



(86) Date de dépôt PCT/PCT Filing Date: 2004/06/25
(87) Date publication PCT/PCT Publication Date: 2005/01/06
(85) Entrée phase nationale/National Entry: 2005/12/20
(86) N° demande PCT/PCT Application No.: JP 2004/009378
(87) N° publication PCT/PCT Publication No.: 2005/001117
(30) Priorités/Priorities: 2003/06/27 (2003-184436) JP;
2004/03/12 (2004-070497) JP

(51) Cl.Int./Int.Cl. *C12Q 1/02* (2006.01),
G01N 33/48 (2006.01), *G01N 15/14* (2006.01),
C12N 15/00 (2006.01), *A61P 35/00* (2006.01),
A61K 39/00 (2006.01)
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(54) Titre : PROCEDE DE SELECTION D'UN PATIENT S'ADAPTANT AU VACCIN ANTI-WT1
(54) Title: METHOD OF SELECTING WT1 VACCINE ADAPTIVE PATIENT

(57) **Abrégé/Abstract:**

A method of selecting a patient of high WT1 vaccine response on the basis of the frequency of WT1-specific CTL precursor cells as an indicator; a diagnostic agent for the selection; etc. In particular, there is provided, for example, a method of selecting a patient of high WT1 vaccine response, comprising the step (a) of isolating a biosample containing CTL precursor cells from a subject patient; the step (b) of measuring the existence frequency or amount of WT1-specific CTL precursor cells present in the biosample (a); and the step (c) of adjudicating on whether or not the results of the measurement (b) are high as compared with those of healthy people and judging the responsiveness to WT1 vaccine.



ABSTRACT

A method of selecting patients highly responsive to WT1 vaccine and a therapeutic method for treating cancer involving said method of selection are provided, which method of selecting patients highly responsive to WT1 vaccine comprises: (a) isolating a biological sample containing CTL precursor cells from a test subject; (b) measuring the existence frequency or amount of WT1-specific CTL precursor cells in the biological sample of (a); and (c) deciding whether or not the measured value of (b) is high by comparison with that of healthy subject, and evaluating the responsiveness to WT1 vaccine.

DESCRIPTION

METHOD OF SELECTING PATIENTS SUITABLE FOR WT1 VACCINE

5 TECHNICAL FIELD

The present invention relates to a method of selecting patients highly responsive to WT1 vaccine and a therapeutic method for treating cancer involving said method of selection. More particularly, the present invention relates to a method of selecting patients highly responsive to WT1 vaccine
10 on the basis of the frequency of WT1-specific CTL precursors as an indicator, and the like.

BACKGROUND ART

The WT1 gene (Wilms' tumor gene 1) has been identified as one of
15 causative genes of Wilms' tumor that is a childhood renal tumor (Cell 60: 509, 1990, Nature 343: 774, 1990). WT1 gene encodes the transcription factor WT1, and WT1 plays an important role in many processes such as proliferation, differentiation and apoptosis of cells, and development of tissues (Int. Rev. Cytol. 181: 151, 1998). The WT1 gene was originally
20 defined as a tumor suppressor gene. However, subsequent studies revealed that WT1 gene is highly expressed in leukemia and various solid cancers including lung cancer and breast cancer, and thus, indicating that WT1 gene rather exerts an oncogenic function promoting cancer growth. In addition, it was demonstrated that stimulation in vitro of peripheral blood
25 mononuclear cells, which cells are positive to HLA-A*0201 or HLA-A*2402, with WT1-derived peptides induces the peptide-specific cytotoxic T-lymphocytes (CTLs), and the CTLs kill leukemia or solid tumor cells endogenously expressing WT1. These results demonstrated that WT1 is a promising target molecule of cancer immunotherapy (Int. J. Hematol 76:
30 127, 2002).

There have been known methods for determining in vitro antigen peptide-specific CTLs, including HLA monomer method, HLA dimer method and HLA tetramer method (Science 274: 94, 1996), HLA pentamer method and ELISPOT method (J. Immunol. Methods 110: 29, 1988), realtime RT-PCR technique (J. Immunol. Methods 210: 195, 1997), limiting dilution method (Br. J. Cancer 77: 1907, 1998), and the like. HLA-tetramer is prepared by biotinylating a complex (HLA monomer) formed by association of HLA α -chain and β 2-microglobulin with a peptide, and allowing the monomer to bind to fluorescence-labeled avidin for tetramerization. The frequencies of CTLs can be measured by staining peptide-specific CTLs with HLA tetramers and analyzing by flow cytometry. Measurement of CTL frequencies by HLA monomer method, HLA dimer method and HLA pentamer method can be carried out on the basis of the same principle.

CTLs not stimulated with vaccine are referred to as "CTL precursor cells". It is considered that the higher the frequency of existence of CTL precursor cells specific for a given cancer antigen, the more efficiently induced specific CTLs can be, when the said antigen is administered as a cancer vaccine, which makes it easier to attain clinical response of cancer vaccine therapy. In other words, if a patient showing high frequency of existence regarding CTL precursor cells specific for a given cancer antigen is selected prior to vaccination, it would be possible to treat more effectively by the use of said cancer antigen.

Frequency of CTL precursor cells were measured using HLA tetramer and peripheral blood mononuclear cells (PBMC) of melanoma patients and reported in several papers; however, they show that the frequency of CTL precursor cells specific for tumor antigen peptide is low (J. Immunother. 24: 66, 2001, Hum. Gene. Ther. 13: 569, 2002). From these results, it has been regarded that the frequency of existence of CTL precursor cells specific for antigen is generally low and that it is difficult to select patients suitable for a cancer vaccine on the basis of the frequency of CTL precursor

cells as an indicator.

DISCLOSURE OF INVENTION

5 The purpose of the present invention is to provide a method of selecting a patient highly responsive to WT1 vaccine on the basis of the frequency of WT1-specific CTL precursor cells as an indicator, and the like.

10 The present inventor prepared an HLA tetramer using a tumor antigen peptide derived from WT1, and used the resultant tetramer in the measurement of frequency of CTL precursor cells in patients of hematopoietic malignancy or lung cancer before administration of vaccine. It was surprisingly found that CTL precursor cells (WT1-specific CTL precursor cells) exist in high frequency than that hitherto known compared to healthy individuals. This result revealed that it is possible to select patients highly responsive to WT1 vaccine or to identify a target molecule of
15 WT1 vaccine on the basis of the frequency of WT1-specific CTL precursor cells as an indicator, as far as tumor antigen WT1 concerns. Also, since patients having various cancers showed high frequency of WT1-specific CTL precursor cells, it became clear that diagnosis of cancer can be done on the basis of the frequency of WT1-specific CTL precursor cells as an
20 indicator.

The present inventor then sorted WT1-specific CTL precursor cells finely regarding function, and found that, in particular, effector-type CTL precursor cell (hereinafter, it may be simply referred to as "effector cell") exists in higher proportion among CTL precursor cells. This result
25 indicated that selection of patients highly responsive to WT1 vaccine or diagnosis of cancer can also be carried out on the basis of the frequency of WT1-specific CTL precursor cells of effector type as an indicator.

In addition, the present inventor measured the frequency of WT1-specific CTLs in patients undergoing treatment with a tumor antigen
30 peptide ("WT1 peptide") derived from WT1 and found that the therapeutic

effect is correlated with the increase of CTL frequency after administration relative to that obtained before administration.

The present invention has been established on the basis of the findings above.

5 Thus, the present invention provides the followings:

(1) A method of selecting a patient highly responsive to WT1 vaccine, comprising the following steps (a), (b) and (c):

 (a) isolating a biological sample containing CTL precursor cells from a test subject;

10 (b) measuring the existence frequency or amount of WT1-specific CTL precursor cells in the biological sample of (a); and

 (c) deciding whether or not the measured value of (b) is high by comparison with that of healthy subject, and evaluating the responsiveness to WT1 vaccine.

15 (2) The method of selection as described in (1) above, wherein the measurement of the existence frequency or amount of WT1-specific CTL precursor cells is carried out by any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique and limiting dilution method.

20 (3) The method of selection as described in (2) above, wherein the measurement is carried out by HLA tetramer method.

 (4) The method of selection as described in (3) above, which comprises the following steps (a), (b), (c) and (d):

25 (a) isolating a biological sample containing CTL precursor cells from a test subject;

 (b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide contact with the biological sample of (a);

 (c) measuring the existence frequency or amount of WT1-specific CTL precursor cells bound to the HLA tetramer; and

30 (d) deciding whether or not the measured value of (c) is high by

comparison with that of healthy subject, and evaluating the responsiveness to WT1 vaccine.

(5) The method of selection as described in (4) above, wherein the step (c) in (4) is carried out by measuring the proportion of HLA tetramer-bound cells among CD8-positive or CD8/CD3-positive CTL precursor cells.

(6) The method of selection as described in (4) or (5) above, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

(7) The method of selection as described in any one of (4) to (6) above, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

(8) The method of selection as described in any one of (1) to (7) above, which is carried out using flow cytometry.

(9) The method of selection as described in (1) to (8) above, wherein the responsiveness to WT1 vaccine is evaluated using as an indicator that the existence frequency or amount of WT1-specific CTL precursor cells is 1.5 times or higher compared to that of healthy subject.

(10) The method of selection as described in (1) above, wherein the CTL precursor cells are CTL precursor cells of effector type.

(11) The method of selection as described in (10) above, which uses any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique and limiting dilution method in the measurement of the existence frequency or amount of WT1-specific CTL precursor cells of effector type.

(12) The method of selection as described in (11) above, which uses the HLA tetramer method.

(13) The method of selection as described in (12) above, which comprises the following steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTL precursor cells from a test subject;

5 (b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide, an anti-CD8 antibody, an anti-CD45RA antibody and an anti-CD27 antibody contact with the biological sample of (a);

(c) measuring the proportion of CD45RA-positive and CD27-negative CTL precursor cells of effector type among CTL precursor cells
10 which are positive for CD8 or CD8/CD3 and positive for binding to HLA tetramer; and

(d) deciding whether or not the measured result of (c) is high by comparison with that of healthy subject, and evaluating the responsiveness to WT1 vaccine.

15 (14) The method of selection as described in (13) above, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

(15) The method of selection as described in (13) or (14) above, wherein the WT1-derived tumor antigen peptide is selected from the following
20 peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

25 (16) The method of selection as described in any one of (10) to (15) above, which is carried out using flow cytometry.

(17) A method of diagnosing cancer, comprising the following steps (a), (b) and (c):

(a) isolating a biological sample containing CTL precursor cells
30 from a test subject;

(b) measuring the existence frequency or amount of WT1-specific CTL precursor cells in the biological sample of (a); and

(c) deciding whether or not the measured result of (b) is high by comparison with that of healthy subject, and evaluating whether the test
5 subject has cancer.

(18) The method of diagnosis as described in (17) above, wherein the measurement of the existence frequency or amount of WT1-specific CTL precursor cells is carried out by any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT
10 method, realtime RT-PCR technique and limiting dilution method.

(19) The method of diagnosis as described in (18) above, wherein the measurement is carried out by HLA tetramer method.

(20) The method of diagnosis as described in (19) above, which comprises the following steps (a), (b), (c) and (d):

15 (a) isolating a biological sample containing CTL precursor cells from a test subject;

(b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide contact with the biological sample of (a);

(c) measuring the existence frequency or amount of WT1-specific
20 CTL precursor cells bound to the HLA tetramer; and

(d) deciding whether or not the measured result of (c) is high by comparison with that of healthy subject, and evaluating whether the test subject has cancer.

(21) The method of diagnosis as described in (20) above, wherein the step
25 (c) in (20) is carried out by measuring the proportion of HLA tetramer-bound cells among CD8-positive or CD8/CD3-positive CTL precursor cells.

(22) The method of diagnosis as described in (20) or (21) above, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

30 (23) The method of diagnosis as described in any one of (20) to (22) above,

wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

5 Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

(24) The method of diagnosis as described in any one of (17) to (23) above, which is carried out using flow cytometry.

10 (25) The method of diagnosis as described in any one of (17) to (24) above, wherein cancer is diagnosed using as an indicator that the existence frequency or amount of WT1-specific CTL precursor cells is 1.5 times or higher compared to that of healthy subject.

(26) The method of diagnosis as described in (17) above, wherein the CTL precursor cells are CTL precursor cells of effector type.

15 (27) The method of diagnosis as described in (26) above, which uses any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique and limiting dilution method in the measurement of the existence frequency or amount of WT1-specific CTL precursor cells of effector type.

20 (28) The method of diagnosis as described in (27) above, which uses the HLA tetramer method.

(29) The method of diagnosis as described in (28) above, which comprises the following steps (a), (b), (c) and (d):

25 (a) isolating a biological sample containing CTL precursor cells from a test subject;

(b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide, an anti-CD8 antibody, an anti-CD45RA antibody and an anti-CD27 antibody contact with the biological sample of (a);

30 (c) measuring the proportion of CD45RA-positive and CD27-negative CTL precursor cells of effector type among CTL precursor cells

which are positive for CD8 or CD8/CD3 and positive for binding to HLA tetramer; and

(d) deciding whether or not the measured value of (c) is high by comparison with that of healthy subject, and evaluating whether the test subject has cancer.

(30) The method of diagnosis as described in (29) above, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

(31) The method of diagnosis as described in (29) or (30) above, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

(32) The method of diagnosis as described in any one of (26) to (31) above, which is carried out using flow cytometry.

(33) A method of identifying a target molecule of WT1 vaccine said molecule being peculiar to a patient, comprising the following steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTL precursor cells from a test patient;

(b) applying each of plural target molecules of WT1 vaccine to the biological sample of (a);

(c) measuring the existence frequency or amount of WT1-specific CTL precursor cells in the respective biological samples of (b) and comparing the results with each other; and

(d) identifying a target molecule of WT1 vaccine effective to the test patient on the basis of the results obtained in (c).

(34) The method of identification as described in (33) above, wherein the

measurement of the existence frequency or amount of WT1-specific CTL precursor cells is carried out by any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique and limiting dilution method.

5 (35) The method of identification as described in (34) above, wherein the measurement is carried out by HLA tetramer method.

(36) The method of identification as described in (35) above, which comprises the following steps (a), (b), (c) and (d):

10 (a) isolating a biological sample containing CTL precursor cells from a test patient;

(b) bringing each of plural HLA tetramers comprising different WT1-derived tumor antigen peptides contact with the biological sample of (a);

15 (c) measuring the existence frequency or amount of WT1-specific CTL precursor cells bound to the respective HLA tetramers, and comparing the results with each other; and

(d) identifying a WT1-derived tumor antigen peptide effective to the test patient on the basis of the results obtained in (c).

20 (37) The method of identification as described in (36) above, wherein the step (c) in (36) is carried out by measuring the proportion of HLA tetramer-bound cells among CD8-positive or CD8/CD3-positive CTL precursor cells.

(38) The method of identification as described in (36) or (37) above, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

25 (39) The method of identification as described in any one of (36) to (38) above, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

30 Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

(40) The method of identification as described in any one of (33) to (39) above, which is carried out using flow cytometry.

(41) A clinical diagnostic agent for selecting a patient highly responsive to WT1 vaccine, which comprises as an ingredient an HLA monomer, an HLA dimer, an HLA tetramer or an HLA pentamer each containing a WT1-derived tumor antigen peptide.

(42) The clinical diagnostic agent as described in (41) above, wherein the HLA antigen as a component of an HLA monomer, an HLA dimer, an HLA tetramer or an HLA pentamer is an HLA-A24 antigen or an HLA-A2 antigen.

(43) The clinical diagnostic agent as described in (41) or (42) above, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

(44) A kit comprising a clinical diagnostic agent as described in any one of (41) to (43) above.

(45) A pharmaceutical composition for treating cancer in a given patient, which comprises a target molecule identified by the method of identification of a target molecule of WT1 vaccine said molecule being peculiar to the patient as described in any one of (33) to (40) above.

(46) A diagnostic agent for cancer, which comprises as an ingredient an HLA monomer, an HLA dimer, an HLA tetramer or an HLA pentamer each containing a WT1-derived tumor antigen peptide.

(47) The diagnostic agent as described in (46) above, wherein the HLA antigen as a component of an HLA monomer, an HLA dimer, an HLA tetramer or an HLA pentamer is an HLA-A24 antigen or an HLA-A2 antigen.

(48) The diagnostic agent as described in (46) or (47) above, wherein the

WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

5 Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

(49) A kit which comprises a diagnostic agent as described in any one of (46) to (48) above.

(50) A method of determining the suitability of a patient for WT1 vaccine, comprising the following steps (a), (b) and (c):

10 (a) isolating a biological sample containing CTLs from a patient after WT1 vaccine administration;

(b) measuring the existence frequency or amount of WT1-specific CTLs in the biological sample of (a);

15 (c) deciding whether or not the measured value of (b) is high by comparison with that of biological sample obtained before WT1 vaccine administration, and evaluating whether the patient is suitable for WT1 vaccine therapy.

(51) The method of determination as described in (50) above, wherein the measurement of the existence frequency or amount of WT1-specific CTLs is carried out by any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique and limiting dilution method.

20 (52) The method of determination as described in (51) above, wherein the measurement is carried out by HLA tetramer method.

25 (53) The method of determination as described in (52) above, which comprises the following steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTLs from a patient after WT1 vaccine administration;

30 (b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide contact with the biological sample of (a);

(c) measuring the existence frequency or amount of WT1-specific CTLs bound to the HLA tetramer; and

(d) deciding whether or not the measured value of (c) is high by comparison with that of biological sample obtained before WT1 vaccine administration, and evaluating whether the patient is suitable for WT1 vaccine therapy.

(54) The method of determination as described in (53) above, wherein the step (c) in (53) is carried out by measuring the proportion of HLA tetramer-bound cells among CD8-positive or CD8/CD3-positive CTLs.

10 (55) The method of determination as described in (53) or (54) above, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

(56) The method of determination as described in any one of (53) to (55) above, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

20 (57) The method of determination as described in any one of (50) to (56) above, which is carried out using flow cytometry.

(58) The method of determination as described in any one of (50) to (57) above, wherein the suitability for WT1 vaccine therapy is evaluated using as an indicator that the existence frequency or amount of WT1-specific CTLs is 1.5 times or higher compared to that in the sample obtained before administration.

25 (59) A clinical diagnostic agent for determining the suitability for WT1 vaccine which comprises as an ingredient an HLA monomer, an HLA dimer, an HLA tetramer or an HLA pentamer each containing a WT1-derived tumor antigen peptide.

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(60) The clinical diagnostic agent as described in (59) above, wherein the HLA antigen as a component of an HLA monomer, a HLA dimer, an HLA tetramer or an HLA pentamer is an HLA-A24 antigen or an HLA-A2 antigen.

(61) The clinical diagnostic agent as described in (59) or (60) above,
5 wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

10 Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

(62) A kit comprising a clinical diagnostic agent as described in any one of (59) to (61) above.

The present invention is further related to the followings:

(63) In relation to the above-mentioned embodiment (1), a method of
15 treating cancer in a patient, which comprises:

selecting a patient highly responsive to WT1 vaccine by the following steps (a), (b) and (c):

(a) isolating a biological sample containing CTL precursor cells from a test subject;

20 (b) measuring the existence frequency or amount of WT1-specific CTL precursor cells in the biological sample of (a); and

(c) deciding whether or not the measured value of (b) is high by comparison with that of healthy subject, and evaluating the responsiveness to WT1 vaccine; and

25 treating the selected patient with WT1 or a WT1-derived tumor antigen peptide; and

a method of treating cancer in a patient selected by a method as described in any one of (2) to (16) above.

(64) In relation to the above-mentioned embodiment (33), a method of
30 treating cancer in a given patient, which comprises administering a target

molecule of WT1 vaccine said molecule being peculiar to the patient and having been identified by a method comprising the following steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTL precursor cells
5 from a test patient;

(b) applying each of plural target molecules of WT1 vaccine to the biological sample of (a);

(c) measuring the existence frequency or amount of WT1-specific CTL precursor cells in the respective biological samples of (b) and
10 comparing the results with each other; and

(d) identifying a target molecule of WT1 vaccine effective to the test patient on the basis of the results obtained in (c); and

a method of treating cancer in a given patient, which comprises administering a target molecule of WT1 vaccine said molecule being
15 peculiar to the patient and having been identified by a method as described in any one of (34) to (40) above.

(65) In relation to the above-mentioned embodiment (50), a method of treatment of cancer, which comprises treating a patient with WT1 or a WT1-derived tumor antigen peptide said patient having been evaluated to
20 be suitable by a method of determination of suitability of a patient for WT1 vaccine, comprising the following steps (a), (b) and (c):

(a) isolating a biological sample containing CTLs from a patient after WT1 vaccine administration;

(b) measuring the existence frequency or amount of WT1-specific
25 CTLs in the biological sample of (b);

(c) deciding whether or not the measured value of (b) is high by comparison with that of biological sample obtained before WT1 vaccine administration, and evaluating whether the patient is suitable for WT1 vaccine therapy; and

30 a method of treating cancer in a patient evaluated to be suitable by the

method as described in any one of (51) to (58) above.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a graph showing the existence frequency of WT1-specific CTL precursors in patients having cancer and healthy individuals. The vertical axis indicates the frequency of CTL precursors (precursor cells). The "blood cancer" shows the results obtained from HLA-A2402-positive patients having hematopoietic malignant tumor. The term "lung cancer" shows the results obtained from HLA-A2402-positive patients having lung cancer, and the term "healthy" the results obtained from HLA-A2402-positive healthy individuals.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention provides a method of selecting a patient highly responsive to WT1 vaccine, comprising the following steps (a), (b) and (c):

- (a) isolating a biological sample containing CTL precursor cells from a test subject;
- (b) measuring the existence frequency or amount of WT1-specific CTL precursor cells in the biological sample of (a); and
- (c) deciding whether or not the measured value of (b) is high by comparison with that of healthy subject, and evaluating the responsiveness to WT1 vaccine, and a method of treating cancer in the patient selected by said method.

The present inventor has found that there exist CTL precursor cells (WT1-specific CTL precursor cells) in a patient before vaccine administration in high frequency than that hitherto known. Accordingly, patients highly responsive to WT1 vaccine can be selected on the basis of the amount or frequency of WT1-specific CTL precursor cells as an indicator.

The test subject in step (a) refers to a person who is suspected or

diagnosed to have cancer, specifically, a person who is suspected or diagnosed to have cancer including blood cancers such as leukemia, myelodysplastic syndrome, multiple myeloma and malignant lymphoma, and solid cancers such as gastric cancer, colon cancer, lung cancer, breast
5 cancer, embryonal cancer, hepatic cancer, skin cancer, bladder cancer, prostate cancer, uterine cancer, cervical cancer, and ovarian cancer. Preferred example is a person who is suspected or diagnosed to have leukemia, myelodysplastic syndrome and lung cancer.

There are no limitations regarding the biological sample isolated
10 from a test subject in step (a) as far as it contains CTL precursor cells. Specific example includes blood, lymph fluid or cultures thereof, peripheral blood mononuclear cells (PBMCs) isolated from blood, or tissues to which T cells infiltrated, and the like. The biological sample can be used as it is, or after dilution or concentration. Preferred example is PBMC, which can be
15 isolated by a conventional manner such as density gradient centrifugation method with Ficoll-Hypaque.

The method of measuring the existence frequency or amount of WT1-specific CTL precursor cells in step (b) can be carried out by any methods known in the art, by which the existence frequency or amount of
20 CTL can be measured. Specific examples include HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique, limiting dilution method, and the like.

As herein used, the HLA tetramer method is a method wherein an
25 antigen peptide-specific CTL is detected using an HLA tetramer prepared by biotinylating an HLA monomer (said monomer has been formed by association of an HLA antigen α -chain and a β 2-microglobulin with an objective antigen peptide), and allowing to bind to fluorescently labeled avidin for tetramerization. Specifically, the amount of CTL can be
30 determined by staining an antigen peptide-specific CTL by said HLA

tetramer and analyzing by flow cytometry. Preparation of an HLA tetramer and detection of CTLs using the same are known and can be carried out according to a method, for example, described in a literature (Science 274: 94, 1996).

5 The HLA monomer method is a method wherein an antigen peptide-specific CTL is detected using an HLA monomer used in the preparation of HLA tetramer, which monomer is formed by association of an HLA antigen α -chain and a β 2-microglobulin with an antigen peptide followed by biotinylation.

10 The HLA dimer method is a method wherein an antigen peptide-specific CTL is detected using an HLA dimer which is prepared by fusing an HLA antigen α -chain and an Ig (immunoglobulin, for example, IgG1), and allowing the resultant fusion to bind to β 2-microglobulin and an antigen peptide (Proc. Natl. Acad. Sci. USA 90:6671-6675 (1993)). The
15 antigen peptide-specific CTLs bound to HLA dimer can be detected by, for example, allowing labeled anti-IgG1 antibody to bind to IgG1.

 The HLA pentamer method is a method that has been developed recently, wherein an antigen peptide-specific CTL is detected using a pentamer wherein five complex molecules of an HLA antigen and an
20 antigen peptide are polymerized through Coiled-Coil domain. Since the HLA antigen-antigen peptide complex can be labeled with fluorescence or the like, the analysis can be carried out by flow cytometry or the like as is the case with HLA tetramer method (see, <http://www.proimmune.co.uk/>).

 The aforementioned HLA-monomer, dimer, tetramer and pentamer
25 are all available by custom production from a manufacture such as ProImmune or BD Biosciences.

 The ELISPOT method is a method wherein CTL in a biological sample is detected by immobilizing an antibody raised against a cytokine such as IFN- γ or GM-CSF on a plate, adding a biological sample stimulated
30 by an intended antigen or antigen peptide, and detecting the cytokine

secreted from activated CTL in the biological sample and bound to the immobilized antibody above as a spot using anti-cytokine antibody. The method of determining CTL by the ELISPOT method is known and can be carried out according to a method described in a literature (e.g., J. Immunol. Methods 110: 29, 1988).

The realtime RT-PCR method is a method wherein the frequency of CTL reactive to an objective antigen or antigen peptide is measured indirectly through the measurement of a gene encoding cytokine such as IFN- γ , GM-CSF or the like produced by activated CTL by means of RT-PCR. The method of determining CTL by the realtime RT-PCR method is known and can be carried out according to a method described in a literature (e.g., J. Immunol. Methods 210: 195, 1997).

The limiting dilution method is a method wherein the frequency of CTL is measured by plating a biological sample containing CTLs in wells at different cell densities, culturing the plate while stimulating with an objective antigen or antigen peptide, measuring the amount of cytokines or cytotoxicity produced by the activated CTLs, and determining the CTL frequency on the basis of the number of positive wells. The method of determining CTL by the limiting dilution method is known and can be carried out according to a method described in a literature (e.g., Br. J. Cancer 77: 1907, 1998).

It is possible to measure the existence frequency or amount of WT1-specific CTL precursors according to the known method for determination of CTL as mentioned above.

The evaluation of responsiveness to WT1 vaccine in step (c) can be carried out by comparing the existence frequency or amount of WT1-specific CTL precursor cells in a test subject obtained in step (b) (hereinafter, referred to as "test subject value"), with that of healthy subject (hereinafter, referred to as "healthy subject value") and deciding the difference between them. In this case, a biological material isolated and

prepared from a healthy subject (blood, lymph fluid, PBMC, etc.) is needed, which can be obtained by collecting a biological sample from a subject not having cancer. As used herein, "healthy subject" means a person who has not been diagnosed to have cancer.

5 The comparison between the test subject values and the healthy subject values can be carried out by measuring the biological sample of a test subject and that of a healthy subject in parallel. When the parallel comparison is not conducted, comparison can also be carried out using a mean value or a statistical intermediate value of healthy subject values
10 calculated from the healthy subject values obtained by measuring plural (at least two, preferably three or more, more preferably five or more) biological samples of a healthy subject under a constant conditions when the parallel measurement is not conducted.

 The evaluation whether or not a test subject is highly responsive to
15 WT1 vaccine can be carried out using as an indicator that the test subject value is 1.5 times or higher, preferably 2 times or higher compared to the healthy subject value. That is, when the test subject value is 1.5 times or higher, preferably, 2 times or higher than the healthy subject value, the responsiveness to WT1 vaccine is evaluated to be high. A patient evaluated
20 to be highly responsive to WT1 vaccine is evaluated to be suitable for WT1 vaccine, in other words, it is possible to apply WT1 vaccine therapy to the patient preferably.

 Among the above-mentioned methods of measuring CTL, the HLA tetramer method is most preferred from the viewpoint of easiness and
25 accuracy. Thus, in a preferred embodiment, the present invention provides a method of selecting and treating a patient highly responsive to WT1 vaccine characterized in that it uses the HLA tetramer method. As mentioned above, the HLA monomer method, HLA dimer method, and HLA pentamer method are principally the same as HLA tetramer method, and
30 are preferred methods for measuring CTL. However, the present invention

will be herein described by taking HLA tetramer method as an example.

The method of selecting and treating a patient highly responsive to WT1 vaccine by the use of HLA tetramer, specifically, comprises the following steps (a), (b), (c) and (d):

5 (a) isolating a biological sample containing CTL precursor cells from a test subject;

(b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide contact with the biological sample of (a);

10 (c) measuring the existence frequency or amount of WT1-specific CTL precursor cells bound to the HLA tetramer; and

(d) deciding whether or not the measured value of (c) is high by comparison with that of healthy subject, and evaluating the responsiveness to WT1 vaccine.

15 In this regard, the “biological sample” and the “test subject” in step (a) are the same as defined above.

The “HLA tetramer” used in step (b) refers to a tetramer prepared by biotinylating a complex (HLA monomer) obtained by association of an HLA antigen α -chain and a β 2-microglobulin with a peptide (antigen peptide), and allowing to bind to avidin for tetramerization (Science 279: 2103- 2106
20 (1998); and Science 274: 94-96 (1996)). The HLA tetramer is preferably labeled with fluorescence so that the CTL precursor cells can be easily sorted out or detected by a known detection measure such as flow cytometry, fluorescent microscopy, and the like. Specific examples include HLA tetramers labeled with phycoerythrin(PE), fluorescein isothiocyanate
25 (FITC), peridynyl chlorophyll protein (PerCP), allophycocyanin (APC), phycoerythrin-texasred (also called ECD), and phycoerythrin-cyanine 5.1 (also called PC5), or the like.

The WT1-derived cancer antigen peptide used as a component of an HLA tetramer above is originated from human WT1 (Cell, 60: 509, 1990,
30 NCBI database Accession No. XP_034418, SEQ ID NO:1), and is able to

form a complex with an HLA antigen and thereby exerting the HLA-restricted cytotoxic T cell (CTL)-inducing activity (immunogenicity).

It has been known that there are many subtypes of HLA molecule and that the amino acid sequence of tumor antigen peptide to which an HLA molecule binds obeys a certain rule (binding motif) (Immunogenetics, 41, p178, 1995; J.Immunol., 155: p4749, 1995). For example, the binding motif for HLA-A24 is known that, in the peptides consisting of 8 - 11 amino acid residues, the amino acid at position 2 is tyrosine (Tyr), phenylalanine (Phe), methionine (Met) or tryptophan (Trp), and the amino acid at the C-terminus is phenylalanine (Phe), leucine (Leu), isoleucine (Ile), tryptophan (Trp) or methionine (Met) (J. Immunol., 152, p3913, 1994, Immunogenetics, 41, p178, 1995, J. Immunol., 155, p4307, 1994).

Regarding the motifs for HLA-A2, the following motifs listed in Table 1 are known (Immunogenetics, 41, p178, 1995; J. Immunol., 155: p4749, 1995).

Table 1

HLA-A2 type	2nd amino acid from N-terminus	amino acid at C-terminus
HLA-A0201	L, M	V, L
HLA-A0204	L	L
HLA-A0205	V, L, I, M	L
HLA-A0206	V, Q	V, L
HLA-A0207	L	L
Peptide length = 8 - 11 amino acids		

Recently, it has become possible to search peptide sequences expected to be capable of binding to HLA antigens via the internet using BIMAS software; NIH (http://bimas.dcrt.nih.gov/molbio/hla_bind/). It is also possible to search for peptide sequences using BIMAS HLA peptide binding prediction analysis (J.Immunol.,152,163, 1994). Specific examples of WT1-derived peptides which have been searched and identified in such a

manner are those listed in Table II - Table XLVI of WO2000/18795.

The above-mentioned WT1-derived peptides may be partially altered by substitution, deletion and/or addition of an amino acid residue(s) inclusive of addition of an amino acid residue(s) at the N- and/or C-terminus of peptide, preferably, by substitution of an amino acid residue(s). The substitution is preferably carried out with an amino acid residue available in view of the motifs mentioned above.

A WT1-derived cancer antigen peptide can be selected by subjecting the WT1-derived peptides (including variants) to a known assay for cancer antigen peptide, for example, a method described in WO02/47474 or Int. J. Cancer: 100, 565-570 (2002).

Specific examples of WT1-derived cancer antigen peptides include the following peptides.

	Cys Met Thr Trp Asn Gln Met Asn Leu	(SEQ ID NO: 2)
15	Cys Tyr Thr Trp Asn Gln Met Asn Leu	(SEQ ID NO: 3)
	Arg Met Phe Pro Asn Ala Pro Tyr Leu	(SEQ ID NO: 4)
	Arg Tyr Pro Ser Cys Gln Lys Lys Phe	(SEQ ID NO: 5)
	Ser Tyr Thr Trp Asn Gln Met Asn Leu	(SEQ ID NO: 6)
	Ala Tyr Thr Trp Asn Gln Met Asn Leu	(SEQ ID NO: 7)
20	Abu Tyr Thr Trp Asn Gln Met Asn Leu	(SEQ ID NO: 8)
	Arg Tyr Thr Trp Asn Gln Met Asn Leu	(SEQ ID NO: 9)
	Lys Tyr Thr Trp Asn Gln Met Asn Leu	(SEQ ID NO: 10)
	Arg Tyr Phe Pro Asn Ala Pro Tyr Leu	(SEQ ID NO: 11)
	Arg Tyr Pro Gly Val Ala Pro Thr Leu	(SEQ ID NO: 12)
25	Ala Tyr Leu Pro Ala Val Pro Ser Leu	(SEQ ID NO: 13)
	Asn Tyr Met Asn Leu Gly Ala Thr Leu	(SEQ ID NO: 14)
	Arg Val Pro Gly Val Ala Pro Thr Leu	(SEQ ID NO: 15)
	Arg Tyr Pro Ser Ser Gln Lys Lys Phe	(SEQ ID NO: 16)
	Arg Tyr Pro Ser Ala Gln Lys Lys Phe	(SEQ ID NO: 17)
30	Arg Tyr Pro Ser Abu Gln Lys Lys Phe	(SEQ ID NO: 18)

In the above, "Abu" refers to "α-aminoacetic acid.

Among them, the peptides set forth in SEQ ID NO: 2 and SEQ ID NO: 4 are HLA-A24 antigen- and HLA-A2 antigen-binding peptides and the other peptides set forth in SEQ ID NO: 3, 5, 6 - 18 are HLA-A24 antigen-binding peptides.

Preferred cancer antigen peptide can be selected from those set forth in SEQ ID NOs: 2, 3, 4 and 5 above.

An HLA tetramer may contain two or more peptides among the above-mentioned ones.

Synthesis of a peptide can be conducted according to processes generally used in the field of peptide chemistry. Such a method can be found in literatures including Peptide Synthesis, Interscience, New York, 1966; The Proteins, Vol. 2, Academic Press Inc., New York, 1976; Peptide Synthesis, Maruzen, Inc., 1975; Peptide-Gosei no Kiso to Jikken, Maruzen, Inc., 1985; and Iyakuhin no Kaihatsu (Zoku), Vol. 14, Peptide Synthesis, Hirokawa-syoten, 1991.

Also, the present invention includes peptides wherein the amino group of the N-terminal amino acid or the carboxyl group of the C-terminal amino acid of the above-described peptides is modified.

The peptides undergone such modification also fall within the scope of the present invention.

Examples of a group for the modification of amino group of the N-terminal amino acid include 1 to 3 groups selected from a C₁ - C₆ alkyl group, a phenyl group, a cycloalkyl group and an acyl group, specifically, a C₁ - C₆ alkanoyl group, a C₁ - C₆ alkanoyl group substituted by phenyl group, a carbonyl group substituted by C₅ - C₇ cycloalkyl group, a C₁ - C₆ alkylsulfonyl group, a phenylsulfonyl group, a C₂ - C₆ alkoxycarbonyl group, an alkoxycarbonyl group substituted by phenyl group, a carbonyl group substituted by C₅ - C₇ cycloalkoxy group, and a phenoxycarbonyl group, and the like.

Examples of a group for the modification of carboxyl group of the C-terminal amino acid include an ester group and an amide group. The ester group specifically includes a C₁ - C₆ alkyl ester group, a C₀ - C₆ alkyl ester group substituted by phenyl group, and a C₅ - C₇ cycloalkyl ester group, and the like. The amide group specifically includes an amide group, an amide group substituted by one or two C₁ - C₆ alkyl groups, an amide group substituted by one or two C₀ - C₆ alkyl groups that are substituted by phenyl group, and an amide group forming 5- to 7-membered azacycloalkane inclusive of nitrogen atom of amide group, and the like.

As an HLA antigen (α -chain of HLA antigen), which is a component of HLA tetramer, an HLA antigen of any subtype can be used; however, it is necessary to use an HLA antigen of the same subtype as that of the subject to be diagnosed or selected. Examples of HLA antigen includes HLA-A24 antigen such as HLA-A*2402, etc.; HLA-A2 antigen such as HLA-A*0201, -A*0204, -A*0205, -A*0206, etc.; HLA-A26 antigen such as HLA-A*2601, etc.; and HLA-A*3101, HLA-A*3303, HLA-A*1101, and the like. Specific examples include HLA-A24 antigen, HLA-A2 antigen and HLA-A26 antigen. The nucleotide and amino acid sequences of these HLA antigens are known. For example, the sequences for HLA-A24 antigen are disclosed in Cancer Res., 55: 4248-4252 (1995) and Genbank Accession No.M64740; those for HLA-A2 antigen in Genbank Accession No.M84379; and those for HLA-A26 antigen in Genbank Accession No.D14350. Accordingly, an α -chain of HLA antigen can be easily cloned on the basis of information regarding these known base sequences in a conventional manner such as PCR.

The α -chain of said HLA antigen is preferably a fragment in the soluble form to make the binding and selection of CTL easy. It is further preferred that the C-terminus of α -chain of said HLA antigen has a structure feasible for biotinylation to enable tetramerization by biotin-avidin binding, that is, has a biotin-binding portion added.

Specifically, in the case of HLA-A2402 (a kind of HLA-A24), cDNA for

a recombinant soluble HLA-A*2402 α -chain which is designed to enable the specific lysine residue in the C-terminal tag to be biotinylated by BirA enzyme is prepared by PCR reaction using, as a template, an HLA-A*2402 (GenBank Acc. No. M64740) expression plasmid and, as a forward primer:

5 5'-CCATGGGCAGCCATTCTATGCGCTATTTTCTACCTCCGT-3' (SEQ ID NO: 19); and, as a reverse primer:
5'-GGATCCTGGCTCCCATCTCAGGGTGAGGGGCTTGGGCAGACCCTC-3'
(SEQ ID NO: 20).

As a β 2-microglobulin which is a component of HLA tetramer,
10 human β 2-microglobulin is preferred. cDNA for said human β 2-microglobulin can be prepared by PCR reaction using, as a template, a human β 2-microglobulin (GenBank Acc. No. ABO21288) expression plasmid and, as a forward primer:

5'-CATATGATCCAGCGTACCCCGAAAATTCAG-3' (SEQ ID NO: 21); and, as
15 a reverse primer:
5'-GGATCCTTACATGTCTCGATCCCCTTAAC-3' (SEQ ID NO: 22).

As an avidin which is a component of HLA tetramer, any avidin heretofore known can be used. However, it is preferred that said avidin is labeled with fluorescence to facilitate the detection by flow cytometry or
20 fluorescent microscopy, and the like. Any known fluorescent pigments can be used without limitation, for example, phycoerythrin (PE), fluorescein isothiocyanate (FITC), peridynyl chlorophyll protein (PerCP), allophycocyanin (APC), phycoerythrin-texasred (also called ECD), and phycoerythrin-cyanine 5.1 (also called PC5), or the like.

25 The process for the preparation of HLA tetramers comprising those components for an HLA tetramer is well known as described in literatures (Science 279: 2103-2106 (1998), Science 274: 94-96 (1996), etc. The preparation will be hereinafter described briefly.

First, an appropriate host cells such as E. coli or mammalian cells
30 capable of expressing a protein is transformed with an HLA α -chain

expression vector and a β 2-microglobulin expression vector, and allowed to express. *E. coli* (e.g., BL21) is preferably used here. The resultant monomer HLA complex and an antigen peptide (WT1-derived cancer antigen peptide) are then mixed to form a soluble HLA-peptide complex.

5 The C-terminal sequence of HLA α -chain of the resultant HLA-peptide complex is biotinylated with BirA enzyme. When a biotinylated HLA-peptide complex and a fluorescently labeled avidin are mixed at the molar ratio of 4:1, an HLA tetramer is formed. It is preferred to purify the resulting protein by gel filtration or the like in each step above.

10 The step (b) above is carried out by bringing an HLA tetramer prepared as mentioned above into contact with a biological sample (a biological sample containing CTL precursors isolated from a test subject). The contact is preferably carried out at 37 °C. Furthermore, the contact is preferably carried out in a normal biological buffer such as phosphate
15 buffer containing serum (PBS).

It is preferred that a negative control is prepared by conducting the same procedures in parallel using fluorescently labeled streptavidin in stead of HLA tetramer.

In step (c) after step (b), the existence frequency or amount of WT1-specific CTL precursor cells bound to an HLA tetramer is measured. The
20 measurement can be carried out by any of heretofore known methods. When an HLA tetramer is fluorescently labeled, CTL precursor cells bound to the HLA tetramer are also labeled with fluorescein. The so labeled CTLs can be detected or isolated by flow cytometry or fluorescent microscopy.

25 The existence frequency of WT1-specific CTL precursor cells bound to HLA tetramer can be obtained by, for example, measuring the proportion (frequency) of HLA tetramer-bound cells among CD8-positive cells (CD8-positive CTL precursor cell) or CD8/CD3-positive cells (CD8/CD3-positive CTL precursor cells).

30 The CD8-positive cells can be labeled and detected using, for

example, fluorescently labeled mouse anti-human CD8 monoclonal antibody. The CD3-positive cells can be labeled and detected using, for example, fluorescently labeled mouse anti-human CD3 monoclonal antibody.

5 A fluorescent pigment used here must be different from that used in the HLA tetramer. That is, fluorescent pigments distinct from each other must be used, for example, when PE-labeled HLA tetramer is used, FITC-labeled mouse anti-human CD8 monoclonal antibody and PerCP-labeled mouse anti-human CD3 monoclonal antibody are usable.

10 The concrete process comprises, when the proportion (frequency) of HLA tetramer-bound cells for CD8-positive cells is measured, for example, bringing PE-labeled HLA tetramer contact with a biological sample, adding FITC-labeled mouse anti-human CD8 monoclonal antibody, allowing to react the mixture, and analyzing the stained cells by flow cytometry or
15 fluorescent microscopy. The CD8-positive cells (CD8⁺) are selected. The proportion (frequency) of CTL precursor cells specific for WT1 antigen peptide can be calculated by subtracting the proportion of avidin-positive cells (CD8⁺avidin⁺) as the negative control from the proportion of tetramer-positive cells (CD8⁺ tetramer⁺) in the selected CD8⁺ cells, as follows:

20

$$\text{WT1 antigen peptide-specific CTL precursor cells (\%)} = 100 \times \{ [(CD8^+ \text{ tetramer}^+ \text{ cells}) / (CD8^+ \text{ cells})] - [(CD8^+ \text{ avidin}^+ \text{ cells}) / (CD8^+ \text{ cells})] \}$$

25 When the proportion (frequency) of HLA tetramer-bound cells for CD8- and CD3-positive cells is measured, for example, bringing PE-labeled HLA tetramer contact with a biological sample, adding FITC-labeled mouse anti-human CD8 monoclonal antibody and PerCP-labeled mouse anti-human CD3 antibody, allowing to react the mixture, and analyzing the stained cells by flow cytometry or fluorescent microscopy. The CD3- and
30 CD8-positive cells (CD3⁺CD8⁺) are selected. The proportion (frequency) of

CTL precursor cells specific for WT1 antigen peptide can be calculated by subtracting the proportion of avidin-positive cells (CD3+CD8+avidin+) as the negative control from the proportion of tetramer-positive cells (CD3+CD8+tetramer+) in the selected CD3+CD8+ cells, as follows:

5

$$\text{WT1 antigen peptide-specific CTL precursor cells (\%)} = 100 \times \left\{ \left(\frac{\text{CD3+CD8+tetramer+ cells}}{\text{CD3+CD8+ cells}} \right) - \left(\frac{\text{CD3+CD8+avidin+ cells}}{\text{CD3+CD8+ cells}} \right) \right\}$$

10

The responsiveness to WT1 vaccine is evaluated on the basis of the results obtained by the measurement above. Specifically, it can be carried out by comparing the existence frequency or amount of WT1-specific CTL precursor cells in a test subject obtained in step (b) (hereinafter, referred to as “test subject value”) with that of healthy subject (hereinafter, referred to as “healthy subject value”), and deciding the difference between them. In this case, a biological material isolated and prepared from a healthy subject is needed (blood, lymph fluid, PBMC, etc.), which can be obtained by collecting a biological sample from a subject not having cancer. As used herein, “healthy subject” means a person who have not diagnosed to have cancer.

20

25

The comparison between the test subject values and the healthy subject values can be carried out by measuring the biological sample of a test subject and that of a healthy subject in parallel. When the parallel comparison is not conducted, the comparison can also be carried out using a mean value or a statistical intermediate value of healthy subject values calculated from the healthy subject values obtained by measuring plural (at least two, preferably three or more, more preferably five or more) biological samples of a healthy subject under a constant conditions when the parallel measurement is not conducted.

30

The evaluation whether or not a test subject is highly responsive to

WT1 vaccine can be carried out using as an indicator that the test subject value is 1.5 times or higher, preferably 2 times or higher compared to the healthy subject value. That is, when the test subject value is 1.5 times or higher, preferably, 2 times or higher than the healthy subject value, the responsiveness to WT1 vaccine is evaluated to be high. A patient evaluated to be highly responsive to WT1 vaccine is evaluated to be suitable for WT1 vaccine, in other words, it is possible to apply WT1 vaccine therapy to the patient preferably.

The method of selecting patients of the present invention as described above can also be used in not only the evaluation of patients before vaccine administration but also diagnosis or confirmation of efficacy after vaccine administration.

The present invention also provides a method of selecting a patient highly responsive to WT1 vaccine, which comprises the following steps (a), (b), and (c):

(a) isolating a biological sample containing CTL precursor cells from a test subject;

(b) measuring the existence frequency or amount of WT1-specific CTL precursor cells of effector type in the biological sample of (a); and

(c) deciding whether or not the measured value of (b) is high by comparison with that of healthy subject, and evaluating the responsiveness to WT1 vaccine, and

a method of treating cancer in the patient selected by said method.

The present inventor sorted WT1-specific CTL precursor cells finely regarding function, and found that, in particular, the effector-type CTL precursor cells exist in higher proportion compared to healthy individuals. Accordingly, patients highly responsive to WT1 vaccine can be selected on the basis of the amount or frequency of WT1-specific CTL precursor cells of effector type as an indicator. The method of selection which uses effector-type CTL precursor cells as an indicator can be useful to carry out more

detailed analysis, when there are no/poor differences between the values of a test subject and those of a healthy subject when measured by the method of selection which uses the existence frequency or amount of CTL precursor cells as an indicator.

5 Specific example of a method of selection comprises the following steps (a), (b), (c) and (d):

 (a) isolating a biological sample containing CTL precursor cells from a test subject;

 (b) bringing an HLA tetramer comprising a WT1-derived tumor
10 antigen peptide, an anti-CD8 antibody, an anti-CD45RA antibody and an anti-CD27 antibody contact with the biological sample of (a);

 (c) measuring the proportion of CD45RA-positive and CD27-negative CTL precursor cells of effector type among CTL precursor cells which are positive for CD8 or CD8/CD3 and positive for binding to HLA
15 tetramer; and

 (d) deciding whether or not the measured value of (c) is high by comparison with that of healthy subject, and evaluating the responsiveness to WT1 vaccine, and
a method of treating cancer in the patient selected by said method.

20 As used herein, the term "effector cell" means CD45RA-positive and CD27-negative CTL precursor cell. The frequency of said effector cells can be obtained by measuring the proportion of CD45RA-positive and CD27-negative CTL precursor cell among CTL precursor cells. Specifically, it can be carried out by bringing a test sample into contact with an HLA tetramer,
25 an anti-CD8 antibody, an anti-CD45RA antibody and an anti-CD27 antibody; and measuring the proportion of CD45RA-positive and CD27-negative cells among CTL precursor cells (WT1-specific CTL precursor cells) which are positive for CD8 or CD8/CD3 and positive for binding to HLA tetramer.

30 The CD45RA-positive cells can be labeled and detected using, for

example, fluorescently labeled mouse anti-human CD45RA monoclonal antibody. The CD27-positive cells can be labeled and detected using, for example, fluorescently labeled mouse anti-human CD27 monoclonal antibody. A fluorescent pigment used here must be different from that
5 used in the HLA tetramer, anti-CD8 antibody or anti-CD3 antibody. That is, fluorescent pigments distinct from each other must be used, for example, when PE-labeled HLA tetramer, FITC-labeled anti-CD8 monoclonal antibody and Per-labeled anti-CD3 monoclonal antibody are used, ECD-labeled mouse anti-human CD45RA monoclonal antibody and
10 PC5-labeled mouse anti-human CD27 monoclonal antibody are usable. Those labeled antibodies can be purchased from Beckman Coulter, and the like.

The concrete process for measuring the precursor cells or the like can be carried out in a similar manner to the method of selection described
15 above wherein the existence frequency or amount of CTL precursor cells is used as an indicator.

The method of selecting patients highly responsive to WT1 vaccine as described above is also applicable to diagnosis of cancer. That is, the present inventor found that the frequency of WT1-specific CTL precursor
20 cells is higher in patients of hematopoietic malignancy or lung cancer compared to healthy individuals. Accordingly, it is possible to diagnose cancer using the frequency of WT1-specific CTL precursor cells or WT1-specific CTL precursor cells of effector type as an indicator. Examples of cancer that can be diagnosed include blood cancers such as leukemia,
25 myelodysplastic syndrome, multiple myeloma and malignant lymphoma, and solid cancers such as gastric cancer, colon cancer, lung cancer, breast cancer, embryonal cancer, hepatic cancer, skin cancer, bladder cancer, prostate cancer, uterine cancer, cervical cancer, and ovarian cancer. Preferred examples are leukemia, myelodysplastic syndrome and lung
30 cancer.

The present invention also provides a method of identifying a target molecule of WT1 vaccine said molecule being peculiar to a patient, comprising the following steps (a), (b), (c) and (d):

5 (a) isolating a biological sample containing CTL precursor cells from a test patient;

(b) applying each of plural target molecules of WT1 vaccine to the biological sample of (a);

10 (c) measuring the existence frequency or amount of WT1-specific CTL precursor cells in the respective biological samples of (b) and comparing the results with each other; and

(d) identifying a target molecule of WT1 vaccine effective to the test patient on the basis of the results obtained in (c).

The present inventor has found that there exist WT1-specific CTL precursor cells in a patient before vaccine administration in high frequency
15 than that hitherto known. Accordingly, it is possible to identify a target molecule (target molecule for therapeutic use) of WT1 vaccine said molecule being peculiar to a patient on the basis of the amount or frequency of WT1-specific CTL precursor cells as an indicator.

20 That is, the identification method above can be used effectively to identify the most suitable target molecule (antigen peptide) for treating a patient who has been evaluated to be highly responsive to WT1 vaccine.

Specifically, the identification can be carried out, similarly to the method of selecting patients highly responsive to WT1 vaccine, by measuring the existence frequency or amount of WT1 specific CTL
25 precursor cell using HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique or limiting dilution method. Preferably, HLA monomer method, HLA dimer method, HLA tetramer method or HLA pentamer method is used. The method of identification will be hereinafter described
30 concretely taking the HLA tetramer method as an example,

The method involving HLA tetramer method comprises the following steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTL precursor cells from a test patient;

5 (b) bringing each of plural HLA tetramers comprising different WT1-derived tumor antigen peptides contact with the biological sample of (a);

(c) measuring the existence frequency or amount of WT1-specific CTL precursor cells bound to the respective HLA tetramers, and comparing
10 the results with each other; and

(d) identifying WT1-derived tumor antigen peptide effective to the test patient on the basis of the results obtained in (c).

Specifically, plural HLA tetramers each containing a WT1-derived cancer antigen peptide as a candidate are prepared. Then each of the HLA
15 tetramer is brought into contact with a biological sample isolated from a test patient, and the existence frequency or amount of WT1-specific CTL precursor cells bound to an HLA tetramer is measured. The values obtained from respective HLA tetramers are compared and a cancer antigen peptide comprised in the HLA tetramer that showed the highest
20 value, which is a cancer antigen peptide that is most readily recognized by CTLs, is identified as the target molecule for WT1 vaccine therapy for the patient, i.e., the target molecule peculiar to the patient.

Any cancer antigen peptide can be used as far as it is derived from WT1, and examples include cancer antigen peptides set forth in SEQ ID
25 NOs: 2 to 18. Preferred cancer antigen peptide is the one set forth in any one of SEQ ID NOs: 2 - 5.

The concrete process of respective steps or method of preparation for each component can be found in the above section regarding a method of selecting a patient highly responsive to WT1 vaccine.

30 According to the present method of identifying a target molecule of

WT1 vaccine, it is possible to identify a target molecule capable of treating cancer peculiar to a patient. Therefore, in a different embodiment, the present invention provides a pharmaceutical composition for treating cancer in a given patient, which comprises a target molecule identified by the method of identification of a target molecule of WT1 vaccine said molecule being peculiar to the patient; and a method of treatment of cancer peculiar to a patient, which comprises administering to the patient the target molecule identified by the method of identification. The pharmaceutical composition of the present invention comprises cancer vaccine and may contain an adjuvant and the like which are known in the art.

The present invention further provides a clinical diagnostic agent for selecting a patient highly responsive to WT1 vaccine, which comprises as an ingredient an HLA monomer, an HLA dimer, an HLA tetramer or an HLA pentamer each containing a WT1-derived tumor antigen peptide. The clinical diagnostic agent of the present invention will be hereinafter described taking HLA tetramer as an example.

The "HLA tetramer" as an ingredient of the clinical diagnostic agent of the present invention refers to, as mentioned above, a tetramer prepared by biotinylating a complex (HLA monomer) obtained by association of an HLA antigen α -chain and a β 2-microglobulin with a WT1-derived cancer antigen peptide, and allowing to bind to avidin for tetramerization (Science 279: 2103- 2106 (1998); and Science 274: 94-96 (1996)).

Any cancer antigen peptide can be used here as far as it is derived from WT1, and examples include cancer antigen peptides set forth in SEQ ID NOs: 2 to 18. Preferred cancer antigen peptide is the one set forth in any one of SEQ ID NOs: 2 - 5.

The process of respective steps or method of preparation for each component can be found in the above section regarding the method of selecting a patient highly responsive to WT1 vaccine.

The clinical diagnostic agent of the present invention can be a component of a kit for selecting a patient highly responsive to WT1 vaccine. The kit may be the one that is composed of a clinical diagnostic agent alone of the present invention or the one that is composed of a clinical
5 diagnostic agent of the present invention and other ingredient(s). Examples of other ingredients in the kit include fluorescently labeled streptavidin, fluorescently labeled mouse anti-human CD8 monoclonal antibody, fluorescently labeled mouse anti-human CD3 monoclonal antibody, and the like. When effector cells are detected, the kit may
10 contain fluorescently labeled mouse anti-human CD45RA monoclonal antibody, fluorescently labeled mouse anti-human CD27 monoclonal antibody.

Examples of fluorescent pigment include phycoerythrin (PE), fluorescein isothiocyanate (FITC), peridynyl chlorophyll protein (PerCP),
15 allophycocyanin (APC), phycoerythrin-texasred (also called ECD), and phycoerythrin-cyanine 5.1 (also called PC5), and the like.

The clinical diagnostic agent and a kit of the present invention can be used in the selection not only a patient highly responsive to WT1 vaccine but also a target molecule of WT1 vaccine (target molecule for
20 treatment). In addition, the agent can be used as a diagnostic agent for cancer without changing the components.

The present invention also provides a method of determining the suitability of a patient for WT1 vaccine, comprising the following steps (a), (b) and (c):

- 25 (a) isolating a biological sample containing CTLs from a patient after WT1 vaccine administration;
- (b) measuring the existence frequency or amount of WT1-specific CTLs in the biological sample of (a);
- (c) deciding whether or not the measured value of (b) is high by
30 comparison with that of biological sample obtained before WT1 vaccine

administration, and evaluating whether the patient is suitable for WT1 vaccine therapy, and

5 a method of treating cancer in a patient, which comprises treating the patient evaluated to be suitable by the method of determination with WT1 or WT1-derived cancer antigen peptide.

10 As described in the Examples below, the present inventor measured the frequency of WT1-specific CTLs in patients undergoing treatment with a WT1-derived tumor antigen peptide ("WT1 vaccine") and found that the therapeutic effect is correlated with the increase of CTL frequency after administration of the peptide relative to that obtained before administration thereof. That is, the case where the existence frequency of WT1-specific CTLs after administration of WT1 peptide is 1.5 times or higher compared to that in the sample obtained before administration is defined as "positive immune response", and the relationship between the
15 immunoresponsiveness and the therapeutic effect was investigated. As a result, positive correlation was recognized between the immunoresponsiveness and the therapeutic effect. This result revealed that it is possible to evaluate whether or not treatment with WT1 vaccine is suitable for a subject patient on the basis of the above-mentioned
20 immunoresponsiveness (increase in the frequency or amount of CTLs) as an indicator.

Thus, the method of determination of the present invention can be used effectively to evaluate the suitability of treatment for a patient undergoing WT1 vaccine therapy, for example, the suitability of continuous
25 treatment with peptide administration.

The concrete procedures in the steps (a) and (b) of the method of determination can be found in the above section regarding the "method of selecting a patient highly responsive to WT1 vaccine". Specifically, it can be carried out by measuring the existence frequency or amount of WT1-specific CTLs by HLA monomer method, HLA dimer method, HLA tetramer
30

method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique, limiting dilution method, or the like.

The evaluation whether or not a patient is suitable for WT1 vaccine therapy in the step (c) is carried out by comparing the existence frequency or amount of WT1-specific CTLs obtained from the patient after administration of WT1 vaccine (hereinafter, referred to as “post-administration value”) and that obtained before administration of WT1 vaccine (hereinafter, referred to as “pre-administration value”), and deciding the difference between the both values.

As used herein, “after administration of vaccine” refers to any time (timing) after one or more times of WT1 vaccine administration. However, in the case of peptide-dosing schedule of two-week-interval, preferred time (timing) is after the first to fifth WT1 vaccine administration, preferably, after the first to third WT1 vaccine administration.

The evaluation whether or not treatment with WT1 vaccine is suitable can be carried out using as an indicator that the post-administration value is 1.5 times or higher compared to the pre-administration value. That is, when the post-administration value is 1.5 times or higher than the pre-administration value, treatment with WT1 vaccine is evaluated to be suitable. On the basis of these findings, the present invention also provides a method of treating cancer in a patient, which comprises treating a patient who has been evaluated to be suitable by the method of determining the suitability of a patient for WT1 vaccine of the present invention with WT1 or WT1-derived tumor antigen peptide.

Among the above-mentioned methods of measuring CTL, the HLA monomer method, HLA dimer method, HLA tetramer method and HLA pentamer method are principally preferred from the viewpoint of easiness of handling and accuracy. The method of determination will be hereinafter described taking the HLA tetramer method as an example,

The method involving HLA tetramer method comprises the following

steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTLs from a patient after WT1 vaccine administration;

5 (b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide contact with the biological sample of (a);

(c) measuring the existence frequency or amount of WT1-specific CTLs bound to the HLA tetramer; and

10 (d) deciding whether or not the measured value of (c) is high by comparison with that of biological sample obtained before WT1 vaccine administration, and evaluating whether the patient is suitable for WT1 vaccine therapy.

The HLA antigen used here as a component of HLA tetramer includes an HLA-A24 antigen or an HLA-A2 antigen.

15 Examples of cancer antigen peptide as a component of an HLA tetramer include: Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2), Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3), Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5). As all the peptides set forth in SEQ ID NO: 2 - 5 are peptides capable of binding to HLA-A24, examples of HLA tetramer used in
20 the above-mentioned method for determination of the present invention includes HLA tetramers comprising any one of peptides set forth in SEQ ID NO: 2 - 5 and an HLA-A24 antigen. In addition, as the peptides set forth in SEQ ID NO: 2 and 4 are also capable of binding to HLA-A2 antigen, HLA tetramers comprising a peptide set forth in SEQ ID NO: 2 or 4 and
25 an HLA-A2 antigen are also included.

In the method of determination or treatment of the present invention, it is preferred to use an HLA tetramer comprising the same peptide as that used in the treatment or a peptide with which CTLs show cross-reaction, said CTLs having been induced by the peptide used in the treatment. For
30 example, when a peptide set forth in SEQ ID NO: 3 is used in the treatment

of a patient, an HLA tetramer comprising a peptide set forth in SEQ ID NO: 2 or 3 and an HLA-A24 antigen is used effectively.

5 The concrete procedures in the steps (a) to (c) of the method of determination or treatment of the present invention can be found in the above section regarding the “method of selecting a patient highly responsive to WT1 vaccine”. Further, the evaluation of step (d) can be carried out on the basis of the comparison of pre-administration values and post-administration values as mentioned above.

10 The specific example of the method of determination or treatment of the present invention will be hereinafter described.

First, blood is collected from a patient having cancer before administration of WT1-derived cancer antigen peptide, and PBMC is separated (pre-administration sample). Then, blood is collected from the patient after treated by peptide administration, and PBMC is separated
15 (post-administration sample). To respective pre- and post-administration samples are added an HLA tetramer, and the frequency of peptide-specific CTLs is measured and calculated according to the methods of analysis which are described in the above section regarding the “method of selecting a patient highly responsive to WT1 vaccine” and Example 3. When the CTL
20 frequency in the post-administration sample is 1.5 times or higher compared to that of pre-administration sample, it is evaluated that treatment with WT1 vaccine is suitable (i.e., WT1 vaccine is expected to be therapeutically effective).

The present invention also provides a clinical diagnostic agent for
25 determining the suitability for WT1 vaccine which comprises as an ingredient an HLA monomer, an HLA dimer, an HLA tetramer or an HLA pentamer each containing a WT1-derived cancer antigen peptide, and a kit comprising said clinical diagnostic agent. The ingredients of said clinical diagnostic agent and kit are as defined in the above section regarding the
30 “diagnostic clinical agent for the selection of a patient highly responsive to

WT1 vaccine”.

EXAMPLES

5 The present invention is further illustrated by the following examples,
but is not limited by these examples in any respect.

Example 1

Preparation of Peripheral Blood Mononuclear Cells

10 After obtaining informed-consent, blood was collected from HLA-A*2402-positive patients having cancer and HLA-A*2402-positive healthy individuals. Among patients, those having hematopoietic malignant tumor were eighteen which composed of acute myelocytic leukemia (AML) (n=11), acute lymphatic leukemia (ALL) (n=2), chronic myelocytic leukemia (CML) (n=1), and myelodysplastic syndrome (MDS) (n=4); and those having lung cancer were seven. There were ten HLA-A*2402-positive healthy
15 individuals.

As for the patients having hematopoietic malignant tumor, significant high expression of WT1 gene was identified in myeloma and peripheral blood samples once or more at the time of diagnosis or in the course of treatment. As for the patients having lung cancer, significant
20 high expression of WT1 gene was identified in biopsy or extracted samples.

Peripheral mononuclear cells (PBMCs) were separated from the collected blood by density gradient centrifugation method (Ficoll-Hypaque) and stored in liquid nitrogen in frozen state.

25 Example 2

Preparation of HLA Tetramer

A tetramer comprising HLA-A*2402 labeled with fluorescent pigment (Phycoerythrin; PE) was prepared using a 9-amino-acid peptide (SEQ ID NO: 2) comprising the amino acid sequence at position 235-243 of WT1
30 protein according to the method described in Int. J. Cancer: 100, 565-570,

2002.

First, cDNA encoding recombinant HLA-A2402 was amplified by PCR using an HLA-A* 2402 expression plasmid (GenBank Acc. No. M64740) as a template and a forward primer:

5 5'-CCATGGGCAGCCATTCTATGCGCTATTTTCTACCTCCGT-3' (SEQ ID NO: 19); and a reverse primer:
5'-GGATCCTGGCTCCCATCTCAGGGTGAGGGGCTTGGGCAGACCCTC-3'
(SEQ ID NO: 20).

10 The reverse primer encodes a BirA recognition sequence so that the frames conforms at the c-terminus. The amplified fragments were cleaved by restriction enzymes NcoI and BamH1, and cloned into pET11d vector (Novagen).

Then cDNA encoding recombinant soluble human β 2 microglobulin was amplified using a human β 2-microglobulin expression plasmid
15 (GenBank Acc. No. ABO21288) as a template and a forward primer:
5'-CATATGATCCAGCGTACCCCGAAAATTCAG-3' (SEQ ID NO: 21); and a reverse primer:
5'-GGATCCTTACATGTCTCGATCCCACTTAAC-3' (SEQ ID NO: 22).

20 The amplified fragments were cleaved by restriction enzymes NdeI and BamH1, and cloned into pET11a vector (Novagen).

The resulting two vectors were allowed to express in E. coli. BL21, and recovered as insoluble fractions of inclusion bodies. The respective inclusion bodies were dissolved in 8M urea solution and diluted by a refolding buffer. To the dilution was added a peptide (SEQ ID NO: 2) to
25 form a soluble HLA-peptide complex. The C-terminal sequence of the HLA-peptide complex was biotinylated with Bir A enzyme and the resulting biotinylated HLA-peptide tetramer was purified by gel filtration technique. The biotinylated HLA-peptide complex and PE-labeled avidin (Molecular Probe) were mixed at molar ratio of 4:1 to prepare HLA tetramer.

Example 3

Analysis of WT1-specific CTL precursor cells with HLA Tetramer

The frozen PBMCs obtained in Example 1 were thawed, and immediately re-suspended in a phosphate buffered saline (PBS) containing 0.5% fetal bovine serum (FCS) at the cell density of 1×10^6 cells/ml. To the suspension was added a solution of tetramer (500 μ g/ μ l, 2 μ l) prepared in Example 2. The suspension was then incubated at 37 °C for 30 minutes. A sample for the negative control was prepared by treating in a similar manner except that PE-labeled streptavidin (Becton Dickinson) was added instead of tetramer. After quenching with ice-cooled water, FITC-labeled mouse anti-human CD8 monoclonal antibody (15 μ l, BD Pharmigen) and PerCP-labeled mouse anti-human CD3 antibody (15 μ l, BD Pharmigen) were added, and the mixture was incubated at 4 °C for 30 minutes. The stained cells were subjected to centrifugal washing with PBS containing 0.5 % FCS (2x), and analyzed by flow cytometer FACSsort (Becton Dickinson). CD3- and CD8-positive cells (CD3⁺ CD8⁺) were selected. The proportion of WT1-antigen peptide specific CTL precursor cells was calculated by subtracting the proportion of PE-labeled streptavidin-positive cells (CD3⁺CD8⁺avidin⁺) in the negative control from the proportion of tetramer-positive cells (CD3⁺CD8⁺tetramer⁺) in the selected CD3⁺CD8⁺ cells, as follows:

$$\begin{aligned} \text{WT1 antigen peptide-specific CTL precursor cells (\%)} = & 100 \times \\ & \{ [(\text{CD3}^+ \text{CD8}^+ \text{tetramer}^+ \text{ cells}) / (\text{CD3}^+ \text{CD8}^+ \text{ cells})] - \\ & [(\text{CD3}^+ \text{CD8}^+ \text{avidin}^+ \text{ cells}) / (\text{CD3}^+ \text{CD8}^+ \text{ cells})] \} \end{aligned}$$

The results of analysis of PBMCs of patients having cancer and healthy individuals are shown in Table 2. The results were plotted for respective diseases as shown in Figure 1. These results showed that the proportion of WT1 antigen peptide-specific CTL precursor cells in CD3/CD8-positive cells was 0.47 to 1.30% (average = 0.82%) for healthy

individuals, 1.04 - 29.45% (average = 5.24%) for patients having malignant hematopoietic tumor, and 0.33 - 5.97% (average = 2.44%) for patients having lung cancer. The statistical analysis revealed that the proportion was significantly increased ($p<0.05$) in patients having malignant hematopoietic tumor or lung cancer compared to healthy individuals.

Table 2

Sample, patient No.	Frequency of CTL precursors (%)
AML, patient No. 1	8.26
AML, patient No. 2	8.01
AML, patient No. 3	5.12
AML, patient No.4	3.84
AML, patient No.5	4.51
AML, patient No.6	3.27
AML, patient No.7	2.68
AML, patient No.8	2.60
AML, patient No.9	1.77
AML, patient No.10	1.04
AML, patient No.11	1.49
ALL, patient No.1	7.32
ALL, patient No.2	1.78
CML, patient 1	2.46
MDS, patient 1	29.45
MDS, patient 2	2.99
MDS, patient 3	2.81
MDS,. patient 4	2.08
lung cancer. patient 1	5.97
lung cancer. patient2	3.83
lung cancer. patient3	2.63
lung cancer. patient4	1.89
lung cancer. patient5	1.69
lung cancer. patient6	0.72
lung cancer. patient7	0.33
healthy individual 1	1.30
healthy individual 2	1.05
healthy individual 3	1.08
healthy individual 4	0.85
healthy individual 5	0.81
healthy individual 6	0.79
healthy individual 7	0.61
healthy individual 8	0.64
healthy individual 9	0.57

healthy individual 10	0.47
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AML: acute myelocytic leukemia
ALL: acute lymphatic leukemia
CML: chronic myelocytic leukemia
MDS: myelodysplastic syndrome

5

Example 4

Analysis of Frequency of WT1-specific CTLs after Peptide Administration

10 The following test was carried out after obtaining approval of ethical committee of Osaka University, Faculty of Medicine, and informed consent of cancer patients.

15 A peptide comprising the amino acid sequence at position 235-243 of WT1 (SEQ ID NO:2) or its variant comprising SEQ ID NO: 3 wherein the methionine at position 2 of SEQ ID NO: 2 is replaced by tyrosine was administered to cancer patients at 0.3 mg, 1 mg or 3 mg per body. The peptide was emulsified with Montanide ISA51 (SEPPIC), and the resulting emulsion was intradermally injected once or plural times at 2-week-intervals. Subject patients were suffering from HLA-A*2402-positive and WT1-positive lung cancer, breast cancer or leukemia.

20 The immune response to the administered peptide was evaluated on the basis of the CTL frequency measured by the HLA tetramer method similar to Example 3. When the frequency of peptide-specific CTLs at any stage after peptide administration is increased by 1.5 times or higher compared to that obtained before peptide administration, it was evaluated to be "positive immune response". Further, when the tumor marker values, 25 the number of tumor cells or the volume of tumor decreased, it was evaluated to be "therapeutically effective". The correlation between the immune response and therapeutic effect was evaluated by chi-square test in cancer patients (n=19) who have been evaluated for the immune response to and therapeutic effect of peptide administration. As a result,

eight (73%) of eleven patients who were positive regarding therapeutic effect showed positive immune response, while only two (25%) of eight patients who were negative regarding therapeutic effect showed positive immune response, indicating that the therapeutic effect and the immune response are positively correlated ($P = 0.0397$). These results indicates that the induction of CTLs specific for the administered peptide is an important factor for the therapeutic effect. In addition, the immunoresponsiveness above can be used as an indicator for the conformation of favorable progress of treatment with peptide administration or the decision whether or not treatment by the peptide administration should be continued.

Example 5

Analysis of Function of WT1-specific CTLs

It has been reported that antigen-peptide specific CTLs positive for the HLA tetramer staining and CD8 can be further sorted finely by staining with anti-CD45RA antibody and anti-CD27 antibody (J. Exp. Med., 186, p1407, 1997). The CD45RA-positive and CD27-positive cells are classified into naive type; CD45RA-negative and CD27-positive, and CD45RA-negative and CD27-negative cells into memory type; and CD45RA-positive and CD27-negative cells into effector type. The effector-type cells represent cell populations of the strongest CTL activity.

PBMCs were collected before peptide administration from HLA-A*2402-positive cancer patients (n=24; 14 blood cancers, 10 solid cancers) who were tested by the clinical research in Example 4 and HLA-A*2402-positive healthy individuals after obtaining informed consent. The PMBCs were used in the analysis of function of WT1-peptide specific CTL precursors which are positive for the HLA tetramer staining and CD8. For the analysis by flow cytometry, cells were stained in a similar manner to Example 3 with HLA tetramer, anti-CD8 antibody, anti-CD45RA antibody,

and anti-CD27 antibody. The proportion of cells belonging to CD45RA-positive/CD27-positive naive type, CD45RA-negative memory type or CD45RA-positive/CD27-negative effector type in HLA tetramer-positive and CD8-positive cell populations was calculated. The proportions for naive-type, memory-type and effector-type cells were 23.7%, 45.5% and 30.8% for the cancer patients. As for healthy individuals, the proportions were 35.9%, 53.8% and 8.9%. The comparison of cancer patients and healthy individuals showed that the proportion of effector-type cells is significantly high ($P < 0.05$) in the cancer patients; however, there are no significant differences between the cancer patients and healthy individuals regarding the proportion of naive-type and memory-type cells. In Example 3, the cancer patients showed increase of WT1-specific CTL precursor cells, and, now the cancer patients revealed to show increase of proportion of CTLs having effector-type function among CTLs. These results demonstrated that the cancer patients can be diagnosed on the basis of the existence frequency of effector type CTL precursor cells.

INDUSTRIAL APPLICABILITY

According to the present invention, a method of selecting a patient highly responsive to WT1 vaccine on the basis of existence frequency of WT1-specific C T L precursor cells as an indicator, a method of treating cancer utilizing the same, and clinical diagnostic agents for the selection, and the like, are provided. According to the method of selection of the present invention, a patient who is expected to be responsive to WT1 vaccine therapy can be selected, which makes it possible to treat cancer more appropriately.

SEQUENCE LISTING

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<120> Method of selecting patients suitable for WT1 vaccine

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<140> PCT/JP2004/009378
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CLAIMS

1. A method of selecting a patient highly responsive to WT1
5 vaccine, comprising the following steps (a), (b) and (c):

(a) isolating a biological sample containing CTL precursor cells
from a test subject;

(b) measuring the existence frequency or amount of WT1-specific
CTL precursor cells in the biological sample of (a); and

10 (c) deciding whether or not the measured value of (b) is high by
comparison with that of healthy subject, and evaluating the responsiveness
to WT1 vaccine.

2. The method of selection according to claim 1, wherein the
measurement of the existence frequency or amount of WT1-specific CTL
15 precursor cells is carried out by any one of HLA monomer method, HLA
dimer method, HLA tetramer method, HLA pentamer method, ELISPOT
method, realtime RT-PCR technique and limiting dilution method.

3. The method of selection according to claim 2, wherein the
measurement is carried out by HLA tetramer method.

20 4 The method of selection according to claim 3, which comprises
the following steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTL precursor cells
from a test subject;

25 (b) bringing an HLA tetramer comprising a WT1-derived tumor
antigen peptide contact with the biological sample of (a);

(c) measuring the existence frequency or amount of WT1-specific
CTL precursor cells bound to the HLA tetramer; and

30 (d) deciding whether or not the measured value of (c) is high by
comparison with that of healthy subject, and evaluating the responsiveness
to WT1 vaccine.

5. The method of selection according to claim 4, wherein the step (c) in claim 4 is carried out by measuring the proportion of HLA tetramer-bound cells among CD8-positive or CD8/CD3-positive CTL precursor cells.

6. The method of selection according to claim 4 or 5 wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

7. The method of selection according to any one of claims 4 to 6, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

10 Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),
Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),
Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and
Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

8. The method of selection according to any one of claims 1 to 7, which is carried out using flow cytometry.

9. The method of selection according to any one of claims 1 to 8, wherein the responsiveness to WT1 vaccine is evaluated using as an indicator that the existence frequency or amount of WT1-specific CTL precursor cells is 1.5 times or higher compared to that of healthy subject.

10 10 The method of selection according to claim 1, wherein the CTL precursor cells are CTL precursor cells of effector type.

11. The method of selection according to claim 10, which uses any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique and limiting dilution method in the measurement of the existence frequency or amount of WT1-specific CTL precursor cells of effector type.

12 The method of selection according to claim 11, which uses the HLA tetramer method.

13. The method of selection according to claim 12, which comprises the following steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTL precursor cells from a test subject;

(b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide, an anti-CD8 antibody, an anti-CD45RA antibody and an anti-CD27 antibody contact with the biological sample of (a);

(c) measuring the proportion of CD45RA-positive and CD27-negative CTL precursor cells of effector type among CTL precursor cells which are positive for CD8 or CD8/CD3 and positive for binding to HLA tetramer; and

(d) deciding whether or not the measured result of (c) is high by comparison with that of healthy subject, and evaluating the responsiveness to WT1 vaccine.

14. The method of selection according to claim 13, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

15. The method of selection according to claim 13 or 14, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

16. The method of selection according to any one of claims 10 to 15, which is carried out using flow cytometry.

17. A method of diagnosing cancer, comprising the following steps (a), (b) and (c):

(a) isolating a biological sample containing CTL precursor cells from a test subject;

(b) measuring the existence frequency or amount of WT1-specific CTL precursor cells in the biological sample of (a); and

(c) deciding whether or not the measured result of (b) is high by comparison with that of healthy subject, and evaluating whether the test subject has cancer.

18. The method of diagnosis according to claim 17, wherein the measurement of the existence frequency or amount of WT1-specific CTL precursor cells is carried out by any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique and limiting dilution method.

19. The method of diagnosis according to claim 18, wherein the measurement is carried out by HLA tetramer method.

20. The method of diagnosis according to claim 19, which comprises the following steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTL precursor cells from a test subject;

(b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide contact with the biological sample of (a);

(c) measuring the existence frequency or amount of WT1-specific CTL precursor cells bound to the HLA tetramer; and

(d) deciding whether or not the measured result of (c) is high by comparison with that of healthy subject, and evaluating whether the test subject has cancer.

21. The method of diagnosis according to claim 20, wherein the step (c) in claim 20 is carried out by measuring the proportion of HLA tetramer-bound cells among CD8-positive or CD8/CD3-positive CTL precursor cells.

22. The method of diagnosis according to claim 20 or 21, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

23. The method of diagnosis according to any one of claims 20 to 22, wherein the WT1-derived tumor antigen peptide is selected from the

following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

5 Arg Tyr Pro Ser Cys Gln Lys Lys Phe(SEQ ID NO: 5).

24. The method of diagnosis according to any one of claims 17 to 23, which is carried out using flow cytometry.

25. The method of diagnosis according to any one of claims 17 to 24, wherein cancer is diagnosed using as an indicator that the existence
10 frequency or amount of WT1-specific CTL precursor cells is 1.5 times or higher compared to that of healthy subject.

26. The method of diagnosis according to claim 17, wherein the CTL precursor cells are CTL precursor cells of effector type.

27. The method of diagnosis according to claim 26, which uses
15 any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique and limiting dilution method in the measurement of the existence frequency or amount of WT1-specific CTL precursor cells of effector type.

20 28. The method of diagnosis according to claim 27, which uses the HLA tetramer method.

29. The method of diagnosis according to claim 28, which comprises the following steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTL precursor cells
25 from a test subject;

(b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide, an anti-CD8 antibody, an anti-CD45RA antibody and an anti-CD27 antibody contact with the biological sample of (a);

(c) measuring the proportion of CD45RA-positive and CD27-
30 negative CTL precursor cells of effector type among CTL precursor cells

which are positive for CD8 or CD8/CD3 and positive for binding to HLA tetramer; and

(d) deciding whether or not the measured value of (c) is high by comparison with that of healthy subject, and evaluating whether the test subject has cancer.

30. The method of diagnosis according to claim 29, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

31. The method of diagnosis according to claim 29 or 30, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

32. The method of diagnosis according to any one of claims 26 to 31, which is carried out using flow cytometry.

33. A method of identifying a target molecule of WT1 vaccine said molecule being peculiar to a patient, comprising the following steps (a), (b), (c) and (d):

(a) isolating a biological sample containing CTL precursor cells from a test patient;

(b) applying each of plural target molecules of WT1 vaccine to the biological sample of (a);

(c) measuring the existence frequency or amount of WT1-specific CTL precursor cells in the respective biological samples of (b) and comparing the results with each other; and

(d) identifying a target molecule of WT1 vaccine effective to the test patient on the basis of the results obtained in (c).

34. The method of identification according to claim 33, wherein

the measurement of the existence frequency or amount of WT1-specific CTL precursor cells is carried out by any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique and limiting dilution method.

5 35. The method of identification according to claim 34, wherein the measurement is carried out by HLA tetramer method.

36. The method of identification according to claim 35, which comprises the following steps (a), (b), (c) and (d):

10 (a) isolating a biological sample containing CTL precursor cells from a test patient;

 (b) bringing each of plural HLA tetramers comprising different WT1-derived tumor antigen peptides contact with the biological sample of (a);

15 (c) measuring the existence frequency or amount of WT1-specific CTL precursor cells bound to the respective HLA tetramers, and comparing the results with each other; and

 (d) identifying a WT1-derived tumor antigen peptide effective to the test patient on the basis of the results obtained in (c).

20 37. The method of identification according to claim 36, wherein the step (c) in claim 36 is carried out by measuring the proportion of HLA tetramer-bound cells among CD8-positive or CD8/CD3-positive CTL precursor cells.

25 38. The method of identification according to claim 36 or 37, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

39. The method of identification according to any one of claims 36 to 38, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

30 Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and
Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

40. The method of identification according to any one of claims 33 to 39, which is carried out using flow cytometry.

5 41. A clinical diagnostic agent for selecting a patient highly responsive to WT1 vaccine, which comprises as an ingredient an HLA monomer, an HLA dimer, an HLA tetramer or an HLA pentamer each containing a WT1-derived tumor antigen peptide.

10 42. The clinical diagnostic agent according to claim 41, wherein the HLA antigen as a component of an HLA monomer, an HLA dimer, an HLA tetramer or an HLA pentamer is an HLA-A24 antigen or an HLA-A2 antigen.

15 43. The clinical diagnostic agent according to claim 41 or 42, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

20 44. A kit comprising a clinical diagnostic agent according to any one of claims 41 to 43.

25 45. A pharmaceutical composition for treating cancer in a given patient, which comprises a target molecule identified by the method of identification of a target molecule of WT1 vaccine said molecule being peculiar to the patient according to any one of claims 33 to 40.

46. A diagnostic agent for cancer, which comprises as an ingredient an HLA monomer, an HLA dimer, an HLA tetramer or an HLA pentamer each containing a WT1-derived tumor antigen peptide.

30 47. The diagnostic agent according to claim 46, wherein the HLA antigen as a component of an HLA monomer, an HLA dimer, an HLA

tetramer or an HLA pentamer is an HLA-A24 antigen or an HLA-A2 antigen.

48. The diagnostic agent according to claim 46 or 47, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

5 Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

49. A kit which comprises a diagnostic agent according to any one of claims 46 to 48.

10 50. A method of determining the suitability of a patient for WT1 vaccine, comprising the following steps (a), (b) and (c):

(a) isolating a biological sample containing CTLs from a patient after WT1 vaccine administration;

15 (b) measuring the existence frequency or amount of WT1-specific CTLs in the biological sample of (a);

(c) deciding whether or not the measured value of (b) is high by comparison with that of biological sample obtained before WT1 vaccine administration, and evaluating whether the patient is suitable for WT1 vaccine therapy.

20 51. The method of determination according to claim 50, wherein the measurement of the existence frequency or amount of WT1-specific CTLs is carried out by any one of HLA monomer method, HLA dimer method, HLA tetramer method, HLA pentamer method, ELISPOT method, realtime RT-PCR technique and limiting dilution method.

25 52. The method of determination according to claim 51, wherein the measurement is carried out by HLA tetramer method.

53 The method of determination according to claim 52, which comprises the following steps (a), (b), (c) and (d):

30 (a) isolating a biological sample containing CTLs from a patient after WT1 vaccine administration;

(b) bringing an HLA tetramer comprising a WT1-derived tumor antigen peptide contact with the biological sample of (a);

(c) measuring the existence frequency or amount of WT1-specific CTLs bound to the HLA tetramer; and

5 (d) deciding whether or not the measured value of (c) is high by comparison with that of biological sample obtained before WT1 vaccine administration, and evaluating whether the patient is suitable for WT1 vaccine therapy.

10 54. The method of determination according to claim 53, wherein the step (c) in claim 53 is carried out by measuring the proportion of HLA tetramer-bound cells among CD8-positive or CD8/CD3-positive CTLs.

55. The method of determination according to claim 53 or 54, wherein the HLA antigen as a component of HLA tetramer is an HLA-A24 antigen or an HLA-A2 antigen.

15 56. The method of determination according to any one of claims 53 to 55, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),

20 Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and

Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

57 The method of determination according to any one of claim 50 to 56, which is carried out using flow cytometry.

25 58. The method of determination according to any one of claims 50 to 57, wherein the suitability for WT1 vaccine therapy is evaluated using as an indicator that the existence frequency or amount of WT1-specific CTLs is 1.5 times or higher compared to that in the sample obtained before administration.

30 59. A clinical diagnostic agent for determining the suitability for WT1 vaccine which comprises as an ingredient an HLA monomer, an HLA

dimer, an HLA tetramer or an HLA pentamer each containing a WT1-derived tumor antigen peptide.

60 The clinical diagnostic agent according to claim 59, wherein the HLA antigen as a component of an HLA monomer, a HLA dimer, an
5 HLA tetramer or an HLA pentamer is an HLA-A24 antigen or an HLA-A2 antigen.

61. The clinical diagnostic agent according to claim 59 or 60, wherein the WT1-derived tumor antigen peptide is selected from the following peptides:

10 Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 2),
Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 3),
Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 4) and
Arg Tyr Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 5).

62. A kit comprising a clinical diagnostic agent according to any
15 one of claims 59 to 61.

FIG. 1

