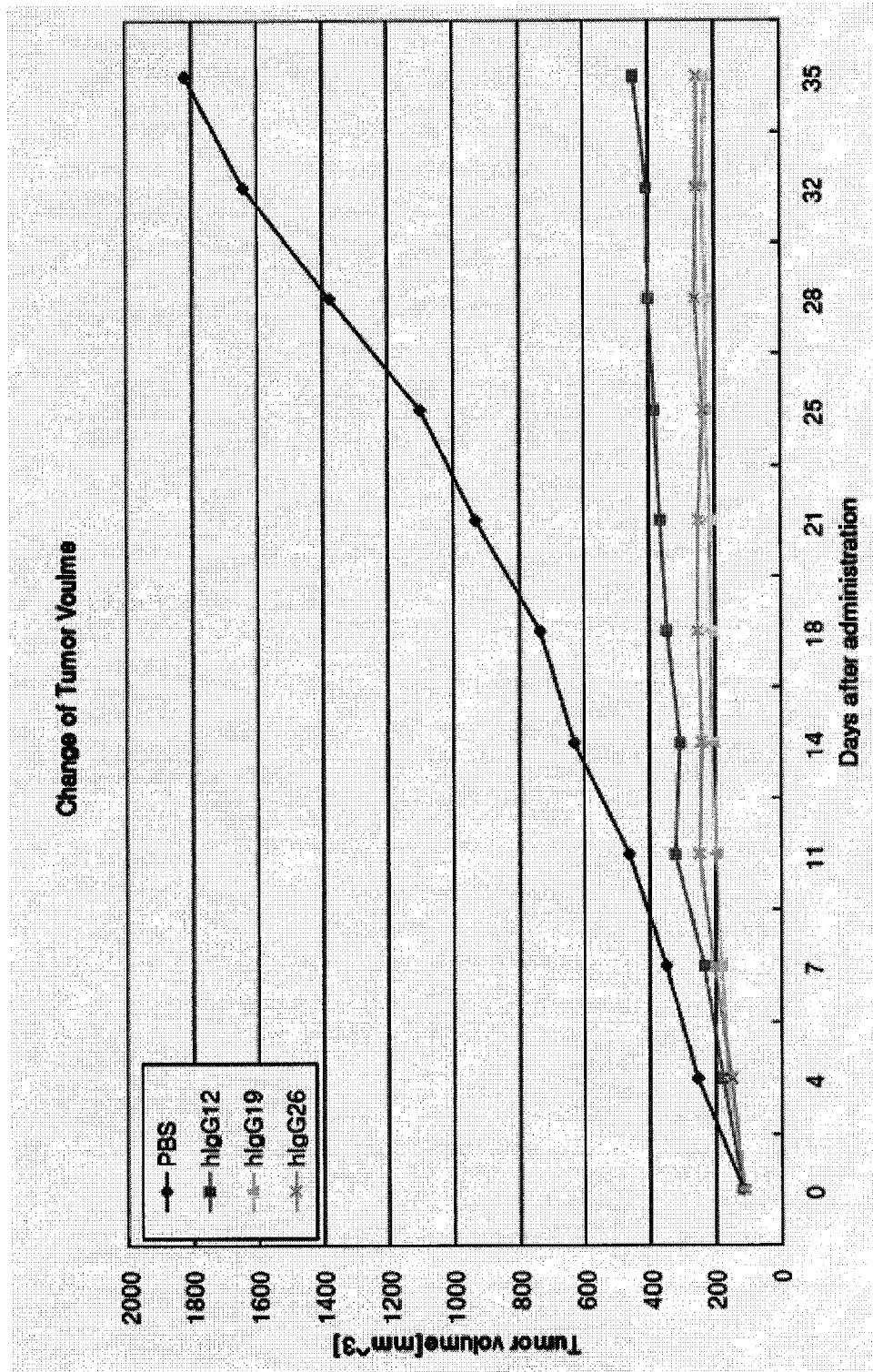


B. in vivo distribution of the CRH412 modified antibody

Biodistribution (¹¹¹In-hlgG26)



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Figures
Fig. 10



A. CLASSIFICATION OF SUBJECT MATTER**C07K 16/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8: C07K 16/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Pubmed, Esp@snet, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 01/88138 A1 (SCANCELL LIMITED) 22 November 2001 See Abstract; examples 1-7; claims	1-15
Y	POPKOV, M. et al. 'Rabbit Immune repertoires as sources for therapeutic monoclonal antibodies: The impact of kappa allotype-correlated variation in cysteine content on antibody libraries selected by phage display' Journal of Molecular Biology, Vol. 325, pp. 325-335, 10 January 2003 See Result and discussion	1-2, 5-15
Y	WORN, A. and PLUCKTHUN, A. 'Different Equilibrium stability behavior of ScFv fragment: Identification, classification, and improvement by protein engineering' Biochemistry, Vol. 38, pp. 8739-8750, 6 July 1999 See Abstract; p. 8746, lines 20-31	3-4, 7-15
Y	WILLUDA, J. et al. 'High thermal stability is essential for tumor targeting of antibody fragments: Engineering of a humanized anti-epithelial glycoprotein-2 (Epithelial cell adhesion molecule) single-chain Fv fragment' Cancer Research, Vol. 59, pp. 5758-5767, 15 November 1999 See p. 5758, lines 31-44; p. 5766, lines 5-11	3-4, 7-15
A	DEMAREST, S. J. et al. 'Optimization of the antibody C(H)3 domain by residue frequency analysis of IgG sequences' Journal of Molecular Biology, Vol. 335, pp. 41-48, 2 January 2004 See the whole document.	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 OCTOBER 2007 (12.10.2007)

Date of mailing of the international search report

12 OCTOBER 2007 (12.10.2007)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

KIN, Ji Yun

Telephone No. 82-42-481-8288



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2007/003294

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



on paper



in electronic form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in electronic form



furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2007/003294**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 16-17
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 16-17 pertain to methods for treatment of the human or animal body, thus relate to a subject-matter which International Searching Authority is not required to search under PCT Art. 17(2)(a)(i) and Rule 39.1(iv).
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2007/003294

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 01/ 88138 A1 EP 1278851 A1			22 - 11 - 2001 29 - 01 - 2003
01 - 2006		EP 1278851 B1	18 -
- 2000		GB 0011981 D0	05 - 07
2003		J P 2003533987 T	18 - 11 -
2004		US 2004091485 A1	13 - 05 -
2003		KR 20030021161 A	12 - 03 -