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(54) **METHOD FOR PREDICTING CLINICAL EFFECT OF IMMUNOTHERAPY**

(57) Provided is a method for predicting a clinical effect on a subject in a WT1 peptide immunotherapy, said method comprising: a) a step for contacting a sample derived from the subject with WT1 antigen peptide or a variant thereof; and b) a step for detecting the binding of the sample to the WT1 antigen peptide or a variant thereof and thus measuring anti-WT1 antigen peptide IgG an-

tibody titer existing in the sample, characterized in that an increase in the anti-WT1 antigen peptide IgG antibody titer in the subject determines the achievement of a favorable clinical effect. Also provided is a kit for performing the method according to the present invention, said kit containing WT1 antigen peptide or a variant thereof.

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Description

Technical Field

5 **[0001]** The present application claims the priority benefit of Japanese Patent Application No. 2013-101566 filed May 13, 2013, and the entire disclosure thereof including specification, claims, drawings and abstract is incorporated herein by reference in its entirety.

[0002] The present application relates to a method for predicting clinical effect in WT1 peptide immunotherapy and the like.

10 Background Art

[0003] Wilms' tumor 1 gene was identified as a responsible gene of Wilms' tumor which is a kidney cancer in childhood (Non Patent Documents 1 and 2), and the gene encodes a transcription factor having a zinc finger structure. Although 15 WT1 gene was considered to be a tumor suppressor gene at first, subsequent studies (Non Patent Documents 3-6) showed that this gene serves as a cancer gene rather than a tumor suppressor gene in hematopoietic organ tumors and solid cancers.

[0004] Recently, WT1 peptide immunotherapy using a WT1 gene product or a fragment thereof has been performed. In order to plan a long-term treatment strategy after the start of immunotherapy, predicting the extent of clinical effect has clinically important significance. Therefore, it is desirable to establish a high-accurate method for predicting clinical 20 effect.

Prior Art Documents

25 Patent Documents

[0005]

- 30 Patent Document 1: Japanese Patent No. 3728439 Non Patent Documents
Non Patent Document 1: Daniel A. Haber et al., Cell. 1990 Jun 29;61(7):1257-69.
Non Patent Document 2: Call KM et al., Cell. 1990 Feb 9;60(3):509-20.
Non Patent Document 3: Menke AL et al., Int Rev Cytol. 1998;181:151-212. Review.
Non Patent Document 4: Yamagami T et al., Blood. 1996 Apr 1;87(7):2878-84.
Non Patent Document 5: Inoue K et al., Blood. 1998 Apr 15; 91 (8) :2969-76.
35 Non Patent Document 6: Tsuboi A et al., Leuk Res. 1999 May; 23 (5) :499-505.
Non Patent Document 7: Fujiki F et al., J Immunother. 2007 Apr;30(3):282-93.

Disclosure of the Invention

40 Problems to be Solved by the Invention

[0006] Objects to be achieved by the present invention include providing a method for predicting a long-term clinical effect of a subject given a WT1 peptide vaccine with higher accuracy. Objects to be achieved by the present invention also include providing a kit for predicting the clinical effect with higher accuracy.

45 Means for Solving the Problems

[0007] The present inventors have established a system of measuring IgG antibody against a WT1 peptide by using a WT1 antigen peptide corresponding to a WT1 peptide vaccine administered to a subject, and thus completed the present invention. In particular, the present inventors have established a system of measuring antibody titers of IgG1, IgG3 and IgG4 against a WT1 antigen peptide and using values thereof as indexes.

[0008] Namely, the present invention provides the following:

55 (1) A method for predicting a clinical effect on a subject in WT1 peptide immunotherapy comprising the following steps:

- a) contacting a sample from the subject with a WT1 antigen peptide or a variant thereof; and
- b) detecting the binding of the sample to the WT1 antigen peptide or the variant thereof, and thereby, measuring an antibody titer of anti-WT1 antigen peptide IgG present in the sample,

wherein an increase in the anti-WT1 antigen peptide IgG antibody titer in the subject means the achievement of a favorable clinical effect;

(2) The method according to (1), wherein the subclasses of the measured anti-WT1 antigen peptide IgG antibody are IgG1, IgG3 and IgG4, and wherein the achievement of a favorable clinical effect is determined when the titer of each of IgG1 and IgG3 is twice or more than twice the titer of IgG4;

(3) The method according to (2), wherein the achievement of a favorable clinical effect is determined when the titer of IgG3 is less than twice the titer of IgG1 and the titer of IgG1 is less than twice the titer of IgG3;

(4) The method according to any of (1)-(3), wherein the anti-WT1 antigen peptide IgG antibody titer at 8 to 14 weeks after the start of administration of a WT1 peptide vaccine is measured;

(5) The method according to (4), wherein the anti-WT1 antigen peptide IgG antibody titer at 12 to 14 weeks after the start of administration of the WT1 peptide vaccine is measured;

(6) The method according to any of (1)-(5), wherein the WT1 peptide vaccine administered to the subject consists of the amino acid sequence of any of SEQ ID NOs: 2-6;

(7) The method according to any of (1)-(6), wherein the WT1 antigen peptide comprises the amino acid sequence of any of SEQ ID NOs: 7-71;

(8) The method according to (7), wherein the WT1 antigen peptide comprises the amino acid sequence of any of SEQ ID NOs: 20, 21, 27, 31, 32, 42, 43 or 57-71;

(9) The method according to any of (1)-(8), wherein the sample is a blood sample, a plasma sample, a serum sample or a urine sample;

(10) The method according to (9), wherein the sample is a serum sample;

(11) The method according to any of (1)-(10), wherein the subject is a patient with a WT1-associated disease;

(12) The method according to (11), wherein the WT1-associated disease is leukemia such as chronic myeloid leukemia, hematopoietic organ tumor such as myelodysplastic syndrome, multiple myeloma, and malignant lymphoma, or solid cancer such as esophageal cancer, stomach cancer, colorectal cancer, pancreatic cancer, lung cancer, breast cancer, germ cell cancer, liver cancer, biliary tract cancer, head and neck cancer, skin cancer, sarcoma, kidney cancer, bladder cancer, prostate cancer, testicular cancer, uterine cancer, cervical cancer, ovarian cancer, thyroid cancer, carcinoid, pneumoblastoma, hepatoblastoma, brain tumor and thymic carcinoma;

(13) The method according to (12), wherein the WT1-associated disease is recurrent malignant glioma, thymic carcinoma or pancreatic cancer; and

(14) A kit for performing the method according to any of (1)-(13), comprising a WT1 antigen peptide or a variant thereof.

Effects of the Invention

[0009] According to the present invention, it is made possible to provide a method for predicting a long-term clinical effect of a subject given a WT1 peptide vaccine with higher accuracy compared with a conventional method. It is also made possible to provide a kit for predicting the clinical effect with higher accuracy. Thereby, it is made possible to more appropriately judge the propriety of a continuous administration of the WT1 peptide vaccine etc.

Brief Description of the Drawings

[0010]

[Fig.1] Figure 1 is a graph depicting the change of the anti-WT1-235 peptide IgG antibody titer in recurrent malignant glioma patients after the start of the administration of WT1₂₃₅ peptide vaccine.

[Fig.2] Figure 2 is a graph depicting the relationship between the increase in the anti-WT1-235 peptide IgG antibody titer at 8-9 weeks after the start of administration of WT1₂₃₅ peptide vaccine and an overall survival rate in recurrent malignant glioma patients. Black squares show a group in which the anti-WT1-235 peptide IgG antibody titer was increased (antibody titer increase group), and white squares show a group in which the anti-WT1-235 peptide IgG antibody titer was not increased (antibody titer non-increase group).

[Fig.3] Figure 3 is a graph depicting the relationship between the increase in the anti-WT1-235 peptide IgG antibody titer at 12 to 14 weeks after the start of administration of WT1₂₃₅ peptide vaccine and an overall survival rate in recurrent malignant glioma patients. Black squares show a group in which the anti-WT1-235 peptide IgG antibody titer was increased (antibody titer increase group), and white squares show a group in which the anti-WT1-235 peptide IgG antibody titer was not increased (antibody titer non-increase group).

[Fig.4] Figure 4 is a graph depicting the relationship between the increase in the anti-WT1-235 peptide IgG antibody titer at 8 to 9 weeks after the start of administration of WT1₂₃₅ peptide vaccine and a progression-free survival rate in recurrent malignant glioma patients. Black squares show a group in which the anti-WT1-235 peptide IgG antibody

titer was increased (antibody titer increase group), and white squares show a group in which the anti-WT1-235 peptide IgG antibody titer was not increased (antibody titer non-increase group).

[Fig.5] Figure 5 is a graph depicting the relationship between the increase in the anti-WT1-235 peptide IgG antibody titer at 12 to 14 weeks after the start of administration of WT1₂₃₅ peptide vaccine and a progression-free survival rate in recurrent malignant glioma patients. Black squares show a group in which the anti-WT1-235 peptide IgG antibody titer was increased (antibody titer increase group), and white squares show a group in which the anti-WT1-235 peptide IgG antibody titer was not increased (antibody titer non-increase group).

[Fig.6] Figure 6 is a graph depicting the relationship between the increase in the anti-WT1-235 peptide IgG antibody titer at 8 to 9 weeks after the start of administration of WT1₂₃₅ peptide vaccine and a continuation rate of the WT1 peptide vaccine administration in recurrent malignant glioma patients. Black squares show a group in which the anti-WT1-235 peptide IgG antibody titer was increased (antibody titer increase group), and white squares show a group in which the anti-WT1-235 peptide IgG antibody titer was not increased (antibody titer non-increase group).

[Fig.7] Figure 7 is a graph depicting the relationship between the increase in the anti-WT1-235 peptide IgG antibody titer at 12 to 14 weeks after the start of administration of WT1₂₃₅ peptide vaccine and a continuation rate of the WT1 peptide vaccine administration in recurrent malignant glioma patients. Black squares show a group in which the anti-WT1-235 peptide IgG antibody titer was increased (antibody titer increase group), and white squares show a group in which the anti-WT1-235 peptide IgG antibody titer was not increased (antibody titer non-increase group).

[Fig.8] Figure 8 is a graph depicting the relationship between the type of anti-WT1-235 peptide IgG antibody at 12 to 14 weeks after the start of administration of WT1₂₃₅ peptide vaccine and an overall survival rate in recurrent malignant glioma patients. Black squares show Th1 type, and white squares show non-Th1 type.

[Fig.9] Figure 9 is a graph depicting the relationship between the type of anti-WT1-235 peptide IgG antibody at 12 to 14 weeks after the start of administration of WT1₂₃₅ peptide vaccine and a progression-free survival rate in recurrent malignant glioma patients. Black squares show Th1 type, and white squares show non-Th1 type.

[Fig.10] Figure 10 is a graph depicting the respective continuation rates of the WT1 peptide vaccine administration of Th1 type and non-Th1 type when the anti-WT1-235 peptide IgG antibody titer at 12 to 14 weeks after the start of administration of WT1₂₃₅ peptide vaccine was measured in recurrent malignant glioma patients. Black squares show Th1 type, and white squares show non-Th1 type.

[Fig.11] Figure 11 is a graph depicting the respective overall survival rates of IgG1 type/IgG3 type/IgG1 & IgG3 type when the antibody titer at 12 to 14 weeks after the start of administration of WT1₂₃₅ peptide vaccine was measured in recurrent malignant glioma patients. Black squares show IgG1 type, black circles show IgG3 type, and black triangles show IgG1 & IgG3 type.

[Fig.12] Figure 12 is a graph depicting the respective progression-free survival rates of IgG1 type/IgG3 type/IgG1 & IgG3 type when the antibody titer at 12 to 14 weeks after the start of administration of WT1₂₃₅ peptide vaccine was measured in recurrent malignant glioma patients. Black squares show IgG1 type, black circles show IgG3 type, and black triangles show IgG1 & IgG3 type.

[Fig.13] Figure 13 is a graph depicting the respective continuation rates of the WT1 peptide vaccine administration of IgG1 type/IgG3 type/IgG1 & IgG3 type when the antibody titer at 12 to 14 weeks after the start of administration of WT1₂₃₅ peptide vaccine was measured in recurrent malignant glioma patients. Black squares show IgG1 type, black circles show IgG3 type, and black triangles show IgG1 & IgG3 type.

[Fig.14] Figure 14 is a graph depicting the respective continuation rates of the WT1 peptide vaccine administration of anti-WT1-235 peptide IgG antibody titer increase group/non-increase group when the antibody titer at 4 to 8 weeks after the start of combined administration of WT1₂₃₅ peptide vaccine and WT1₃₃₂ (helper) peptide vaccine was measured in recurrent malignant glioma patients. Black squares show a group in which the anti-WT1-235 peptide IgG antibody titer was increased (antibody titer increase group), and white squares show a group in which the anti-WT1-235 peptide IgG antibody titer was not increased (antibody titer non-increase group).

[Fig.15] Figure 15 is a graph depicting the respective continuation rates of the WT1 peptide vaccine administration of anti-WT1-325 peptide IgG antibody titer increase group/non-increase group when the antibody titer at 4 to 8 weeks after the start of combined administration of WT1₂₃₅ peptide vaccine and WT1₃₃₂ (helper) peptide vaccine was measured in recurrent malignant glioma patients. Black squares show a group in which the anti-WT1-325 peptide IgG antibody titer was increased (antibody titer increase group), and white squares show a group in which the anti-WT1-325 peptide IgG antibody titer was not increased (antibody titer non-increase group).

[Fig.16] Figure 16 is a graph depicting the respective continuation rates of the WT1 peptide vaccine administration of anti-WT1-325 peptide IgG antibody titer increase group/non-increase group when the antibody titer at 12 to 14 weeks after the start of administration of WT1₃₃₂ (helper) peptide vaccine was measured in recurrent malignant glioma patients. Black squares show a group in which the anti-WT1-325 peptide IgG antibody titer was increased (antibody titer increase group), and white squares show a group in which the anti-WT1-325 peptide IgG antibody titer was not increased (antibody titer non-increase group).

[Fig.17] Figure 17 is a graph depicting the relationship between the increase in the anti-WT1-235 peptide IgG

antibody titer at 12 to 14 weeks after the start of administration of WT1₂₃₅ peptide vaccine and a continuation rate of the WT1 peptide vaccine administration in thymic carcinoma patients. Black squares show a group in which the anti-WT1-235 peptide IgG antibody titer was increased (antibody titer increase group), and white squares show a group in which the anti-WT1-235 peptide IgG antibody titer was not increased (antibody titer non-increase group).

[Fig.18] Figure 18 is a graph depicting the measured results of the anti-WT1₃₃₂ peptide IgG antibody titer by using variant WT1 antigen peptides in a recurrent malignant glioma patient given WT1₃₃₂ (helper) peptide vaccine. White squares show results when WT1-frg3 was used, black circles show results when WT325-342 was used, white triangles show results when WT332-347 was used, black squares show results when WT334-342 was used, and white circles show results when WT332-338 was used, as an antigen for ELISA.

[Fig.19] Figure 19 is a graph depicting the measured results of the anti-WT1₂₃₅ peptide IgG antibody titer by using variant WT1 antigen peptides in a thymic carcinoma patient given WT1₂₃₅ peptide vaccine. White squares show results when WT1-frg2 was used, black circles show results when WT235-252 was used, white triangles show results when WK235-243 was used, and black squares show results when WT237-243 was used, as an antigen for ELISA.

[Fig.20] Figure 20 is a graph depicting the measured results of the anti-WT1₁₂₆ peptide IgG antibody titer by using variant WT1 antigen peptides in a pancreatic cancer patient receiving a combination therapy with WT1₁₂₆ peptide vaccine and an anticancer agent. White squares show results when WT1-frg1 was used, black circles show results when WT118-135 was used, black triangles show results when WK126-134 was used, black squares show results when WT126-131 was used, white circles show results when WT129-134 was used, and white triangles show results when WT126-130 was used, as an antigen for ELISA.

Modes for Carrying Out the Invention

[0011] In one aspect, the present invention relates to a method for predicting a clinical effect on a subject in WT1 peptide immunotherapy comprising the following steps: a) contacting a sample from the subject with a WT1 antigen peptide or a variant thereof; and b) detecting the binding of the sample to the WT1 antigen peptide or the variant thereof, and thereby, measuring an antibody titer of anti-WT1 antigen peptide IgG present in the sample, wherein an increase in the anti-WT1 antigen peptide IgG antibody titer in the subject means the achievement of a favorable clinical effect.

[0012] In a further aspect, the present invention relates to the method for predicting the clinical effect on the subject in WT1 peptide immunotherapy comprising the steps a) and b), wherein the subclasses of the measured anti-WT1 antigen peptide IgG antibody are IgG1, IgG3 and IgG4, and wherein the achievement of a favorable clinical effect is determined when the titer of each of IgG1 and IgG3 is twice or more than twice the titer of IgG4. Preferably, the method is characterized in that the achievement of a favorable clinical effect is determined when the titer of IgG3 is less than twice the titer of IgG1 and the titer of IgG1 is less than twice the titer of IgG3.

[0013] In a preferable embodiment, the anti-WT1 antigen peptide IgG antibody titer at 8 to 14 weeks after the start of administration of a WT1 peptide vaccine is measured. In a more preferable embodiment, the anti-WT1 antigen peptide IgG antibody titer at 12 to 14 weeks after the start of administration of the WT1 peptide vaccine is measured.

[0014] In the present invention, the term "WT1 peptide vaccine" refers to a peptide derived from WT1 gene product (SEQ ID NO: 1) or a modified peptide thereof that is administered to a subject as vaccine in WT1 peptide immunotherapy. The WT1 peptide vaccine includes, for example, WT1₂₃₅ peptide vaccine (SEQ ID NO: 2), WT1-CTL peptide (modified mp235-243) vaccine (SEQ ID NO: 3) (Patent Document 1), WT1₁₂₆ peptide vaccine (SEQ ID NO: 4), WT1₁₈₇ peptide vaccine (SEQ ID NO: 5) and the like. Alternatively, the WT1 peptide vaccine may be WT1₃₃₂ helper peptide vaccine (SEQ ID NO: 6) (Non Patent Document 7). The WT1 peptide vaccine is not particularly limited, and a peptide which is well-known as the vaccine used in WT1 peptide immunotherapy or will be used in future may be used.

[0015] In the present invention, the terms "WT1 antigen peptide corresponding to WT1 peptide vaccine" and "WT1 antigen peptide" are used interchangeably. A WT1 antigen peptide is a peptide which comprises an amino acid sequence consisting of contiguous amino acids derived from an amino acid sequence of a WT1 peptide vaccine administered to a subject and can detect an antibody against the WT1 peptide vaccine. The contiguous amino acids are several contiguous amino acids, for example, 5, 6, 7, 8, 9 or more contiguous amino acids. As long as the WT1 antigen peptide in the present invention has the above feature, the amino acid sequence and length thereof are not particularly limited, and the length is, for example, 5-200 amino acids, 5-190 amino acids, 5-185 amino acids, 5-184 amino acids, 5-183 amino acids, 5-182 amino acids, 5-181 amino acids, 5-180 amino acids, 5-170 amino acids, 5-160 amino acids, 5-150 amino acids, 5-140 amino acids, 5-130 amino acids, 5-120 amino acids, 5-110 amino acids, 5-100 amino acids, 5-90 amino acids, 5-80 amino acids, 5-70 amino acids, 5-60 amino acids, 5-50 amino acids, 5-40 amino acids, 5-30 amino acids, 6-27 amino acids, or 7-24 amino acids. "WT1 antigen peptide" may be a protein or peptide comprising the same amino acid sequence as that of a WT1 peptide vaccine administered to a subject. Alternatively, the WT1 antigen peptide may be a peptide consisting of the same amino acid sequence as that of a WT1 peptide vaccine administered to a subject. Namely, WT1 antigen peptide may be a peptide per se consisting of the amino acid sequence of a WT1 peptide vaccine

administered to a subject, or may be a peptide comprising all or part of the amino acid sequence of the WT1 peptide vaccine.

[0016] In the present invention, the WT1 antigen peptide further includes a variant of a WT1 peptide vaccine administered to a subject. The variant may include a peptide having an amino acid sequence in which several amino acids, for example, 10, 9, 8, 7, 6, 5, preferably, 4, 3, more preferably, 2, even more preferably, 1 amino acid(s) are/is substituted and/or deleted, and/or 200, 190, 180, 170, 160, 150, 140, 130, 120, 110, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 9, 8, 7, 6, 5, preferably, 4, 3, more preferably, 2, even more preferably, 1 amino acid(s) are/is added in the amino acid sequence of the WT1 peptide vaccine. The variant is a protein or peptide having an amino acid sequence having 50% or more, preferably, 60% or more, further preferably, 70% or more, more preferably, 80% or more, even more preferably, 85% or more, particularly preferably, 90% or more (for example, 95, 96, 97, 98 or 99% or more) homology when Local Alignment is performed against the amino acid sequence of the WT1 peptide vaccine administered to the subject. The homology between amino acid sequences can be determined, for example, using FASTA, BLAST, DNASIS (Hitachi Software Engineering Co., Ltd.), or GENETYX (Genetyx Corporation). Alternatively, the homology can be calculated by simply comparing their amino acid sequences. The length of the amino acid sequence of the variant is, for example, but not particularly limited to, 5-200 amino acids, 5-190 amino acids, 5-185 amino acids, 5-184 amino acids, 5-183 amino acids, 5-182 amino acids, 5-181 amino acids, 5-180 amino acids, 5-170 amino acids, 5-160 amino acids, 5-150 amino acids, 5-140 amino acids, 5-130 amino acids, 5-120 amino acids, 5-110 amino acids, 5-100 amino acids, 5-90 amino acids, 5-80 amino acids, 5-70 amino acids, 5-60 amino acids, 5-50 amino acids, 5-40 amino acids, 5-30 amino acids, 6-27 amino acids, or 7-24 amino acids. In this specification, such a variant is also referred to as "variant WT1 antigen peptide".

[0017] Furthermore, in the present invention, a variant of a WT1 antigen peptide of the present invention may be used as the WT1 antigen peptide. Such a variant may comprise a peptide having an amino acid sequence in which, for example, several amino acids, for example, 10, 9, 8, 7, 6, 5, preferably, 4, 3, more preferably, 2, even more preferably, 1 amino acid(s) are/is substituted, deleted, and/or added in the amino acid sequence of the WT1 antigen peptide of the present invention.

[0018] In a preferred embodiment, the WT1 antigen peptide is a peptide comprising the amino acid sequence of any of SEQ ID NOs: 7-71, for example, a peptide consisting of the amino acid sequence of any of SEQ ID NOs: 7-71. In a more preferable embodiment, the WT1 antigen peptide is a peptide comprising the amino acid sequence of any of SEQ ID NOs: 20, 21, 27, 31, 32, 42, 43, or 57-71, for example, a peptide consisting of the amino acid sequence of any of SEQ ID NOs: 20, 21, 27, 31, 32, 42, 43, or 57-71. In an even more preferable embodiment, the WT1 antigen peptide is a peptide comprising the amino acid sequence of any of SEQ ID NOs: 20, 32, 42, or 57-71, for example, a peptide consisting of the amino acid sequence of any of SEQ ID NOs: 20, 32, 42, or 57-71. In the present invention, any amino acid of amino acids composing the WT1 antigen peptide may be appropriately modified. The modification of an amino acid residue can be performed by a known method.

[0019] In this specification, "anti-WT1 antigen peptide IgG antibody titer was increased" means a case where (1) an anti-WT1 antigen peptide IgG antibody titer after the start of administration of a WT1 vaccine became not less than a predetermined value, or (2) an anti-WT1 antigen peptide IgG antibody titer after the start of administration of a WT1 vaccine was increased to a predetermined value or higher, compared with a titer before the administration of the WT1 vaccine. This value in the present invention varies depending on conditions such as a measuring object (the number of subject, age, sexuality, body weight, condition, etc.) and a method for measurement, measurement conditions, and a statistical method, and therefore, the value needs to be predetermined. Such a value can be empirically determined based on accumulated data. Accordingly, those skilled in the art in the field to which the present invention pertains will appreciate that a particular value can be selected based on a desired specificity and sensitivity, a type of sample used, and a preparation method of the sample, and other factors described herein by performing a routine experiment. For example, such a particular value can be determined by measuring an antibody titer of anti-WT1 peptide IgG using a sample of which antibody titer of anti-WT1 peptide IgG is considered negative, and referring to the average value plus twice the standard deviation calculated based on the result. Those skilled in the art will also appreciate that the measurement of the anti-WT1 antigen peptide antibody using the above-mentioned WT1 antigen peptide means the measurement of an antibody against the WT1 peptide vaccine to which the WT1 antigen peptide corresponds.

[0020] In the method of the present invention, a body fluid, which is a sample derived from a subject and is generally known that an antibody exists therein, may be used as a sample. Preferably, the sample is blood sample, plasma sample, serum sample, or urine sample. More preferably, the sample is serum sample. The sample may be prepared to a condition suitable for using in the method of the present invention, for example, by using a buffer.

[0021] In one aspect of the present invention, a subject is a patient with WT1-associated disease. In a preferred embodiment, a subject is a patient with leukemia such as chronic myeloid leukemia, hematopoietic organ tumor such as myelodysplastic syndrome, multiple myeloma, and malignant lymphoma, or solid cancer such as esophageal cancer, stomach cancer, colon cancer, pancreatic cancer, lung cancer, breast cancer, germ cell cancer, liver cancer, biliary tract cancer, head and neck cancer, skin cancer, sarcoma, kidney cancer, bladder cancer, prostate cancer, testicular cancer,

uterine cancer, cervical cancer, ovarian cancer, thyroid cancer, carcinoid, pneumoblastoma, hepatoblastoma, brain tumor and thymic carcinoma. In a more preferred embodiment, a subject is a patient with recurrent malignant glioblastoma (GBM), thymic carcinoma, or pancreatic cancer.

5 [0022] The measurement of an antibody titer in the method of the present invention can be performed by using various methods that are commonly used in an antibody measurement technique. Such methods include an immunoassay. Concrete examples of the immunoassay include ELISA, radioimmunoassay (RIA), etc.

10 [0023] For example, ELISA is performed as follows. First, a WT1 antigen peptide capable of specifically antigen-antibody reacting with anti-WT1 antigen peptide antibody to be measured is immobilized. A sample is added thereto. Thereby, the antigen-antibody reaction occurs between the immobilized WT1 antigen peptide and an antibody in the sample, and an anti-WT1 antigen peptide antibody present in the sample binds to the immobilized WT1 antigen peptide. Next, the bound anti-WT1 antigen peptide antibody is detected by using an antibody detection reagent to measure an amount of the antibody present in the sample.

15 [0024] Alternatively, an antibody detection reagent is immobilized, and thereby, capturing the antibodies in the sample. Then, a WT1 antigen peptide is added thereto to bind the peptide with an anti-WT1 antigen peptide antibody in the captured antibodies. Furthermore, a labeled antibody specific to the antigen is bound thereto. This can detect and assay the objective anti-WT1 antigen peptide antibody present in the sample.

20 [0025] Selections, modifications, or the like of each process in these measuring methods are well-known to those skilled in the art. In the present invention, various methods may be employed without particularly being limited to the above-mentioned methods (see, for example, "Rinsho Kensa-ho Teiyo (Kanai's manual of clinical laboratory medicine)", the revised 33th edition, KANEHARA & Co., LTD., 2010).

25 [0026] An antibody detection reagent for detecting an anti-WT1 antigen peptide antibody is not particularly limited, and various reagents that are generally used may be used. For example, a preparation comprising an anti-human IgG antibody, an anti-human IgG1 antibody, an anti-human IgG3 antibody, and/or an anti-human IgG4 antibody, which specifically binds to human IgG, IgG1, IgG3, and/or IgG4 to be measured, may be used. These are commercially available, or may be prepared. Methods for preparing such an antibody detection reagent are well-known to those skilled in the art. In a preferable embodiment, the antibody detection reagent used in the present invention is a labeled secondary antibody. In a more preferable embodiment, the antibody detection reagent used in the present invention is a combination of a labeled secondary antibody and a labeled tertiary antibody which detects the secondary antibody.

30 [0027] In the method of the present invention, a WT1 antigen peptide or a variant thereof may be chemically synthesized, for example, according to a solid phase method or a liquid phase method. When a solid phase method is used, a peptide is synthesized on a solid phase with the operations of activating an N-terminally protected amino acid, coupling, washing, deblocking, activating being repeated until the desired peptide is finished. Said product is removed from the solid phase, purified by HPLC or the like. Subsequently said product is transferred to further investigations such as sequence verification and biological tests.

35 [0028] Alternatively, the WT1 antigen peptide or the variant thereof may be synthesized by cell-free translation system. Alternatively, it may be produced by genetic engineering based on the nucleotide sequence encoding the WT1 antigen peptide. Alternatively, it may also be obtained by combining these methods. The production of a WT1 antigen peptide by a genetic engineering technique may be performed according to usual gene recombination technology. More specifically, a recombinant DNA capable of expressing a desired gene encoding the WT1 antigen peptide in a host cell is prepared, the recombinant DNA is introduced into host cells for transformation, and the transformant is cultured. The transformant can produce a desired polypeptide intracellularly or extracellularly as an expression product of the transformant.

40 [0029] Each operation employed here, for example, chemical synthesis of gene fragments, enzyme treatment for cleavage, deletion, addition, or bonding thereof, isolation, purification, selection, and the like, introduction of a recombinant DNA into a host cell, and culture of the transformant are well-known to those skilled in the art (see, for example, Molecular Cloning, by T. Maniatis et al., Cold Spring Harbor Laboratory (1982)).

45 [0030] If desired, the WT1 antigen peptide or the variant thereof may also be isolated and purified from the above-mentioned expression product by various separation procedures utilizing the physical and chemical properties of the polypeptide.

50 [0031] Furthermore, in the method of the present invention, known methods, means, measuring reagents used therein or the like may be suitably utilized for measuring an amount of an anti-WT1 antigen peptide antibody in a sample.

55 [0032] For example, when a solid phase method is used in the above-mentioned measuring method, an antigen or an antibody for the measurement system is immobilized on a solid phase according to the method well-known to those skilled in the art. As the solid phase, insoluble inactive carrier that is usually used may be widely used. Examples of the carrier include sticks, beads, microplates, and test tubes made of various materials such as glass, cellulose powder, sephadex, sepharose, polystyrene, filter paper, carboxymethyl cellulose, ion exchange resins, dextran, plastic films, plastic tubes, nylon, glass beads, silk, polyamine-methyl vinyl ether-maleic acid copolymers, amino acid copolymers, and ethylene-maleic acid copolymers.

[0033] The immobilization of the antigen or the antibody is not particularly limited, and both physical bonding and chemical bonding may be used. Typical examples of the immobilization include methods using a chemical reaction as covalent bonding methods, for example, diazo methods, peptide methods (such as acid amide derivative method, carboxyl chloride resin method, carbodiimide resin method, maleic anhydride derivative method, isocyanate derivative method, bromocyan activated polysaccharide method, cellulose carbonate derivative method, condensing reagent method), alkylation method, crosslinking reagent coupling method (using, for example, glutaraldehyde or hexamethylene isocyanate as the crosslinking reagent), and Ugi reaction coupling method; ionic binding methods using supports such as ion exchange resins; and physical adsorption methods using porous glass supports such as glass beads.

[0034] The labeling reagent in each measurement system is not particularly limited, and any well-known reagent or any reagent expected to come into use in future can be used. Specific examples thereof include, but not particularly limited to, radioisotopes commonly used in immunoassay methods; enzymes such as alkaline phosphatase (ALP) and peroxidase (PDX); fluorescent substances such as fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (RITC); and 1N-(2,2,6,6-tetramethyl-1-oxyl-4-piperidyl)-5N-(aspartate)-2,4-dinitrobenzene (TOPA). In addition, microperoxidase, chymotrypsinogen, procarboxypeptidase, glyceroaldehyde-3-phosphate dehydrogenase, amylase, phosphorylase, D-Nase, P-Nase, or the like may be used. Labeling method using these labeling materials may be performed according to a known method.

[0035] The enzyme activity can be measured according to a known method depending on the type of the enzyme used. For example, in a case of using peroxidase as a labeling enzyme, ABTSJ (2,2'-azino-bis(3'-ethylbenzthiazoline sulfonic acid) is used as the substrate; in a case of using alkaline phosphatase, p-nitrophenyl phosphate is used as the substrate, and the decomposition of each substrate is measured with, for example, a spectrophotometer.

[0036] When a radioisotope, fluorescent material or the like is used instead of the above-mentioned enzyme label as a label, the label can also be measured according to a known method.

[0037] In the measurement system, any solvent that is usually used and does not adversely affect the reaction can be used. Specifically, a buffer solution having a pH of about 5 to 9, such as a citrate buffer solution, a phosphate buffer solution, a Tris-hydrochloric acid buffer solution, or an acetate buffer solution can be preferably used.

[0038] Immune reaction (binding) conditions are not particularly limited, and usual conditions that are generally used in these assays are employed. In general, a reaction may be performed at a temperature of 45° C or less, preferably about 4 to 40° C, for about 1 to 40 hours.

[0039] As a main characteristic of the method of the present invention, an anti-WT1 antigen peptide IgG antibody titer in a sample that is measured as described above is used as a clinical index for predicting a clinical effect in WT1 peptide immunotherapy.

[0040] In particular, the present inventors found that an anti-WT1 peptide IgG antibody was produced against the administered WT1 peptide in a subject receiving WT1 peptide immunotherapy and an anti-WT1 peptide IgG antibody titer at a certain point of time was correlated with a clinical effect. Namely, the present inventors found that an anti-WT1 antigen peptide IgG antibody titer, which was measured by using a WT1 antigen peptide corresponding to an administered WT1 peptide, or a variant thereof, could be an effective index for predicting a clinical effect in WT1 peptide immunotherapy. In addition, the present inventors analyzed the subclasses of the produced anti-WT1 peptide IgG antibody and found that a clinical effect in WT1 peptide immunotherapy was better in Th1 type than non-Th1 type. Furthermore, the present inventors analyzed Th1 type in more detail and found that a clinical effect in WT1 peptide immunotherapy was better in IgG1 & IgG3 type than IgG1 type and IgG3 type.

[0041] The term "Th1 type" refers to one in which IgG1 antibody or IgG3 antibody, which is Th1 type subclass, is twice or more than twice IgG4 antibody, which is Th2-type subclass. Namely, "Th1 type" satisfies the following formula (I) and formula (II).

- (I) Anti-WT1 antigen peptide IgG1 antibody titer / Anti-WT1 antigen peptide IgG4 antibody titer ≥ 2.0
 (II) Anti-WT1 antigen peptide IgG3 antibody titer / Anti-WT1 antigen peptide IgG4 antibody titer ≥ 2.0

[0042] The term "non-Th1 type" refers to one in which both the above formula (I) and formula (II) are not satisfied.

[0043] The term "IgG1 type" refers to one in which IgG1 antibody is twice or more than twice IgG3 antibody, among Th1 type. Namely, "IgG1 type" among Th1 type satisfies the following formula (III).

- (III) Anti-WT1 antigen peptide IgG1 antibody titer / Anti-WT1 antigen peptide IgG3 antibody titer ≥ 2.0

[0044] The term "IgG3 type" refers to one in which IgG3 antibody is twice or more than twice IgG1 antibody, among Th1 type. Namely, "IgG3 type" among Th1 type satisfies the following formula (IV).

- (IV) Anti-WT1 antigen peptide IgG3 antibody titer / Anti-WT1 antigen peptide IgG1 antibody titer ≥ 2.0

[0045] The term "IgG1 & IgG3 type" refers to one in which both the above formula (III) and formula (IV) are not satisfied, among Th1 type.

[0046] In a further aspect, the present invention provides a kit for performing the method of the present invention, comprising a WT1 antigen peptide. Such a kit comprises a WT1 antigen peptide as an active ingredient, and the WT1 antigen peptide generates antigen-antibody reaction with anti-WT1 antigen peptide IgG antibody to be measured. Such a kit may also comprise any reagent such as an antibody detection reagent used in the measurement system in the method of the present invention. The kit may also comprise appropriate reagents for easily carrying out the measurement, for example, an antibody diluent, a reaction diluent, a buffer, a washing agent, and a reagent for detecting a label. Furthermore, the kit may also comprise materials such as instructions necessary for performing the method of the present invention. The kit measures antibody titer of IgG, preferably based on an immunoassay. More preferably, the kit measures antibody titer of IgG based on ELISA. The variant of the WT1 antigen peptide described above may be used for such a kit. In addition, a peptide in which any amino acid of amino acids composing the WT1 antigen peptide or the variant is appropriately modified may be used. The modification of an amino acid residue can be performed by a known method in the art.

[0047] The present invention will be described in detail and specifically by way of examples, but they should not be construed as limiting the present invention.

Example 1

Synthesis of WT1 antigen peptides

[0048] WT1 peptides shown in Tables 1-1 and 1-2 were synthesized in Kabushiki Kaisha PHJapan. In Tables 1-1 and 1-2, "starting site" and "terminating site" show the positions of the corresponding amino acid residues in the amino acid sequence of wild-type human WT1 protein (SEQ ID NO: 1).

[Table 1-1]

| Antigen peptide | Starting site (aa) | Terminating site (aa) | Amino acid sequence | SEQ ID NO: |
|-----------------|--------------------|-----------------------|---------------------|------------|
| WT1-01 | 1 | 18 | MGSDVRDLNALLPAVPSL | 7 |
| WT1-10 | 10 | 27 | ALLPAVPSLGGGGGALP | 8 |
| WT1-19 | 19 | 36 | GGGGGALPVSGAAQWAP | 9 |
| WT1-28 | 28 | 45 | VSGAAQWAPVLDFAAPGA | 10 |
| WT1-37 | 37 | 54 | VLDFAAPGASAYGSLGGP | 11 |
| WT1-46 | 46 | 63 | SAYGSLGGPAPPPAPPPP | 12 |
| WT1-55 | 55 | 72 | APPPAPPPPPPPPHSFI | 13 |
| WT1-64 | 64 | 81 | PPPPPHSFIKQEPSWGGA | 14 |
| WT1-73 | 73 | 90 | KQEPSWGGAEPHEEQCLS | 15 |
| WT1-82 | 82 | 99 | EPHEEQCLSAFTVHFSGQ | 16 |
| WT1-91 | 91 | 108 | AFTVHFSGQFTGTAGACR | 17 |
| WT1-100 | 100 | 117 | FTGTAGACRYGPFPPPPP | 18 |
| WT1-109 | 109 | 126 | YGPFPPPPSQASSGQAR | 19 |
| WT1-118 | 118 | 135 | SQASSGQARMFPNAPYLP | 20 |
| WT1-127 | 127 | 144 | MFPNAPYLPSCLESQPAI | 21 |
| WT1-136 | 136 | 153 | SCLESQPAIRNQGYSTVT | 22 |
| WT1-145 | 145 | 162 | RNQGYSTVTFDGTPSYGH | 23 |
| WT1-154 | 154 | 171 | FDGTPSYGHTPSHHAAQF | 24 |
| WT1-163 | 163 | 180 | TPSHHAAQFPNHSFKHED | 25 |
| WT1-172 | 172 | 189 | PNHSFKHEDPMGQQGSLG | 26 |
| WT1-181 | 181 | 198 | PMGQQGSLGEQQYSVPPP | 27 |
| WT1-199 | 199 | 216 | VYGCHTPTDCTGSQALL | 28 |
| WT1-208 | 208 | 225 | SCTGSQALLLRTPYSSDN | 29 |
| WT1-217 | 217 | 234 | LRTPYSSDNLYQMTSQLE | 30 |

[Table 1-2]

| | Antigen peptide | Starting site (aa) | Terminating site (aa) | Amino acid sequence | SEQ ID NO: |
|----|-----------------|--------------------|-----------------------|---------------------|------------|
| 5 | WT1-226 | 226 | 243 | LYQMTSQLECMTWNQMNL | 31 |
| | WT1-235 | 235 | 252 | CMTWNQMNLGATLKGVA | 32 |
| | WT1-244 | 244 | 261 | GATLKGVAAGSSSSVKWT | 33 |
| | WT1-253 | 253 | 270 | GSSSSVKWTEGQSNHSTG | 34 |
| | WT1-262 | 262 | 279 | EGQSNHSTGYESDNHSTP | 35 |
| 10 | WT1-271 | 271 | 288 | YESDNHSTPILCGAQYRI | 36 |
| | WT1-280 | 280 | 297 | ILCGAQYRIHTHGVRGI | 37 |
| | WT1-289 | 289 | 306 | HTHGVRGIQDVRVPGV | 38 |
| | WT1-298 | 298 | 315 | QDVRVPGVAPTLVRSAS | 39 |
| | WT1-307 | 307 | 324 | APTLVRSASETSEKRPFM | 40 |
| 15 | WT1-316 | 316 | 333 | ETSEKRPFMCAYPGCNKR | 41 |
| | WT1-325 | 325 | 342 | CAYPGCNKRYFKLSHLQM | 42 |
| | WT1-334 | 334 | 351 | YFKLSHLQMHSRKHTGEK | 43 |
| | WT1-343 | 343 | 360 | HSRKHTGEKPYQCDFKDC | 44 |
| 20 | WT1-352 | 352 | 369 | PYQCDFKDCERRFSRSDQ | 45 |
| | WT1-361 | 361 | 378 | ERRFSRSDQLKRHQRRT | 46 |
| | WT1-370 | 370 | 387 | LKRHQRRTGVKPFQCKT | 47 |
| | WT1-379 | 379 | 396 | GVKPFQCKTCQRKFSRSD | 48 |
| | WT1-388 | 388 | 405 | CQRKFSRSDHLKTHTRTH | 49 |
| 25 | WT1-397 | 397 | 414 | HLKTHTRTHGKTSEKPF | 50 |
| | WT1-406 | 406 | 423 | TGKTSEKPFSCRWPSCQK | 51 |
| | WT1-415 | 415 | 432 | SCRWPSCQKFFARSDELV | 52 |
| | WT1-424 | 424 | 441 | KFARSDELVRHHNMHQRN | 53 |
| | WT1-433 | 433 | 449 | RHHNMHQRNMTKLQAL | 54 |
| 30 | WT1-E5min | 241 | | MNLGATLKGHSTGYESDN | 55 |
| | WT1-KTSmin | 399 | | KTHTRTHGKPFSCRWP | 56 |

Example 2

35 Anti-WT1 antigen peptide antibody titer after start of administration of WT1 peptide vaccine and clinical effects in recurrent malignant glioblastoma (GBM) patients

40 **[0049]** The present inventors conducted the following study to confirm the relationship between an anti-WT1 antigen peptide IgG antibody titer after the start of administration of a WT1 peptide vaccine and clinical effects regarding GBM patients.

1. Materials and methods

45 **[0050]** 1-1 WT1 peptide immunotherapy was carried out in 72 GBM patients using WT1₂₃₅ peptide (WT1-CTL peptide (modified mp235-243) (SEQ ID NO: 3)) as a WT1 peptide vaccine. 3 mg of WT1₂₃₅ peptide (WT1-CTL peptide (modified mp235-243)) was mixed with an incomplete adjuvant Montanide ISA51 at a weight ratio of 1:1 to prepare an emulsion. This emulsion was administered to each patient once a week for 12 weeks by intracutaneous administration. Blood was collected from the patient before the start of administration of the vaccine and at given times after the start of administration and centrifuged to obtain serum. The obtained serum was cryopreserved at -80°C or lower and thawed for use at the time of assay. When the effects were observed, the administration of the WT1 peptide vaccine was continued at 2- to 50 4-week intervals over 12 weeks.

1-2 Assay of anti-WT1-235 peptide IgG antibody (anti-WT1₂₃₅ peptide IgG antibody) (ELISA)

55 **[0051]** To a 96-well reaction plate supplied with Peptide Coating Kit (Takara Bio Inc.) having amino groups attached to the bottom surface of the wells, a solution (4 µg/mL) of the WT1-235 peptide dissolved in a reaction buffer supplied with Peptide Coating Kit was added at 50 µL/well. A coupling reagent was added thereto at 30 µL/well and reacted at

room temperature for 2 hours. The wells were washed with distilled water to immobilize the antigen thereon. Blocking was performed by shaking at room temperature for 2 hours using Blocking One (Nacalai Tesque, Inc.). Subsequently, the wells were washed with 0.05% TBST (40 mM Tris, 0.15 M sodium chloride, and 0.05% Tween-20, pH 8.0). Then, the serum diluted 100-fold with a blocking solution supplied with Peptide Coating Kit was added thereto at 100 μ L/well and reacted overnight at 4°C. After washing with 0.05% TBST, the plate was reacted with a secondary antibody at room temperature for 2 hours. The secondary antibody used was a peroxidase-labeled rabbit anti-human IgG antibody (sc-2769, Santa Cruz Biotechnology, Inc., 400 μ g/mL) diluted 1000-fold with a blocking solution supplied with Peptide Coating Kit. Subsequently, the wells were washed with 0.05% TBST. Then, the plate was reacted with a tertiary antibody at room temperature for 2 hours. The tertiary antibody used was a peroxidase-labeled goat anti-rabbit IgG antibody (sc-2004, Santa Cruz Biotechnology, Inc., 400 μ g/mL) diluted 1000-fold with 0.05% TBST. After washing with 0.05% TBST, a color was developed using a TMB kit (KPL, Kirkegaard & Perry Laboratories, Inc.). The reaction was terminated with 1 N HCl, and the absorbance at 450 nm was then measured using a microplate reader (CORONA ELECTRIC MTP-310Lab).

[0052] The present inventors further analyzed the IgG subclass of an anti-WT1 antigen peptide IgG antibody by the following method to confirm whether the induction of WT1-specific immune response detected with increase in anti-WT1 antigen peptide IgG antibody level was of Th1 type or of non-Th1 type.

1-3 Assay of IgG1, IgG3, and IgG4 against WT1-235 peptide (ELISA)

[0053] ELISA was conducted in the same way as in the preceding paragraph 1-2. Each secondary antibody used was a peroxidase-labeled mouse anti-human IgG1 (#9052-05 mouse mAb clone 4E3, Southern Biotech), a peroxidase-labeled mouse anti-human IgG3 (#9210-05 mouse mAb clone HP6050, Southern Biotech), or a peroxidase-labeled mouse anti-human IgG4 antibody (#9190-05 mouse mAb clone HP6023, Southern Biotech). For use, the peroxidase-labeled mouse anti-human IgG1 and the peroxidase-labeled mouse anti-human IgG4 were each diluted 2000-fold with a blocking solution supplied with Peptide Coating Kit, and the peroxidase-labeled mouse anti-human IgG3 was diluted 1000-fold with a blocking solution supplied with Peptide Coating Kit. The tertiary antibody used was a peroxidase-labeled goat anti-mouse IgG antibody (Promega K.K., W4028, 1 mg/mL) diluted 2500-fold with 0.05% TBST.

2. Statistical analysis

[0054] The comparison between two groups was conducted using the Mann-Whitney test. As for the difference in the rate of increase in anti-WT1 antigen peptide antibody level between a responder group and a nonresponder group, the relationship between two categorical variables was tested by using the Fisher's exact calculation method. Also, the comparison of a continuation rate of the WT1 peptide vaccine administration, a progression-free survival rate, and an overall survival rate was conducted using the log rank test. The anti-WT1 antigen peptide antibody titer was measured using the serum negative for the anti-WT1 antigen peptide antibody (patient's serum before the start of WT1 peptide immunotherapy) and the average value plus twice the standard deviation = 0.045. Thus, (1) when the anti-WT1 antigen peptide IgG antibody titer was 0.05 or higher after the start of administration of the WT1 vaccine or (2) when the anti-WT1 antigen peptide IgG antibody titer increased by 0.05 or higher after the start of administration of the WT1 vaccine as compared with before the start of administration of the WT1 vaccine, this event was defined by the phrase "anti-WT1 antigen peptide antibody titer was increased".

3. Results

(I) Increase in anti-WT1-235 peptide IgG antibody titer (anti-WT1₂₃₅ peptide IgG antibody titer) and clinical effects

(i) Change in anti-WT1-235 peptide IgG antibody titer (Figure 1)

[0055] Figure 1 shows a graph depicting the anti-WT1-235 peptide IgG antibody titer after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)). The anti-WT1-235 peptide IgG antibody titer was increased with the passage of days (progression of treatment) after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)) as compared with before the start of administration thereof. The antibody titer was significantly increased 2 months and 3 months after the start of administration as compared with before the start of administration.

(ii) Increase in anti-WT1-235 peptide IgG antibody titer and overall survival rate (Figures 2 and 3)

[0056] Figures 2 and 3 each show a graph depicting the relationship between increase in the anti-WT1-235 peptide

IgG antibody titer after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)) and an overall survival rate. During the observation period, the antibody titer increase group exhibited a significantly higher overall survival rate than that of the antibody titer non-increase group both in the case of using the antibody titer at 8 to 9 weeks after the start of administration as an index (Figure 2) and in the case of using the antibody titer at 12 to 14 weeks after the start of administration as an index (Figure 3). Moreover, the case of using the antibody titer at 12 to 14 weeks after the start of administration as an index offered more distinct difference between the antibody titer increase group and the antibody titer non-increase group than that offered by the case of using the antibody titer at 8 to 9 weeks after the start of administration as an index.

(iii) Increase in anti-WT1-235 peptide IgG antibody titer and progression-free survival rate (Figures 4 and 5)

[0057] Figures 4 and 5 each show a graph depicting the relationship between increase in the anti-WT1-235 peptide IgG antibody titer after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)) and a progression-free survival rate. During the observation period, the antibody titer increase group tended to exhibit a higher progression-free survival rate than that of the antibody titer non-increase group in the case of using the antibody titer at 8 to 9 weeks after the start of administration as an index (Figure 4). The antibody titer increase group exhibited a significantly higher progression-free survival rate than that of the antibody titer non-increase group in the case of using the antibody titer at 12 to 14 weeks after the start of administration as an index (Figure 5).

(iv) Increase in anti-WT1-235 peptide IgG antibody titer and continuation rate of WT1 peptide vaccine administration (Figures 6 and 7)

[0058] Figures 6 and 7 each show a graph depicting the relationship between increase in the anti-WT1-235 peptide IgG antibody titer after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)) and a continuation rate of the WT1 peptide vaccine administration. During the observation period, the antibody titer increase group tended to exhibit a higher continuation rate of the vaccine administration than that of the antibody titer non-increase group in the case of using the antibody titer at 8 to 9 weeks after the start of administration as an index (Figure 6). The antibody titer increase group exhibited a significantly higher continuation rate of the vaccine administration than that of the antibody titer non-increase group in the case of using the antibody titer at 12 to 14 weeks after the start of administration as an index (Figure 7).

(II) Type (Th1 type/non-Th1 type) of anti-WT1-235 peptide IgG antibody (anti-WT1₂₃₅ peptide IgG antibody) and clinical effects

(i) Th1 type/non-Th1 type and overall survival rate (Figure 8)

[0059] Figure 8 shows the respective overall survival rates of Th1 type and non-Th1 type in the case of using, as an index, the anti-WT1-235 peptide IgG antibody titer at 12 to 14 weeks after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)). During the observation period, the Th1 type tended to exhibit a higher overall survival rate than that of the non-Th1 type.

(ii) Th1 type/non-Th1 type and progression-free survival rate (Figure 9)

[0060] Figure 9 shows the respective progression-free survival rates of Th1 type and non-Th1 type in the case of using, as an index, the anti-WT1-235 peptide IgG antibody titer at 12 to 14 weeks after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)). During the observation period, the Th1 type tended to exhibit a higher progression-free survival rate than that of the non-Th1 type.

(iii) Th1 type/non-Th1 type and continuation rate of WT1 peptide vaccine administration (Figure 10)

[0061] Figure 10 shows the respective continuation rates of the WT1 peptide vaccine administration of Th1 type and non-Th1 type in the case of using, as an index, the anti-WT1-235 peptide IgG antibody titer at 12 to 14 weeks after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)). During the observation period, the Th1 type exhibited a significantly higher continuation rate of the vaccine administration than that of the non-Th1 type (Figure 10).

(III) Analysis of IgG subclass

(i) Increase in anti-WT1-235 peptide IgG subclass antibody titer and overall survival rate (Figure 11)

5 **[0062]** Figure 11 shows the respective overall survival rates of IgG1 type, IgG3 type, and IgG1 & IgG3 type in the case of using, as an index, the antibody titer at 12 to 14 weeks after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)). During the observation period, the IgG1 & IgG3 type exhibited a significantly higher overall survival rate.

10 (ii) Increase in anti-WT1-235 peptide IgG subclass antibody titer and progression-free survival rate (Figure 12)

[0063] Figure 12 shows the respective progression-free survival rates of IgG1 type, IgG3 type, and IgG1 & IgG3 type in the case of using, as an index, the antibody titer at 12 to 14 weeks after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)). During the observation period, the IgG1 & IgG3 type tended to exhibit a higher progression-free survival rate.

(iii) Increase in anti-WT1-235 peptide IgG subclass antibody titer and continuation rate of WT1 peptide vaccine administration (Figure 13)

20 **[0064]** Figure 13 shows the respective continuation rates of the WT1 peptide vaccine administration of IgG1 type, IgG3 type, and IgG1 & IgG3 type in the case of using, as an index, the antibody titer at 12 to 14 weeks after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)). During the observation period, the IgG1 & IgG3 type exhibited a significantly higher continuation rate of the WT1 peptide vaccine administration.

25 Example 3

Anti-WT1 antigen peptide antibody titer after start of combined administration of WT1 peptide vaccine and WT1 helper peptide vaccine and clinical effects in recurrent malignant glioblastoma (GBM) patients

30 **[0065]** The present inventors conducted the following study to confirm the relationship between an anti-WT1 antigen peptide IgG antibody titer after the start of combined administration of a WT1 peptide vaccine and a helper peptide vaccine and clinical effects regarding GBM patients.

1. Materials and methods

35 **[0066]** 1-1 WT1 peptide immunotherapy was carried out in 15 GBM patients using WT1-CTL peptide (modified mp235-243) (SEQ ID NO: 3) as a WT1 peptide vaccine and WT1₃₃₂ peptide (SEQ ID NO: 6) as a helper peptide vaccine. The WT1-CTL peptide (modified mp235-243) was administered at 3 mg/body to each patient a total of 5 times once a week by intracutaneous administration. The WT1₃₃₂ helper peptide was applied to the dose escalation of 0.75 mg (n = 7), 1.5 mg (n = 4), or 3 mg (n = 4) and administered to each patient a total of 3 times once every two weeks by intracutaneous administration. The administration of the WT1 helper peptide vaccine was performed as a mixture with the WT1-CTL peptide. Blood was collected from the patient before the start of administration of the vaccine and at given times after the start of administration and centrifuged to obtain serum. The obtained serum was cryopreserved at -80°C or lower and thawed for use at the time of assay. When the effects were observed, the administration of the WT1-CTL peptide (modified mp235-243) alone and the combined administration of the WT1-CTL peptide (modified mp235-243) and the WT1₃₃₂ helper peptide were subsequently repeated alternately at 2- to 4-week intervals.

1-2 Assay of anti-WT1-235 peptide IgG antibody (anti-WT1₂₃₅ peptide IgG antibody) and anti-WT1-325 peptide IgG antibody (anti-WT1₃₃₂ peptide IgG antibody) (ELISA)

50 **[0067]** The antibody titer was measured in the same way as in Example 2. For assaying the anti-WT1-235 peptide IgG antibody, a WT1-235 peptide was used as a WT1 antigen peptide. For assaying the anti-WT1-325 peptide IgG antibody, a WT1-325 peptide was used as a WT1 antigen peptide. The WT1-325 peptide is a peptide containing a portion (the 1st to 11th amino acids) of the administered WT1₃₃₂ peptide. Thus, the assay of the anti-WT1-325 peptide IgG antibody means the assay of an antibody against the administered WT1₃₃₂ peptide (anti-WT1₃₃₂ peptide IgG antibody).

2. Statistical analysis

[0068] The statistical analysis was conducted in the same way as in Example 2. This analysis was conducted for 11 cases excluding clinical trial dropout cases (3 cases in the 0.75 mg administration group and 1 case in the 3 mg administration group). As in Example 2, (1) when the anti-WT1 antigen peptide IgG antibody titer was 0.05 or higher after the start of administration of the WT1 vaccine or (2) when the anti-WT1 antigen peptide IgG antibody titer increased by 0.05 or higher after the start of administration of the WT1 vaccine as compared with before the start of administration of the WT1 vaccine, this event was defined by the phrase "anti-WT1 antigen peptide antibody titer was increased".

3. Results

(I) Increase in anti-WT1-235 peptide IgG antibody titer (anti-WT1₂₃₅ peptide IgG antibody titer) and clinical effects

(i) Increase in anti-WT1-235 peptide IgG antibody titer and continuation rate of WT1 peptide vaccine administration (Figure 14)

[0069] Figure 14 shows the respective continuation rates of the WT1 peptide vaccine administration of the anti-WT1-235 peptide IgG antibody titer increase group and the non-increase group in the case of using, as an index, the antibody titer at 4 to 8 weeks after the start of combined administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)) and the WT1₃₃₂ (helper) peptide vaccine. During the observation period, the antibody titer increase group tended to exhibit a higher continuation rate of the WT1 peptide vaccine administration than that of the non-increase group.

(II) Increase in anti-WT1-325 peptide IgG antibody titer (anti-WT1₃₃₂ peptide IgG antibody titer) and clinical effects

(i) Increase in anti-WT1-325 peptide IgG antibody titer and continuation rate of WT1 peptide vaccine administration (Figure 15)

[0070] Figure 15 shows the respective continuation rates of the WT1 peptide vaccine administration of the anti-WT1-325 peptide IgG antibody titer increase group and the non-increase group in the case of using, as an index, the antibody titer at 4 to 8 weeks after the start of combined administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)) and the WT1₃₃₂ (helper) peptide vaccine. During the observation period, the increase group tended to exhibit a higher continuation rate of the WT1 peptide vaccine administration than that of the non-increase group.

Example 4

Anti-WT1 antigen peptide antibody titer after start of administration of WT1 helper peptide vaccine and clinical effects in recurrent malignant glioblastoma (GBM) patients

[0071] The present inventors further conducted the following study to confirm the relationship between an anti-WT1 antigen peptide IgG antibody titer after the start of administration of a WT1 helper peptide vaccine and clinical effects regarding GBM patients.

1. Materials and methods

[0072] 1-1 WT1 peptide immunotherapy was carried out in 14 GBM patients using WT1₃₃₂ peptide as a WT1 helper peptide vaccine. 0.75 mg (n = 4), 1.5 mg (n = 4), or 3 mg (n = 6) of the WT1₃₃₂ peptide was mixed with an incomplete adjuvant Montanide ISA51 at a weight ratio of 1:1 to prepare an emulsion. This emulsion was administered to each patient a total of 3 times once every two weeks by intracutaneous administration. Blood was collected from the patient before the start of administration of the vaccine and at given times after the start of administration and centrifuged to obtain serum. The obtained serum was cryopreserved at -80°C or lower and thawed for use at the time of assay. When the effects were observed, the administration of the WT1₃₃₂ helper peptide was subsequently continued at 2- to 4-week intervals.

1-2 Assay of anti-WT1-325 peptide IgG antibody (anti-WT1₃₃₂ peptide IgG antibody) (ELISA)

[0073] The antibody titer was measured in the same way as in Example 2. A WT1-325 peptide was used as a WT1 antigen peptide. The WT1-325 peptide is a peptide containing a portion (the 1st to 11th amino acids) of the administered WT1₃₃₂ peptide. Thus, the assay of the anti-WT1-325 peptide IgG antibody means the assay of an antibody against the

administered WT1₃₃₂ peptide (anti-WT1₃₃₂ peptide IgG antibody).

2. Statistical analysis

5 **[0074]** The statistical analysis was conducted in the same way as in Example 2. This analysis was conducted for 8 cases excluding clinical trial dropout cases (2 cases in the 1.5 mg administration group and 4 cases in the 3 mg administration group). As in Example 2, (1) when the anti-WT1 antigen peptide IgG antibody titer was 0.05 or higher after the start of administration of the WT1 vaccine or (2) when the anti-WT1 antigen peptide IgG antibody titer increased by 0.05 or higher after the start of administration of the WT1 vaccine as compared with before the start of administration of the
10 WT1 vaccine, this event was defined by the phrase "anti-WT1 antigen peptide antibody titer was increased".

3. Results

15 (I) Increase in anti-WT1-325 peptide IgG antibody titer (anti-WT1₃₃₂ peptide IgG antibody titer) and clinical effects

(i) Increase in anti-WT1-325 peptide IgG antibody titer and continuation rate of WT1 peptide vaccine administration (Figure 16)

20 **[0075]** Figure 16 shows the respective continuation rates of the WT1 peptide vaccine administration of the anti-WT1-325 peptide IgG antibody titer increase group and the non-increase group in the case of using, as an index, the antibody titer at 12 to 14 weeks after the start of administration of the WT1₃₃₂ (helper) peptide vaccine. During the observation period, the increase group tended to exhibit a higher continuation rate of the WT1 peptide vaccine administration than that of the non-increase group.

25 Example 5

Anti-WT1 antigen peptide antibody titer after start of administration of WT1 peptide vaccine and clinical effects in thymic carcinoma patient

30 **[0076]** The present inventors similarly conducted the following study to confirm the relationship between an anti-WT1 antigen peptide IgG antibody titer after the start of administration of a WT1 peptide vaccine and clinical effects regarding thymic carcinoma patients.

35 1. Materials and methods

[0077] 1-1 As in Example 2, WT1 peptide immunotherapy was carried out in 10 thymic carcinoma patients using WT1-CTL peptide (modified mp235-243) (SEQ ID NO: 3) as a WT1 peptide vaccine. Blood was collected from the patient before the start of administration of the vaccine and at given times after the start of administration and centrifuged to obtain serum. The anti-WT1-235 peptide IgG antibody titer in the obtained serum was measured in the same way as in
40 Example 2 (ELISA).

2. Statistical analysis

45 **[0078]** The statistical analysis was conducted in the same way as in Example 2. As in Example 2, (1) when the anti-WT1 antigen peptide IgG antibody titer was 0.05 or higher after the start of administration of the WT1 vaccine or (2) when the anti-WT1 antigen peptide IgG antibody titer increased by 0.05 or higher after the start of administration of the WT1 vaccine as compared with before the start of administration of the WT1 vaccine, this event was defined by the phrase "anti-WT1 antigen peptide antibody titer was increased".

50 3. Results

(I) Increase in anti-WT1-235 peptide IgG antibody titer (anti-WT1₂₃₅ peptide IgG antibody titer) and clinical effects

55 (i) Increase in anti-WT1-235 peptide IgG antibody titer and continuation rate of WT1 peptide vaccine administration (Figure 17)

[0079] Figure 17 shows the respective continuation rates of the WT1 peptide vaccine administration of the anti-WT1-235 peptide IgG antibody titer increase group and the non-increase group in the case of using, as an index, the anti-WT1-235

peptide IgG antibody titer at 12 to 14 weeks after the start of administration of the WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243)). During the observation period, the antibody titer increase group tended to exhibit a higher continuation rate of the vaccine administration than that of the antibody titer non-increase group (Figure 17).

5 Discussion

[0080] In the WT1 peptide immunotherapy using the WT1₂₃₅ peptide (WT1-CTL peptide (modified mp235-243)), it was revealed that the group that exhibited increase in anti-WT1-235 peptide IgG antibody titer after the start of administration of the WT1₂₃₅ peptide vaccine exhibited favorable clinical effects. This also held true both for the antibody titer at 8 to 9 weeks after the start of vaccine administration and for the antibody titer at 12 to 14 weeks after the start of vaccine administration. Among them, the correlation with clinical effects was found higher when based on the antibody titer at 12 to 14 weeks after the start of vaccine administration.

[0081] This experiment further revealed that the group having the more increased level of IgG1 or IgG3 than that of IgG4 among the anti-WT1-235 peptide IgG antibodies (Th1 type) exhibited favorable clinical effects. Among them, the IgG1 & IgG3 type was found to exhibit more favorable clinical effects.

[0082] Similar results were found to be obtained in the case of the combined administration of the WT1₃₃₂ helper peptide and the WT1₂₃₅ peptide (WT1-CTL peptide (modified mp235-243)) vaccine or in the case of the administration of the WT1₃₃₂ helper peptide alone.

20 Example 6

Assay of anti-WT1₃₃₂ peptide IgG antibody using variant WT1 antigen peptide (Figure 18)

[0083] The present inventors conducted the following study to confirm whether an anti-WT1₃₃₂ peptide IgG antibody could be assayed even using a variant WT1 antigen peptide.

1. Materials and methods

1-1 Serum

[0084] From one malignant glioblastoma patient given WT1₃₃₂ peptide vaccine (SEQ ID NO: 6), a serum sample was obtained before the start of administration of the vaccine, at 4 weeks after the start of administration, and at 1 year and 2 months after the start of administration. From one malignant thymic carcinoma patient given WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243) (SEQ ID NO: 3)) alone, a serum sample was obtained as a negative control in the same way as above. Each serum sample was stored at -20°C until assay.

1-2 Preparation of variant WT1 antigen peptides

[0085] The variant WT1 antigen peptides shown in the following table were prepared.

[Table 2]

| Variant WT1 antigen peptide | Amino acid sequence | Amino acid length | SEQ ID NO | Feature |
|-----------------------------|--|-------------------|-----------|--|
| WT1-frg3 | SEKRPFMCAYPGCN KRYFKLSHLQMH SRKHTGEKPYQCDFKDCERRFRSDQ LKRHQRRHTGVKPFQCKTCQRKFSRS DHLKTHTRHTGKTSEKPFSCRWPSC QKKFARSEDELVRHHNMHQ ^R NMTKLQL AL | 132 | 57 | Long-chain peptide containing the whole length of the administered WT1 ₃₃₂ peptide |
| WT325-342 (WT1-325) | CAYPGCN KRYFKLSHLQM | 18 | 42 | Peptide containing a portion (1st-11th amino acids) of the administered WT1 ₃₃₂ peptide |

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(continued)

| Variant WT1 antigen peptide | Amino acid sequence | Amino acid length | SEQ ID NO | Feature |
|----------------------------------|-------------------------|-------------------|-----------|--|
| WT332-347 | KRYFKLSHLQMHSRKH | 16 | 58 | Peptide consisting of the whole length of the administered WT1 ₃₃₂ peptide |
| WT334-342 | YFKLSHLQM | 9 | 59 | Peptide containing a portion (3rd-11th amino acids) of the administered WT1 ₃₃₂ peptide |
| WT332-338 | KRYFKLS | 7 | 60 | Peptide containing a portion (1st-7th amino acids) of the administered WT1 ₃₃₂ peptide |
| Administered WT1 peptide vaccine | Amino acid sequence | Amino acid length | SEQ ID NO | |
| WT1 ₃₃₂ peptide | KRYFKLSHLQMHSRKH | 16 | | 6 |

[0086] WT332-347 (SEQ ID NO: 58), WT334-342 (SEQ ID NO: 59), and WT332-338 (SEQ ID NO: 60) were synthesized by GL Biochem (Shanghai) Corporation Ltd. WT1-frag3 (SEQ ID NO: 57) was prepared by the following method.

(Method)

[0087] A vector expressing WT1-frag3 (SEQ ID NO: 57) as a GST-tagged protein was constructed using a pGEX-5X-3 vector (GE Healthcare Japan Corp.). The prepared vector was transferred to competent cells DH5 α by heat shock. The vector-transferred *E. coli* was cultured. At the point in time when the OD₆₀₀ value became 0.4 to 0.6, isopropyl- β -D-thiogalactoside (IPTG) was added at a final concentration of 1 mM to the medium to induce protein expression. The cells were further cultured for 3 hours. The bacterial cells were collected by centrifugation and then lysed in an SDS sample buffer (0.125 M Tris-HCl, 0.1 M DTT, 4% SDS, 10% sucrose, and bromophenol blue, pH 6.8). This lysate was fractionated on the basis of molecular weights by SDS-PAGE using a disc-type electrophoresis apparatus NA-1800 (Nihon Eido Corp.). A portion of each fraction thus collected by SDS-PAGE was stained with CBB, and a fraction containing WT1 was recovered. The purified protein was dissolved in an immobilizing buffer for ELISA (10 mM NaCO₃, 30 mM NaHCO₃, and 0.02% NaN₃, pH 9.6) and used as an antigen for ELISA.

1-3 Measurement of antibody titer

1-3-1 Measurement of antibody titer using WT1-frag3 (SEQ ID NO: 57)

[0088] The GST-WT1 frag1 protein solution (10 ng/ μ L) was added at 100 μ L/well to a 96-well plate for ELISA. The plate was left overnight at 37°C to immobilize the antigen thereon. After washing with 0.05% TBST, blocking was performed by shaking at room temperature for 2 hours using a blocking buffer (1% gelatin/0.05% TBST). Then, the serum diluted 100-fold with 0.05% TBST was added thereto at 100 μ L/well and reacted overnight at 4°C. The plate was reacted with a peroxidase-labeled goat anti-human IgG antibody (Santa Cruz Biotechnology, Inc.) as a secondary antibody at room temperature for 2 hours. Then, a color was developed using a TMB kit (KPL, Kirkegaard & Perry Laboratories, Inc.). The reaction was terminated with 1 N HCl, and the absorbance at 450 nm was then measured using a microplate reader (CORONA ELECTRIC MTP-32).

1-3-2 Measurement of antibody titer using WT332-347 (SEQ ID NO: 58), WT334-342 (SEQ ID NO: 59), or WT332-338 (SEQ ID NO: 60)

[0089] To a 96-well reaction plate supplied with Peptide Coating Kit (Takara Bio Inc.) having amino groups (-NH₂) attached to the bottom surface of the wells, a solution (4 μg/mL) of the WT1 peptide dissolved in a reaction buffer supplied with Peptide Coating Kit was added at 50 μL/well. A coupling reagent was added thereto at 30 μL/well and reacted at room temperature for 2 hours. The wells were washed with distilled water to immobilize the antigen thereon. Blocking was performed by shaking at room temperature for 2 hours using Blocking One (Nacalai Tesque, Inc.). Subsequently, the wells were washed with 0.05% TBST. Then, the serum diluted 100-fold with a blocking solution supplied with Peptide Coating Kit was added thereto at 100 μL/well and reacted overnight at 4°C. After washing with 0.05% TBST, the plate was reacted with a peroxidase-labeled rabbit anti-human IgG antibody (sc-2769, Santa Cruz Biotechnology, Inc., 400 μg/mL) diluted 1000-fold with a blocking solution supplied with Peptide Coating Kit as a secondary antibody at room temperature for 2 hours. After washing with 0.05% TBST, a color was developed using a TMB kit (KPL, Kirkegaard & Perry Laboratories, Inc.). The reaction was terminated with 1 N HCl, and the absorbance at 450 nm was then measured using a microplate reader (CORONA ELECTRIC MTP-310Lab).

2. Results

(I) Measurement of antibody titer using WT1-frg3 (SEQ ID NO: 57)

[0090] The anti-WT1₃₃₂ IgG antibody titer in the serum before immunization and at given times after immunization was measured by ELISA using WT1-frg3 (SEQ ID NO: 57) as an antigen for one patient immunized with the WT1₃₃₂ peptide and one patient immunized with the WT1₂₃₅ peptide (WT1-CTL peptide (modified mp235-243) (SEQ ID NO: 3)) alone. As a result, in the patient immunized with the WT1₃₃₂ peptide, the anti-WT1₃₃₂ IgG antibody titer was increased after immunization (Figure 18). On the other hand, in the patient unimmunized with the WT1₃₃₂ peptide, the anti-WT1₃₃₂ IgG antibody titer was not increased. These results demonstrated that ELISA using WT1-frg3 (SEQ ID NO: 57) as a WT1 antigen peptide could measure an anti-WT1₃₃₂ IgG antibody level in patient's serum.

(II) Measurement of antibody titer using WT332-347 (SEQ ID NO: 58), WT334-342 (SEQ ID NO: 59), or WT332-338 (SEQ ID NO: 60)

[0091] In measurement by ELISA using any of the peptides, in the patient immunized with the WT1₃₃₂ peptide, the anti-WT1₃₃₂ IgG antibody titer was increased after immunization (Figure 18). On the other hand, in the patient unimmunized with the WT1₃₃₂ peptide, the anti-WT1₃₃₂ IgG antibody titer was not increased. These results demonstrated that ELISA using WT332-347 (SEQ ID NO: 58), WT334-342 (SEQ ID NO: 59), or WT332-338 (SEQ ID NO: 60) as a WT1 antigen peptide could measure an anti-WT1₃₃₂ IgG antibody level in patient's serum.

Example 7

Assay of anti-WT1₂₃₅ peptide IgG antibody using variant WT1 antigen peptide (Figure 19)

[0092] The present inventors conducted the following study to confirm whether an anti-WT1₂₃₅ peptide IgG antibody could be assayed even using a variant WT1 antigen peptide.

1. Materials and methods

1-1 Serum

[0093] From one malignant thymic carcinoma patient given a WT1₂₃₅ peptide vaccine (WT1-CTL peptide (modified mp235-243) (SEQ ID NO: 3)), a serum sample was obtained at 4 weeks after the start of administration of the vaccine and at 7 months after the start of administration. Each serum sample was stored at -20°C until assay.

1-2 Preparation of variant WT1 antigen peptides

[0094] The variant WT1 antigen peptides shown in the following table were prepared.

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[Table 3]

| Variant WT1 antigen peptide | Amino acid sequence | Amino acid length | SEQ ID NO | Feature |
|----------------------------------|--|-------------------|-----------|--|
| WT1-frg2 | DPMGQQGSLGEQQYSVPPPVYGCHTP TDSC TGSQALLLRTPYSSDNLYQMTS QLE <u>CMTWNQMNL</u> GATLKGVAAGSSSS VKWTEGQSNHSTGYESDNHTTPIICG AQYRIHTHG VFRGIQD VRRVPGVAPT LVRSA SETSEKRPFM | 145 | 61 | Long-chain peptide containing the whole length (in which the 2nd amino acid Y was substituted by M) of the administered WT1 ₂₃₅ peptide |
| WT235-252 (WT1-235) | <u>CMTWNQMNL</u> GATLKGVA A | 18 | 32 | Peptide containing the whole length (in which the 2nd amino acid Y was substituted by M) of the administered WT1 ₂₃₅ peptide |
| WK235-243 | <u>CMTWNQM</u> <u>NK</u> | 9 | 62 | Peptide consisting of the whole length (in which the 2nd amino acid Y was substituted by M and the 9th amino acid L was substituted by K) of the administered WT1 ₂₃₅ peptide |
| WT237-243 | <u>TWNQMNL</u> | 7 | 63 | Peptide containing a portion (3rd-9th amino acids) of the administered WT1 ₂₃₅ peptide |
| Administered WT1 peptide vaccine | Amino acid sequence | Amino acid length | SEQ ID NO | |
| WT1 ₂₃₅ peptide | CYTWNQMNL | 9 | | 3 |

[0095] WK235-243 (SEQ ID NO: 62) and WT237-243 (SEQ ID NO: 63) were synthesized by GL Biochem (Shanghai) Corporation Ltd. WT1-frg2 (SEQ ID NO: 61) was prepared in the same way as in the aforementioned WT1-frg3 (SEQ ID NO: 57).

1-3 Measurement of antibody titer

[0096] The measurement of the antibody titer using WT1-frg2 (SEQ ID NO: 61) was performed in the same way as in the aforementioned WT1-frg3 (SEQ ID NO: 57). The measurement of the antibody titer using WK235-243 (SEQ ID NO: 62) or WT237-243 (SEQ ID NO: 63) was performed in the same way as in the aforementioned WT332-347 (SEQ ID NO: 58), etc.

2. Results

(I) Measurement of antibody titer using WT1-frg2 (SEQ ID NO: 61)

5 **[0097]** The anti-WT1₂₃₅ IgG antibody titer in the patient given the WT1₂₃₅ peptide was measured by ELISA using WT1-frg2 (SEQ ID NO: 61) as an antigen. As a result, increase in anti-WT1₂₃₅ IgG antibody titer was detected (Figure 19). Also, similar results as in the case of using the WT1-235 peptide (WT235-243) used in the aforementioned Example as an antigen were shown. These results demonstrated that ELISA using WT1-frg2 (SEQ ID NO: 61) as a WT1 antigen peptide could measure an anti-WT1₂₃₅ IgG antibody level in patient's serum.

10 (II) Measurement of antibody titer using WK235-243 (SEQ ID NO: 62) or WT237-243 (SEQ ID NO: 63)

15 **[0098]** Also in the case of using each of these peptides as an antigen, increase in anti-WT1₂₃₅ IgG antibody titer was detected (Figure 19). Also, similar results as in the case of using the WT1-235 peptide (WT235-243) used in the aforementioned Example as an antigen were shown. These results demonstrated that ELISA using WK235-243 (SEQ ID NO: 62) or WT237-243 (SEQ ID NO: 63) as a WT1 antigen peptide could measure an anti-WT1₂₃₅ IgG antibody level in patient's serum.

20 Example 8

Assay of anti-WT1₁₂₆ peptide IgG antibody using variant WT1 antigen peptide (Figure 20)

25 **[0099]** The present inventors conducted the following study to confirm whether an anti-WT1₁₂₆ peptide IgG antibody could be assayed even using a variant WT1 antigen peptide.

1. Materials and methods

1-1 Serum

30 **[0100]** From a pancreatic cancer patient receiving a combination therapy with WT1₁₂₆ peptide and an anticancer agent, a serum sample was obtained before the start of administration of the vaccine and at 7 months after the start of administration. Each serum sample was stored at -20°C until assay.

1-2 Preparation of variant WT1 antigen peptides

35 **[0101]** The variant WT1 antigen peptides shown in the following tables were prepared.

[Table 4-1]

| Variant WT1 antigen peptide | Amino acid sequence | Amino acid length | SEQ ID NO | Feature |
|-----------------------------|---|-------------------|-----------|---|
| WT1-frg1 | MGSDVRDLNALLPAVPSLGGGGGCAL PVSGAAQWAPVLDFAFPGASAYGSLG GPAPPPAPPPPPPPPHSFYKQEPSW GGAEPHEEQCLSAFTVHFSGQFTGTA GACRYGPFPPPPPSQASSGQARMFPN APYL PSCLESQPAIRNQGYSTVTFDG TPSYGHTPSHHAAQFPNHSFKHEDPM | 182 | 64 | Long-chain peptide containing the whole length of the administered WT1 ₁₂₆ peptide |
| WT118-135 (WT1-118) | SQASSGQARMFPNAPYLP | 18 | 20 | Peptide containing the whole length of the administered WT1 ₁₂₆ peptide |

(continued)

| Variant WT1 antigen peptide | Amino acid sequence | Amino acid length | SEQ ID NO | Feature |
|-----------------------------|---------------------|-------------------|-----------|--|
| WT126-134 | RMFPNAPYL | 9 | 65 | Peptide consisting of the whole length of the administered WT1 ₁₂₆ peptide |
| WT126-134 | RMFPNAPYK | 9 | 66 | Peptide consisting of the whole length (in which the 9th amino acid L was substituted by K) of the administered WT1 ₁₂₆ peptide |

[Table 4-2]

| Variant WT1 antigen peptide | Amino acid sequence | Amino acid length | SEQ ID NO | Feature |
|----------------------------------|---------------------|-------------------|-----------|---|
| WT128-134 | FPNAPYL | 7 | 67 | Peptide containing a portion (3rd-9th amino acids) of the administered WT1 ₁₂₆ peptide |
| WT126-132 | RMFPNAP | 7 | 68 | Peptide containing a portion (1st-7th amino acids) of the administered WT1 ₁₂₆ peptide |
| WT129-134 | PNAPYL | 6 | 69 | Peptide containing a portion (4th-9th amino acids) of the administered WT1 ₁₂₆ peptide |
| WT126-131 | RMFPNA | 6 | 70 | Peptide containing a portion (1st-6th amino acids) of the administered WT1 ₁₂₆ peptide |
| WT126-130 | RMFPN | 5 | 71 | Peptide containing a portion (1st-5th amino acids) of the administered WT1 ₁₂₆ peptide |
| Administered WT1 peptide vaccine | Amino acid sequence | Amino acid length | SEQ ID NO | |
| WT1 ₁₂₆ peptide | RMFPNAPYL | 9 | 4 | |

[0102] WT126-134 (SEQ ID NO: 65), WK126-134 (SEQ ID NO: 66), WT128-134 (SEQ ID NO: 67), WT126-132 (SEQ ID NO: 68), WT129-134 (SEQ ID NO: 69), WT126-131 (SEQ ID NO: 70), and WT126-130 (SEQ ID NO: 71) were synthesized by GL Biochem (Shanghai) Corporation Ltd. WT1-frg1 (SEQ ID NO: 64) was prepared in the same way as in the aforementioned WT1-frg3 (SEQ ID NO: 57).

1-3 Measurement of antibody titer

[0103] The measurement of the antibody titer using WT1-frg1 (SEQ ID NO: 64) was performed in the same way as in the aforementioned WT1-frg3 (SEQ ID NO: 57). The measurement of the antibody titer using WT126-134 (SEQ ID NO: 65), WK126-134 (SEQ ID NO: 66), WT128-134 (SEQ ID NO: 67), WT126-132 (SEQ ID NO: 68), WT129-134 (SEQ ID NO: 69), WT126-131 (SEQ ID NO: 70), or WT126-130 (SEQ ID NO: 71) was performed in the same way as in the aforementioned WT332-347 (SEQ ID NO: 58), etc.

2. Results

(I) Measurement of antibody titer using WT1-frg1 (SEQ ID NO: 64)

5 [0104] The anti-WT1₁₂₆ IgG antibody titer in the patient given the WT1₁₂₆ peptide was measured by ELISA using WT1-frg1 (SEQ ID NO: 64) as an antigen. As a result, increase in anti-WT1₁₂₆ IgG antibody titer was detected after the start of administration of the vaccine (Figure 20). These results demonstrated that ELISA using WT1-frg1 (SEQ ID NO: 64) as a WT1 antigen peptide could measure an anti-WT1₁₂₆ IgG antibody level in patient's serum.

10 (II) Measurement of antibody titer using WT126-134 (SEQ ID NO: 65), WK126-134 (SEQ ID NO: 66), WT128-134 (SEQ ID NO: 67), WT126-132 (SEQ ID NO: 68), WT129-134 (SEQ ID NO: 69), WT126-131 (SEQ ID NO: 70), or WT126-130 (SEQ ID NO: 71)

15 [0105] Also in the case of using each of these peptides as an antigen, increase in anti-WT1₁₂₆ IgG antibody titer was detected after the start of administration of the vaccine (Figure 20). These results demonstrated that ELISA using each of these peptides as a WT1 antigen peptide could measure an anti-WT1₁₂₆ IgG antibody level in patient's serum.

20 [0106] The results described above demonstrated that the clinical effects of WT1 vaccine therapy could be predicted after the start of administration of a WT1 peptide vaccine, by measuring the antibody titer of an anti-WT1 antigen peptide IgG antibody using a WT1 antigen peptide corresponding to the administered WT1 peptide vaccine. These results also demonstrated that more accurate prediction could be achieved by measuring the IgG antibody on a subclass (IgG1, IgG3, and IgG4) basis. These results further demonstrated that use of a variant WT1 antigen peptide also allows the antibody titer of the anti-WT1 antigen peptide IgG antibody to be measured and also clinical effects of WT1 vaccine therapy to be predicted.

25 Industrial Applicability

30 [0107] The present invention can provide a more accurate prediction method for predicting the clinical effects of WT1 vaccine therapy. More accurate prediction can be achieved by assaying an anti-WT1 antigen peptide antibody in a test subject and using the obtained value as an index. Thus, the present invention can be utilized in the testing of the applicability of WT1 vaccine therapy to WT1-related disease patients and the prediction of its clinical effects.

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Claims

- 45 1. A method for predicting a clinical effect on a subject in WT1 peptide immunotherapy comprising the following steps:
- a) contacting a sample from the subject with a WT1 antigen peptide or a variant thereof; and
b) detecting the binding of the sample to the WT1 antigen peptide or the variant thereof, and thereby, measuring an antibody titer of anti-WT1 antigen peptide IgG present in the sample,
50 wherein an increase in the anti-WT1 antigen peptide IgG antibody titer in the subject means the achievement of a favorable clinical effect.
2. The method according to claim 1, wherein the subclasses of the measured anti-WT1 antigen peptide IgG antibody are IgG1, IgG3 and IgG4, and wherein the achievement of a favorable clinical effect is determined when the titer of
55 each of IgG1 and IgG3 is twice or more than twice the titer of IgG4.
3. The method according to claim 2, wherein the achievement of a favorable clinical effect is determined when the titer of IgG3 is less than twice the titer of IgG1 and the titer of IgG1 is less than twice the titer of IgG3.

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4. The method according to any of claims 1-3, wherein the anti-WT1 antigen peptide IgG antibody titer at 8 to 14 weeks after the start of administration of a WT1 peptide vaccine is measured.
5. The method according to claim 4, wherein the anti-WT1 antigen peptide IgG antibody titer at 12 to 14 weeks after the start of administration of the WT1 peptide vaccine is measured.
6. The method according to any of claims 1-5, wherein the WT1 peptide vaccine administered to the subject consists of the amino acid sequence of any of SEQ ID NOs: 2-6.
7. The method according to any of claims 1-6, wherein the WT1 antigen peptide comprises the amino acid sequence of any of SEQ ID NOs: 7-71.
8. The method according to claim 7, wherein the WT1 antigen peptide comprises the amino acid sequence of any of SEQ ID NOs: 20, 21, 27, 31, 32, 42, 43 or 57-71.
9. The method according to any of claims 1-8, wherein the sample is a blood sample, a plasma sample, a serum sample or a urine sample.
10. The method according to claim 9, wherein the sample is a serum sample.
11. The method according to any of claims 1-10, wherein the subject is a patient with a WT1-associated disease.
12. The method according to claim 11, wherein the WT1-associated disease is leukemia such as chronic myeloid leukemia, hematopoietic organ tumor such as myelodysplastic syndrome, multiple myeloma, and malignant lymphoma, or solid cancer such as esophageal cancer, stomach cancer, colorectal cancer, pancreatic cancer, lung cancer, breast cancer, germ cell cancer, liver cancer, biliary tract cancer, head and neck cancer, skin cancer, sarcoma, kidney cancer, bladder cancer, prostate cancer, testicular cancer, uterine cancer, cervical cancer, ovarian cancer, thyroid cancer, carcinoid, pneumoblastoma, hepatoblastoma, brain tumor and thymic carcinoma.
13. The method according to claim 12, wherein the WT1-associated disease is recurrent malignant glioma, thymic carcinoma or pancreatic cancer.
14. A kit for performing the method according to any of claims 1-13, comprising a WT1 antigen peptide or a variant thereof.

Fig. 1

Anti-WT1-235 peptide IgG antibody

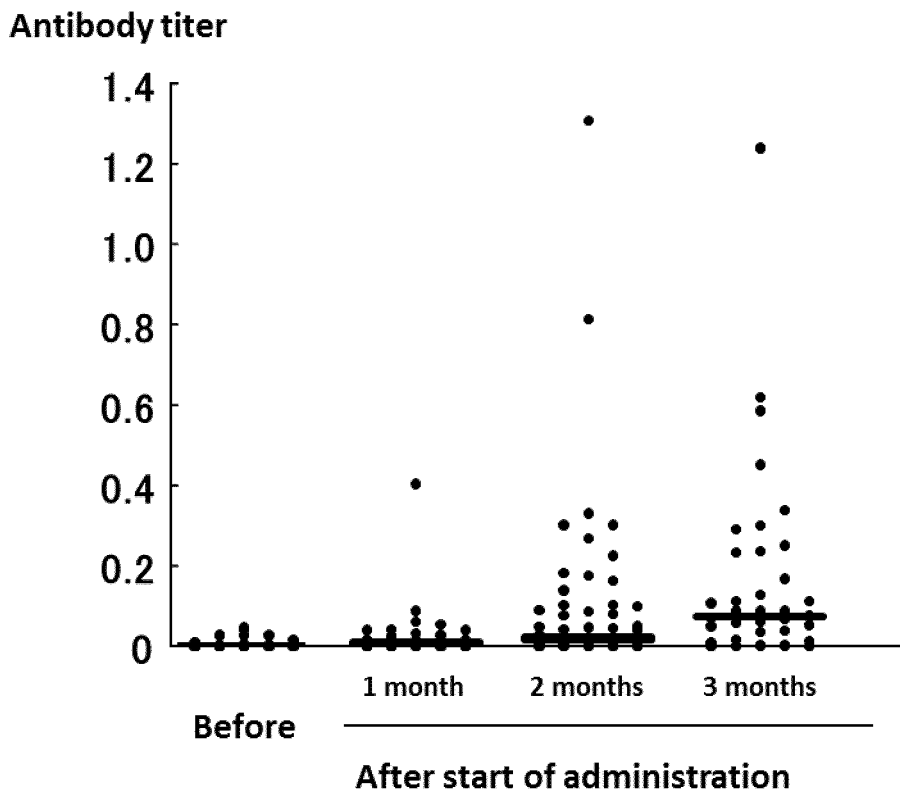


Fig. 2

**Increase in anti-WT1-235 peptide IgG antibody titer
at 8-9 weeks after start of administration of WT1₂₃₅ peptide
and overall survival rate**

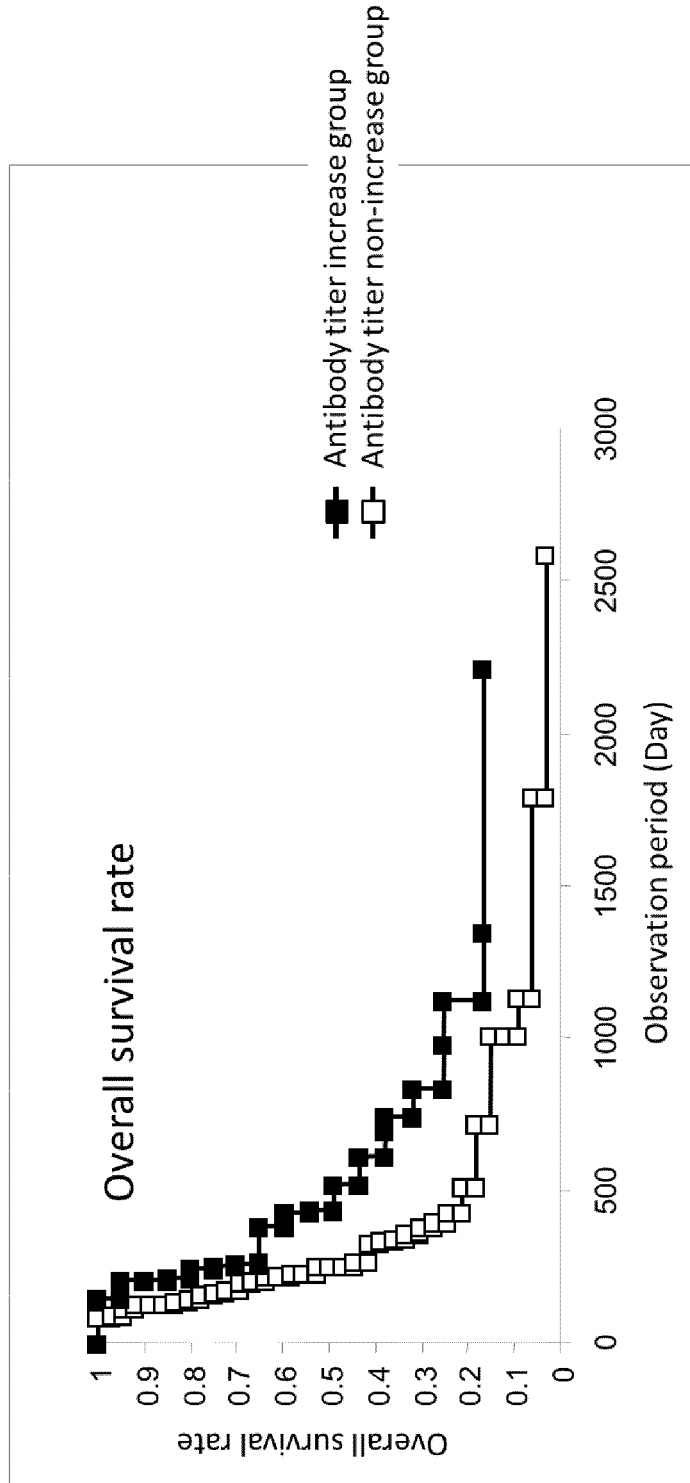


Fig. 3

**Increase in anti-WT1-235 peptide IgG antibody titer
at 12-14 weeks after start of administration of WT1₂₃₅ peptide
and overall survival rate**

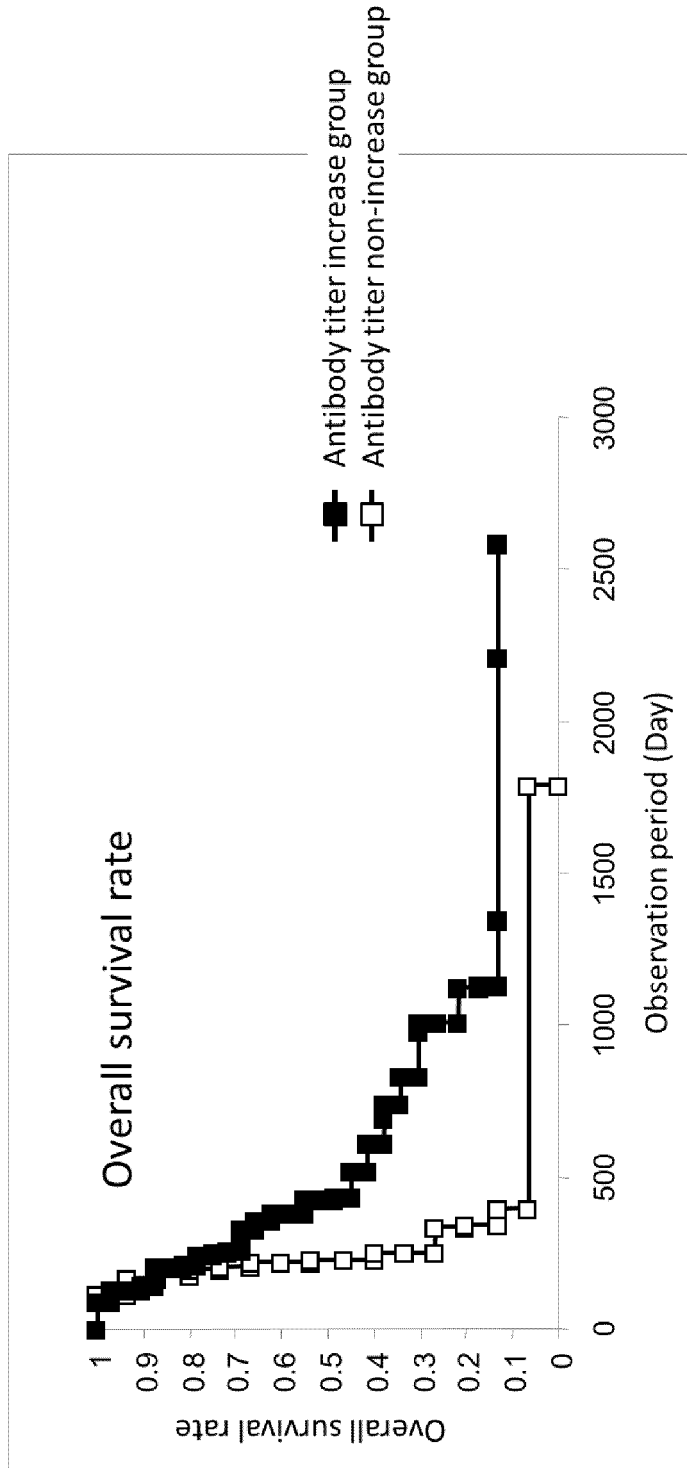


Fig. 4

**Increase in anti-WT1-235 peptide IgG antibody titer
at 8-9 weeks after start of administration of WT1₂₃₅ peptide
and progression-free survival rate**

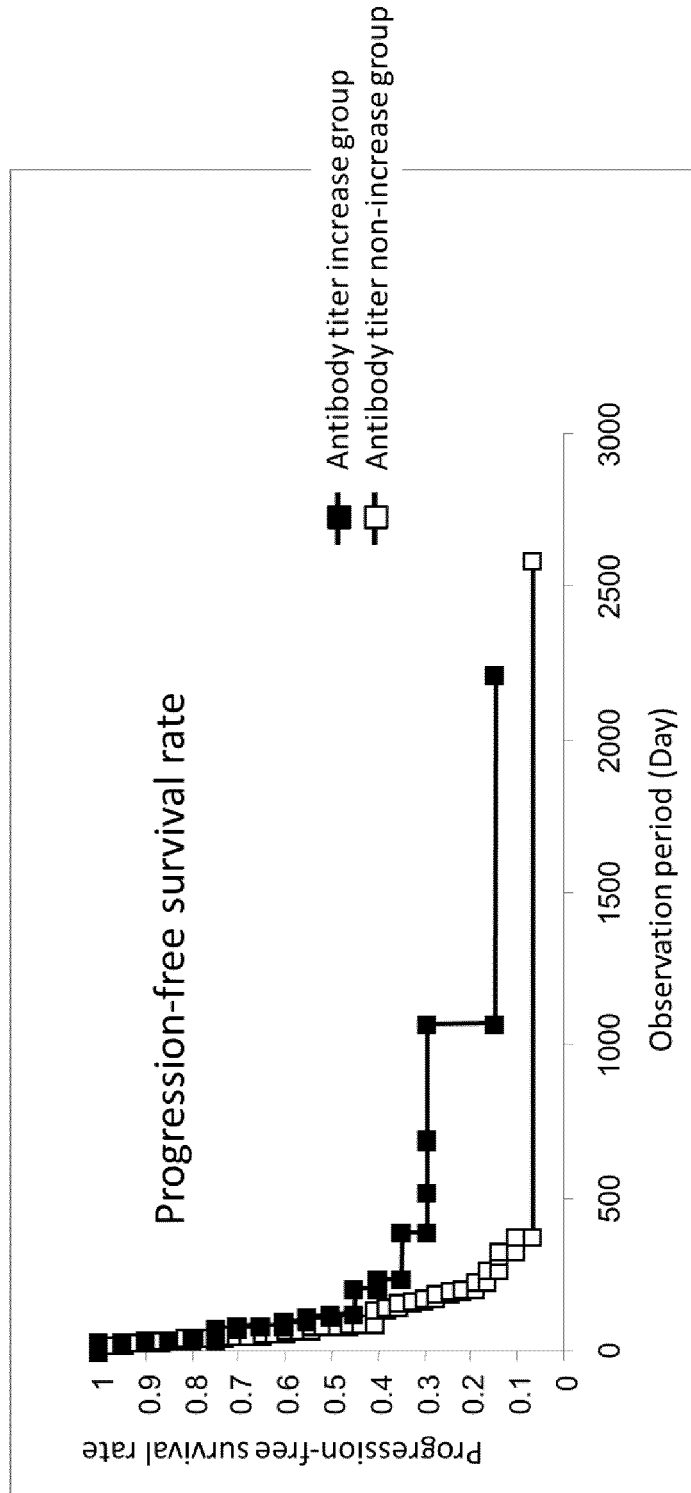


Fig. 5

**Increase in anti-WT1-235 peptide IgG antibody titer
at 12-14 weeks after start of administration of WT1₂₃₅ peptide
and progression-free survival rate**

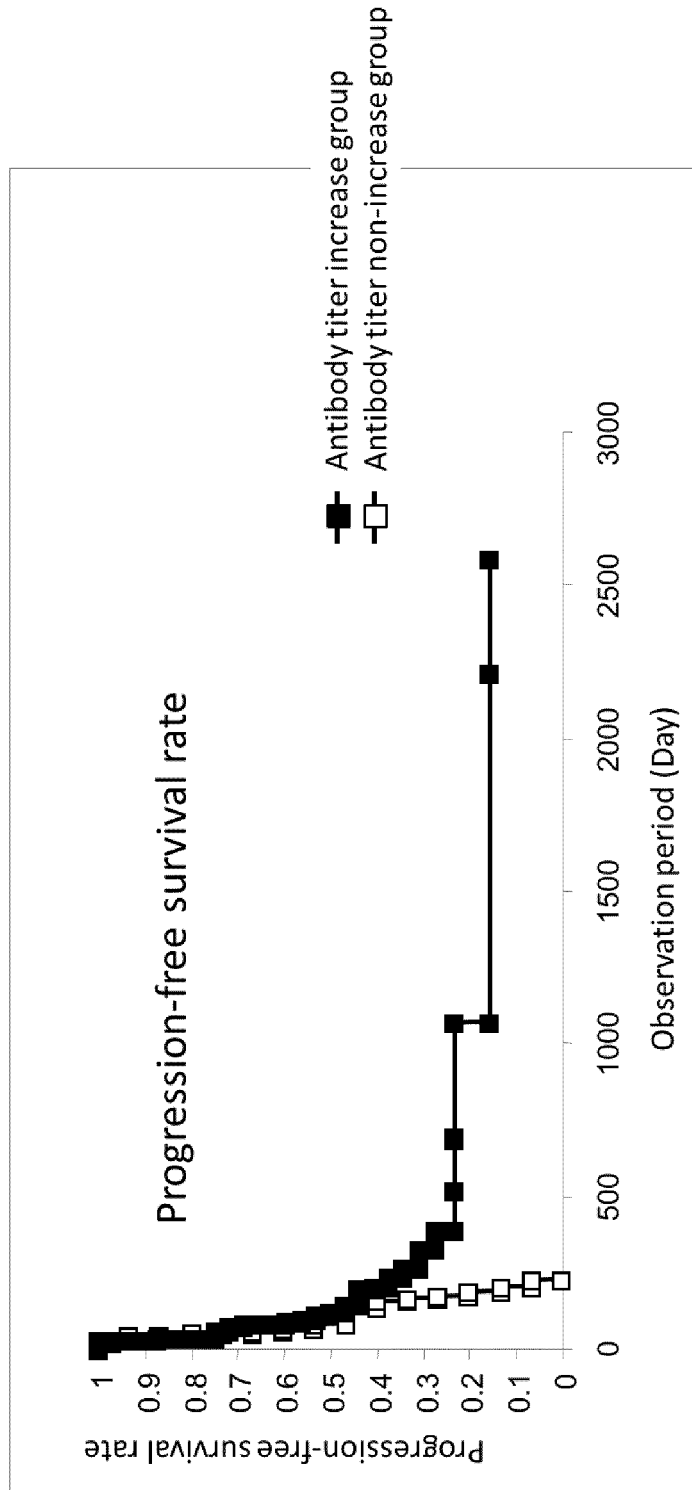


Fig. 6

Increase in anti-WT1-235 peptide IgG antibody titer at 8-9 weeks after start of administration of WT1₂₃₅ peptide and continuation rate of WT1 vaccine administration

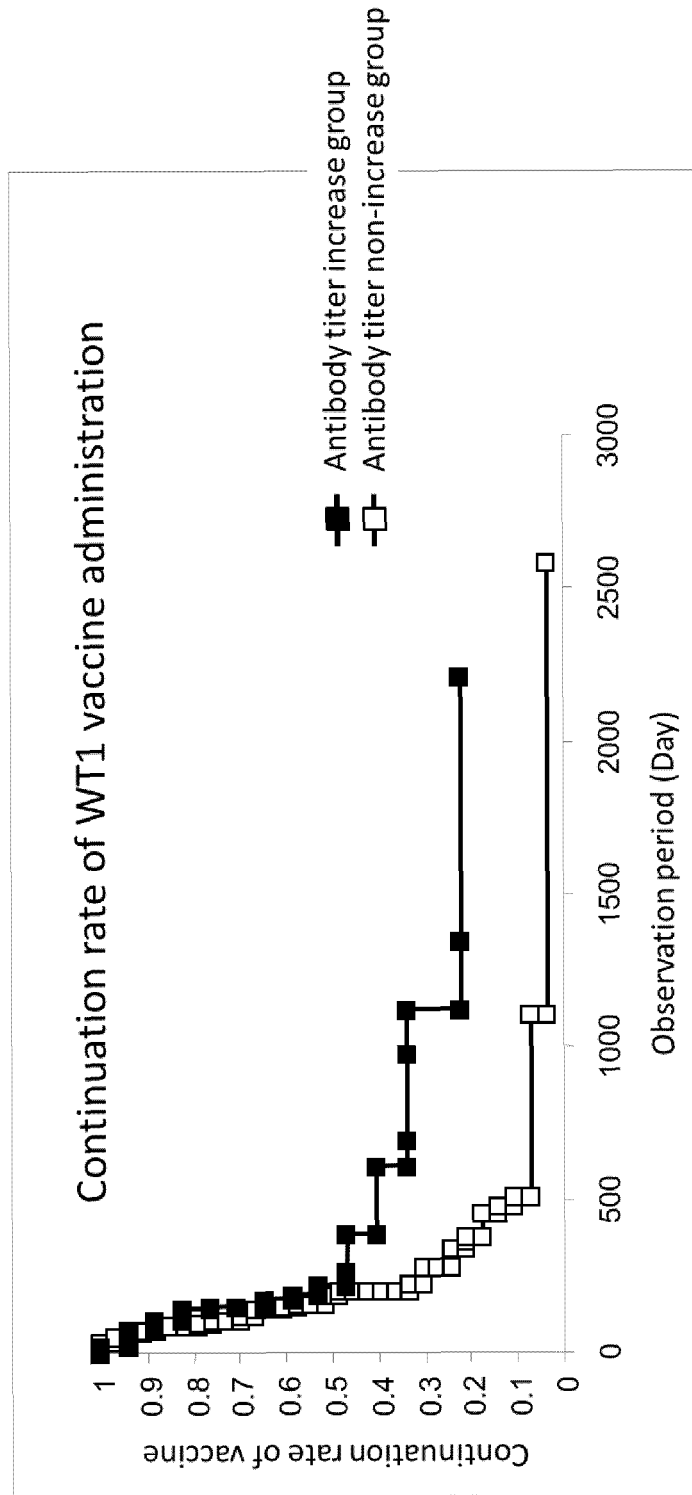


Fig. 7

Increase in anti-WT1-235 peptide IgG antibody titer at 12-14 weeks after start of administration of WT1₂₃₅ peptide and continuation rate of WT1 vaccine administration

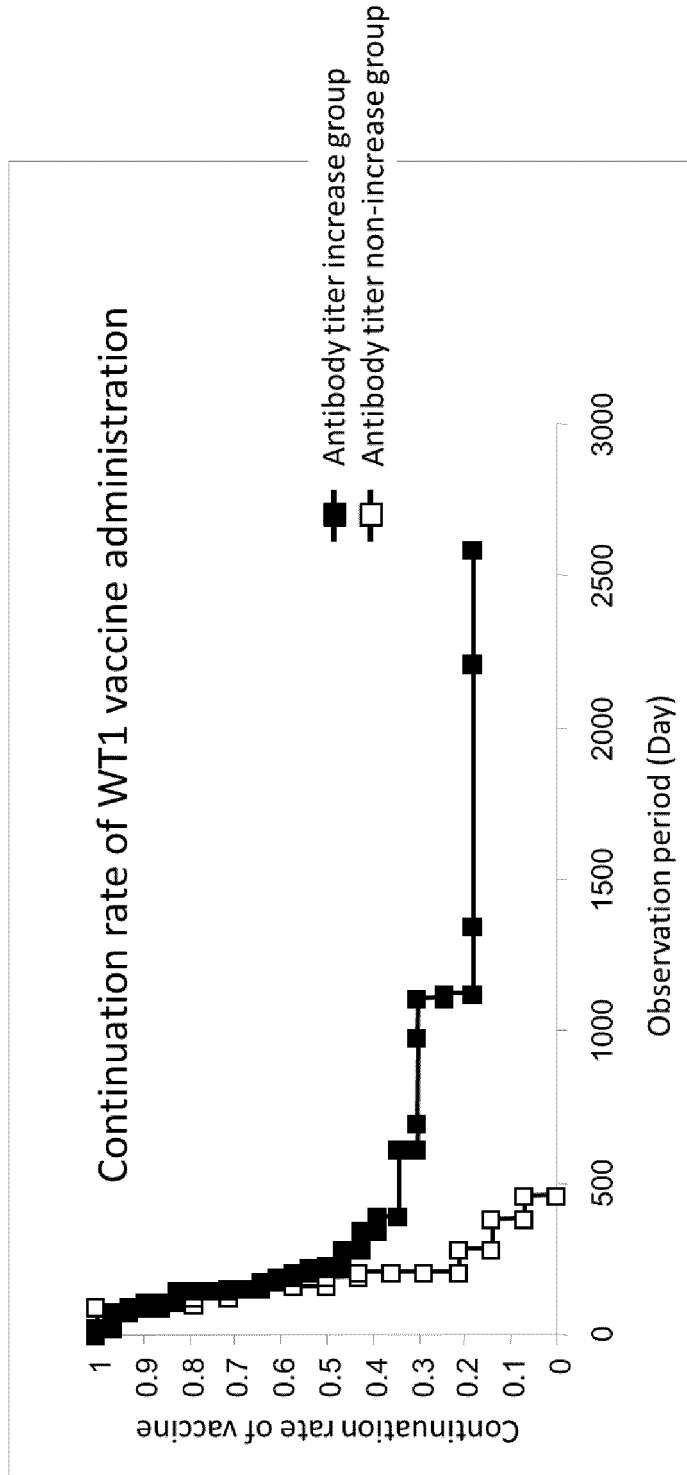


Fig. 8

**Type (Th1/non-Th1) of anti-WT1-235 peptide IgG antibody
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and overall survival rate**

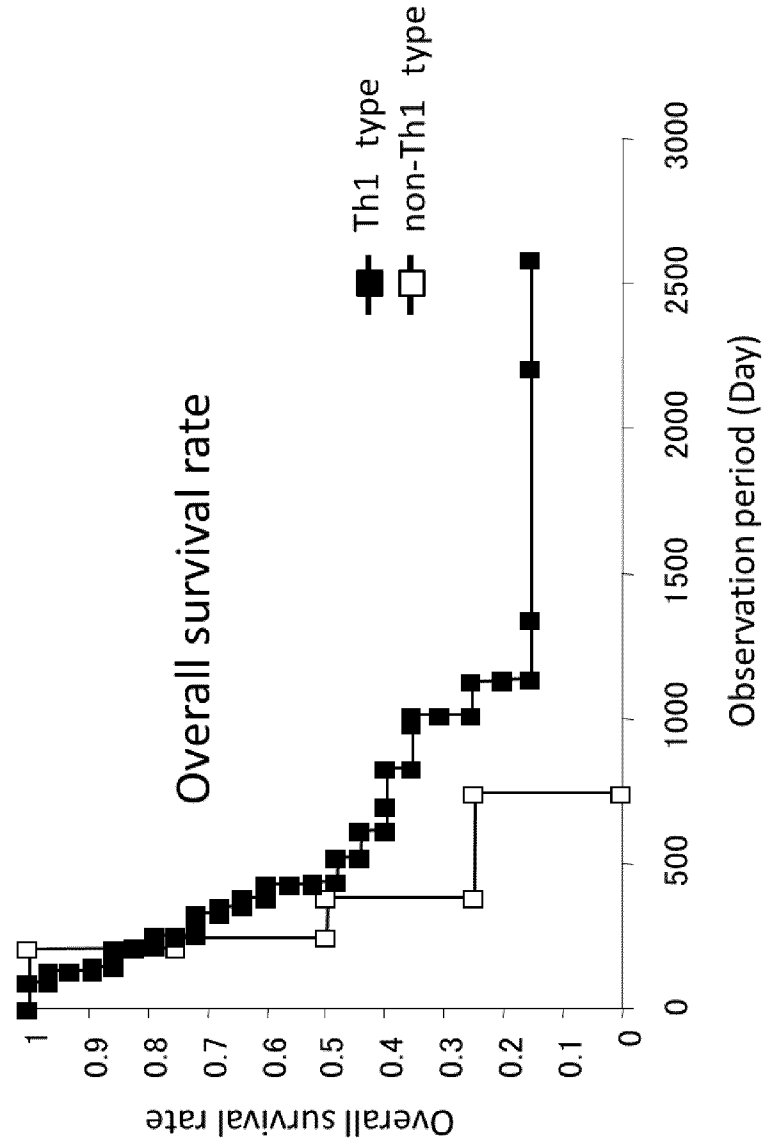


Fig. 9

Type (Th1/non-Th1) of anti-WT1-235 peptide IgG antibody at 12-14 weeks after start of administration of WT1₂₃₅ peptide and progression-free survival rate

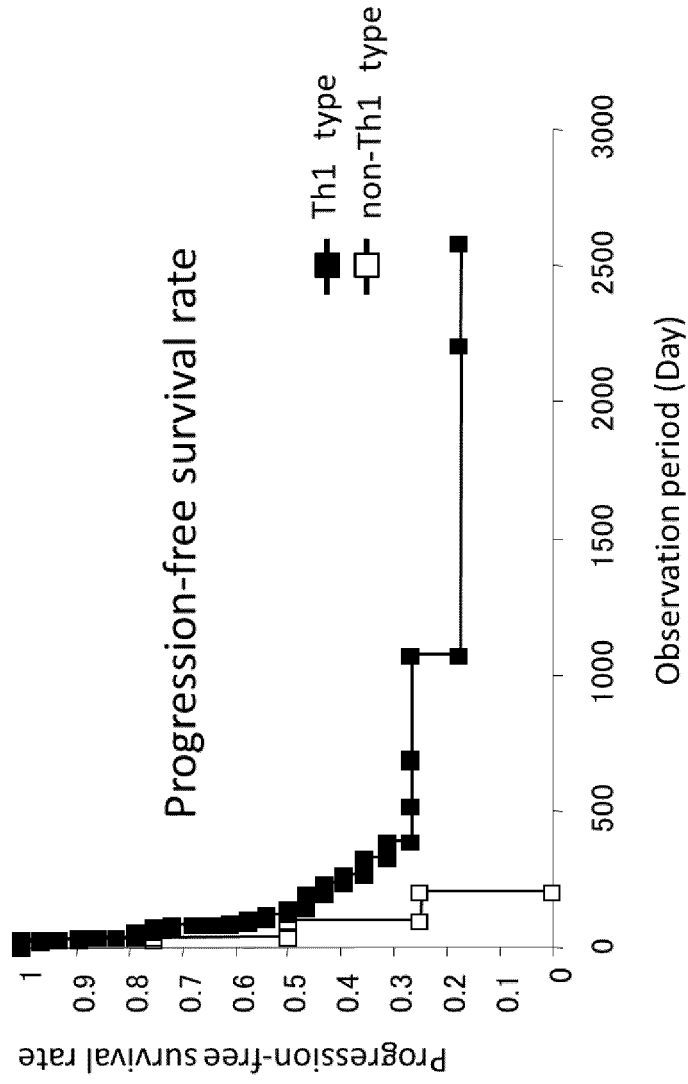


Fig. 10

Type (Th1/non-Th1) of anti-WT1-235 peptide IgG antibody at 12-14 weeks after start of administration of WT1₂₃₅ peptide and continuation rate of WT1 vaccine administration

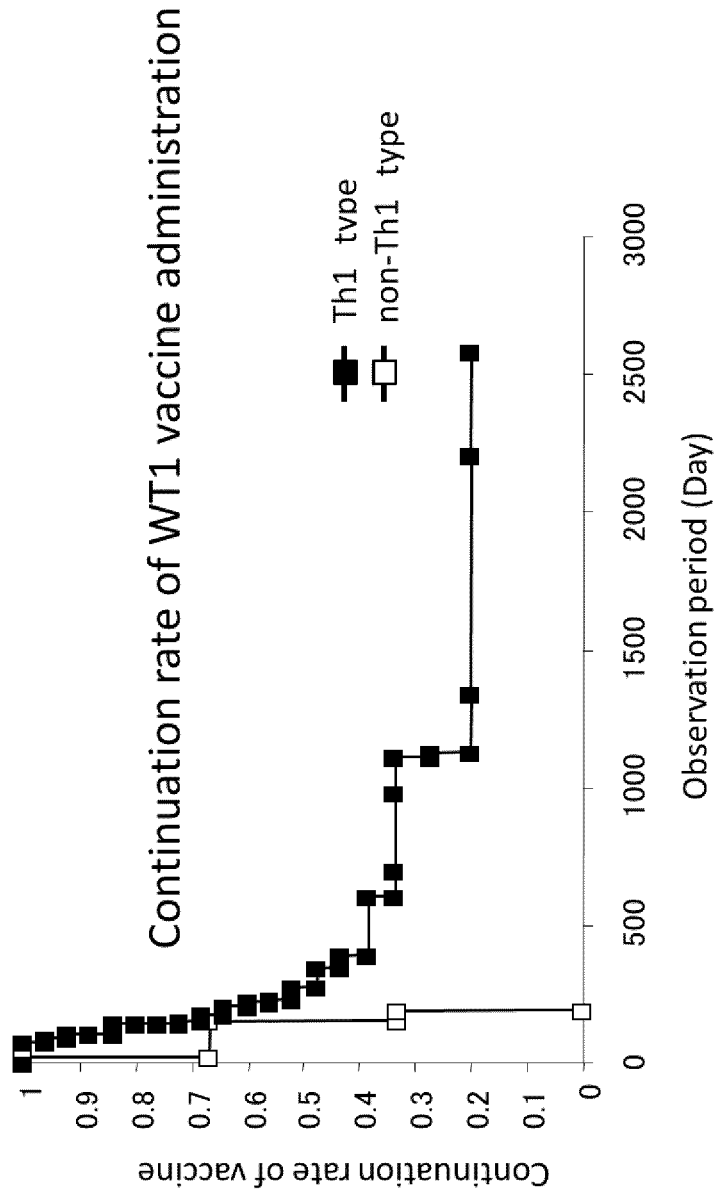


Fig. 11

**Increase in anti-WT1-235 peptide IgG subclass antibody titer
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and overall survival rate**

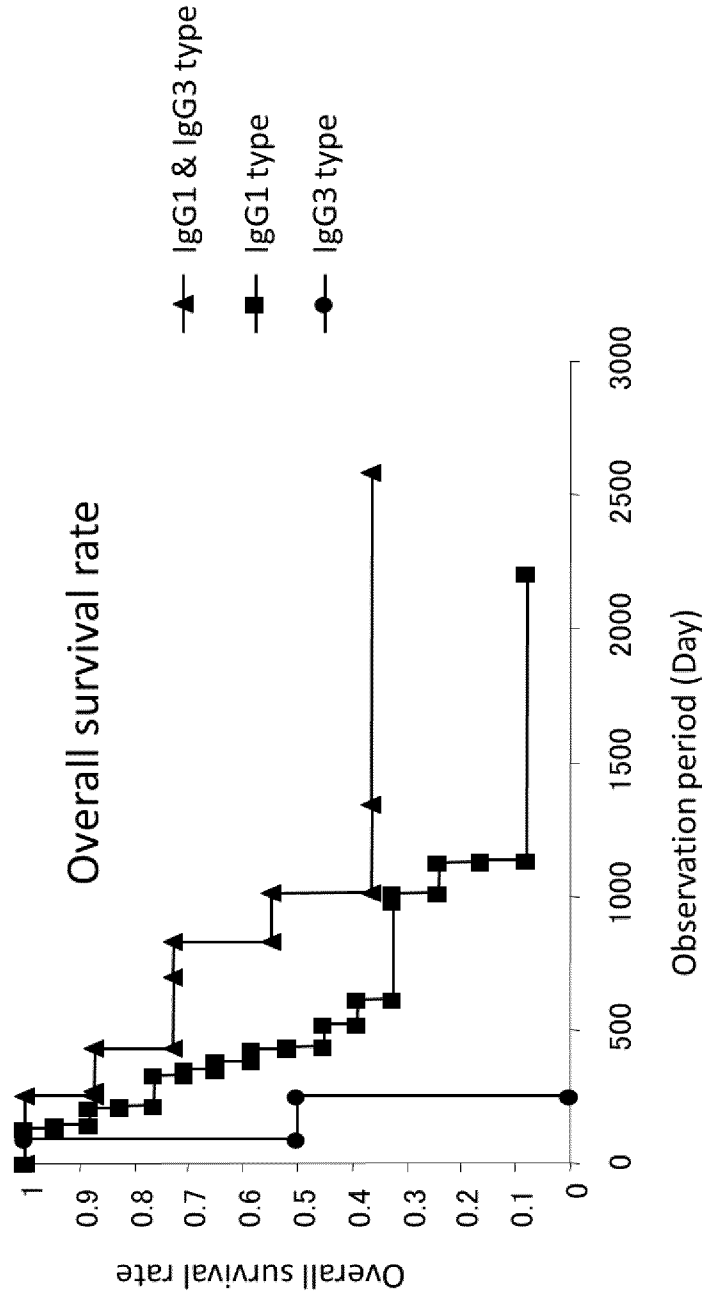


Fig. 12

**Increase in anti-WT1-235 peptide IgG subclass antibody titer
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and progression-free survival rate**

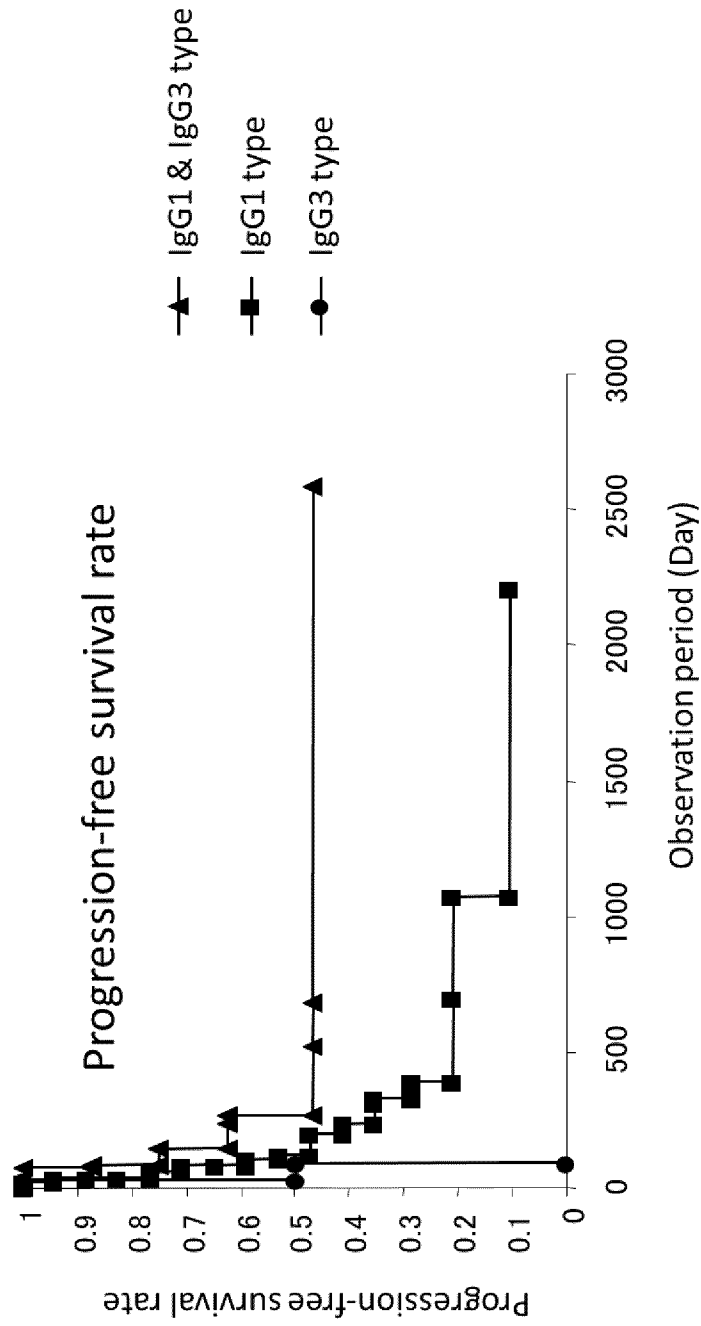


Fig. 13

Increase in anti-WT1-235 peptide IgG subclass antibody titer at 12-14 weeks after start of administration of WT1₂₃₅ peptide and continuation rate of WT1 vaccine

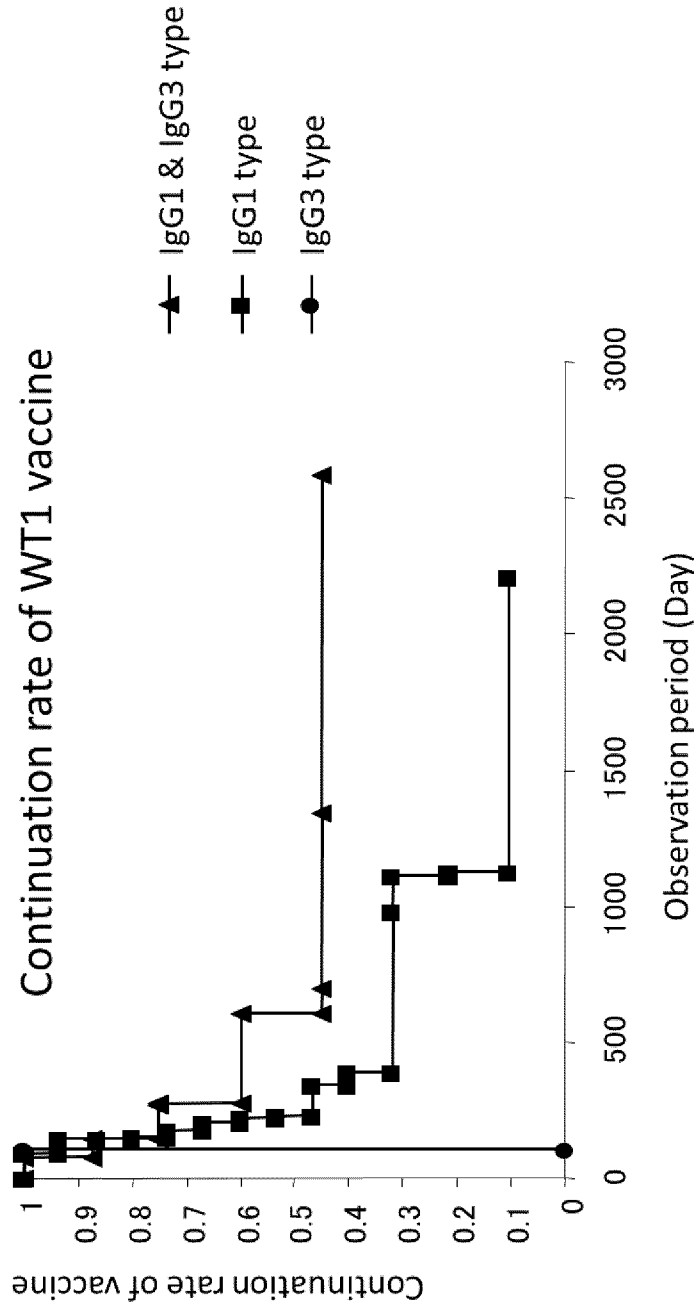


Fig. 14

Anti-WT1-235 peptide IgG antibody titer 4-8 weeks

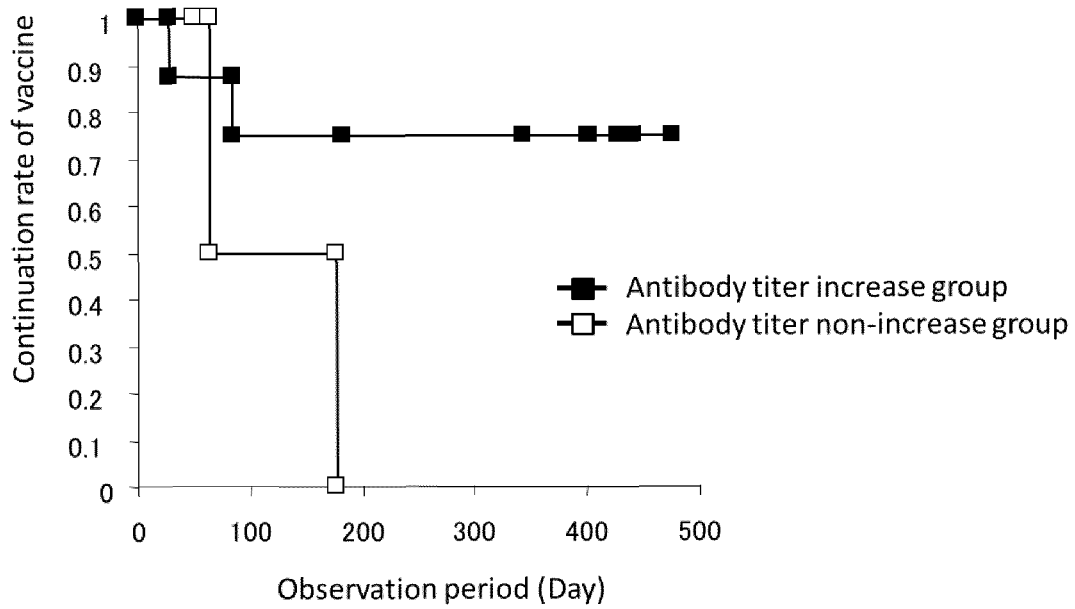


Fig. 15

Anti-WT1-325 peptide IgG antibody titer 4-8 weeks

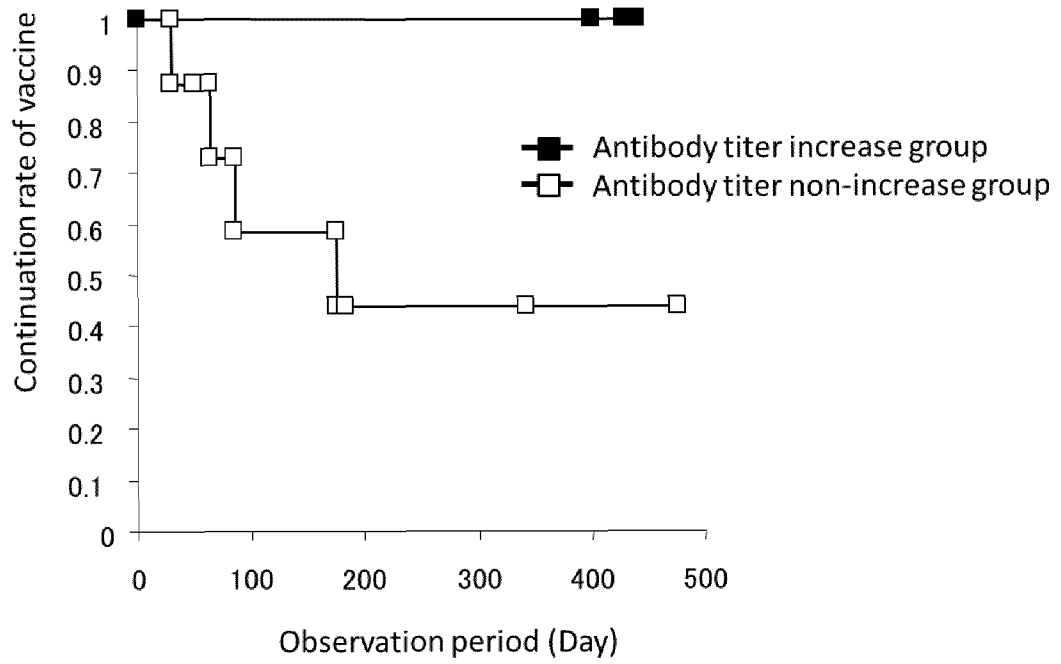


Fig. 16

Anti-WT1-325 peptide IgG antibody titer 12-14 weeks

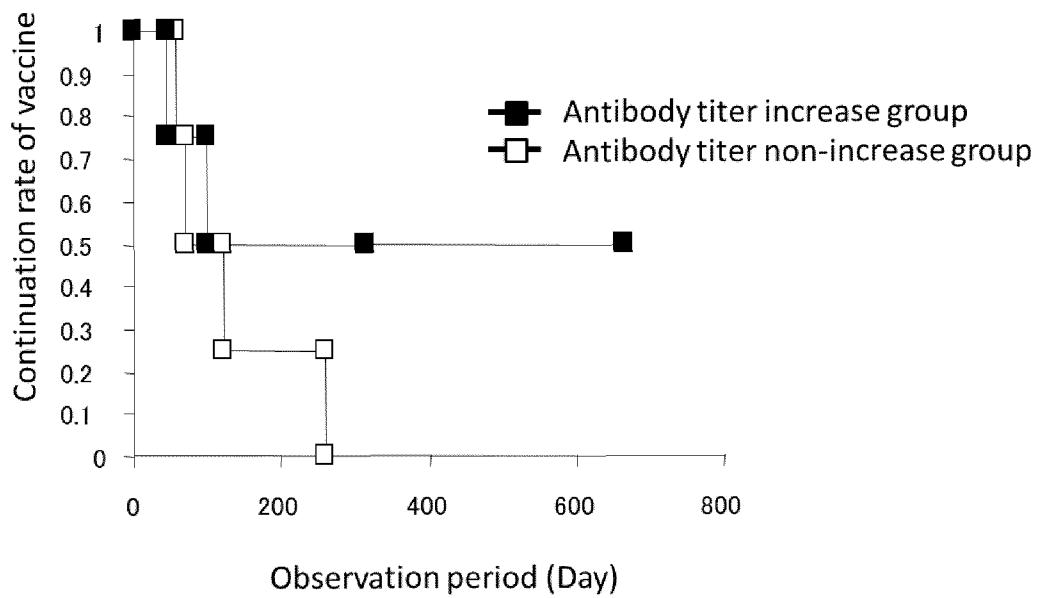


Fig. 17

Increase in anti-WT1-235 peptide antibody titer at 12-14 weeks after start of administration of WT1₂₃₅ peptide and continuation rate of WT1 vaccine

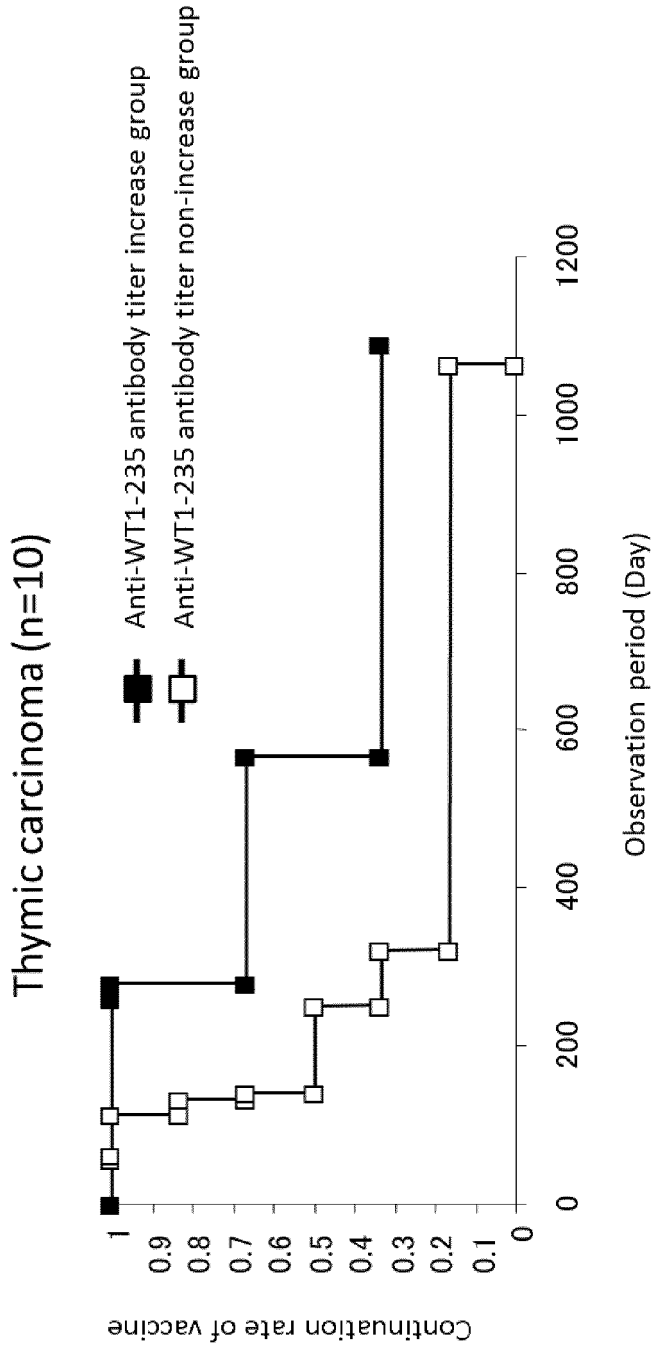


Fig. 18

Change of antibody titer in recurrent malignant glioma patient given WT1₃₃₂ peptide vaccine

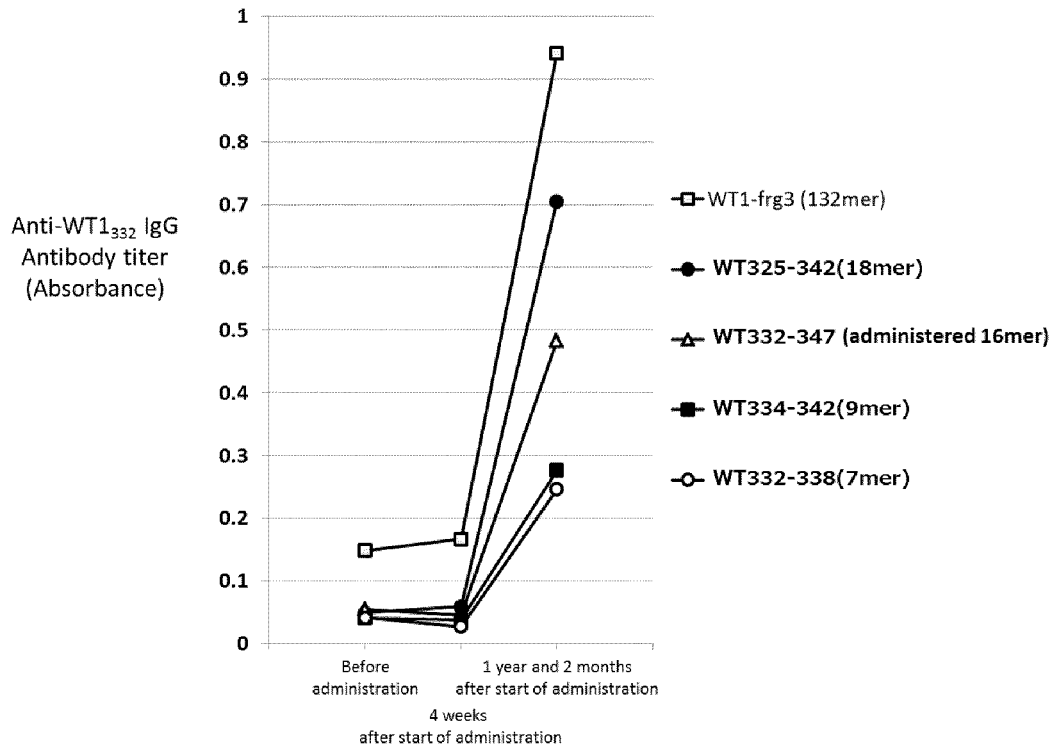


Fig. 19

Change of antibody titer in thymic carcinoma patient given WT1₂₃₅ peptide vaccine

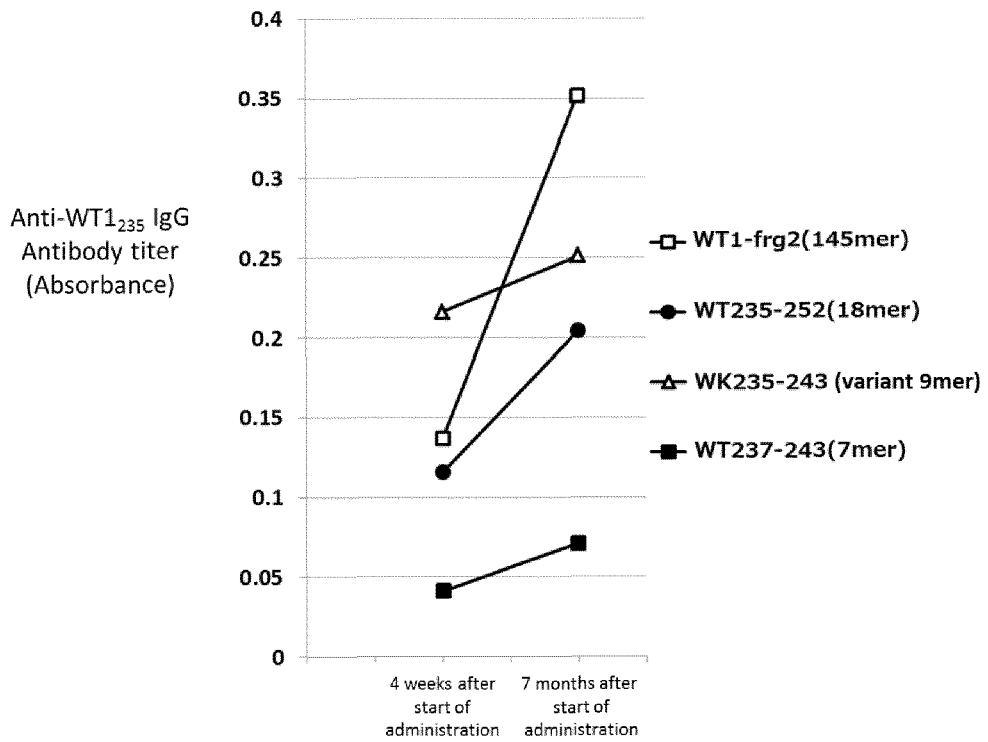
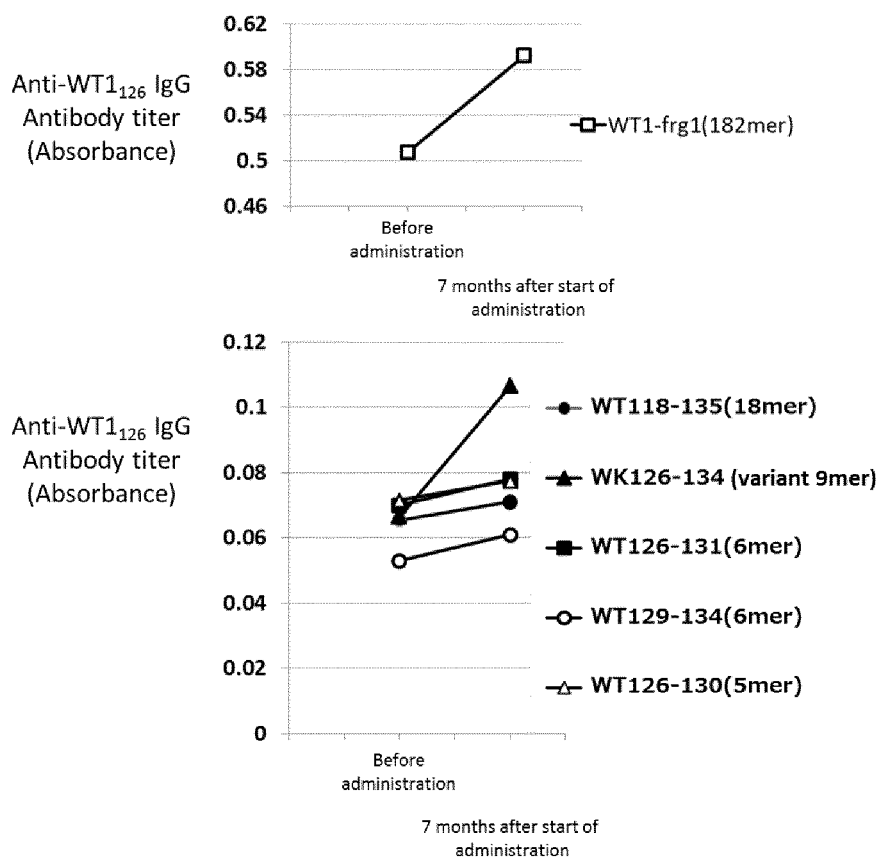


Fig. 20

Change of antibody titer in pancreatic cancer patient receiving combination therapy of WT1₁₂₆ peptide vaccine and anticancer agent



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2014/062630

A. CLASSIFICATION OF SUBJECT MATTER

G01N33/53(2006.01)i, A61K39/00(2006.01)i, A61P35/00(2006.01)i, A61P35/02(2006.01)i, G01N33/574(2006.01)i, C07K7/08(2006.01)n, C07K14/82(2006.01)n

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N33/53, A61K39/00, A61P35/00, A61P35/02, G01N33/574, C07K7/08, C07K14/82

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

Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2014
Kokai Jitsuyo Shinan Koho 1971-2014 Toroku Jitsuyo Shinan Koho 1994-2014

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

JSTPlus/JMEDPlus/JST7580 (JDreamIII), PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-------------|--|--------------------------|
| X Y A | WO 2013/039166 A1 (Osaka University), 21 March 2013 (21.03.2013), paragraphs [0043], [0046], [0065] & CN 103797369 A & CA 2846479 A1 | 1, 4-5, 7-14 6 2-3 |
| X Y A | JP 2006-267124 A (Haruo SUGIYAMA), 05 October 2006 (05.10.2006), paragraph [0038]; examples & US 2003/0138863 A1 & EP 1288661 A1 & WO 2001/090750 A1 | 1, 7-14 6 2-3 |
| Y | WO 2009/072610 A1 (International Institute of Cancer Immunology, Inc.), 11 June 2009 (11.06.2009), claims & US 2011/0070251 A1 & EP 2228072 A1 | 6 |

Further documents are listed in the continuation of Box C. See patent family annex.

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Date of the actual completion of the international search
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2014/062630

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|-----------------------|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | Wu F et al, Th1-biased humoral immune responses against Wilms tumor gene WT1 product in the patients with hematopoietic malignancies, Leukemia, 2005.02, 19(2), 268-274 | 2 |

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REFERENCES CITED IN THE DESCRIPTION

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| [标]发明人 | SUGIYAMA HARUO OJI YUSUKE | | |
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| CPC分类号 | A61P35/00 A61P35/02 C07K14/4748 G01N33/57407 G01N2500/00 G01N33/57496 G01N2333/47 | | |
| 优先权 | 2013101566 2013-05-13 JP | | |
| 其他公开文献 | EP2998740A4 | | |
| 外部链接 | Espacenet | | |

摘要(译)

提供了用于预测WT1肽免疫疗法中对受试者的临床效果的方法, 所述方法包括: a) 使来自受试者的样品与WT1抗原肽或其变体接触的步骤; b) 检测样品与WT1抗原肽或其变体结合的步骤, 从而测量样品中存在的抗WT1抗原肽IgG抗体滴度, 其特征在于抗WT1抗原肽IgG的增加受试者中的抗体滴度决定了有利的临床效果的实现。还提供了用于实施根据本发明的方法的试剂盒, 所述试剂盒包含WT1抗原肽或其变体。

SEKRPFMCAYPGCN**KRYFKLSHLQMH**
SRKHTGEKPYQCDFKDCERRFSRSDQ
LKRHQRRHTGVKPFQCKTCQRKFSRS
DHLKTHTRTHTGKTSEKPFSCRWPSC
QKKFARSDDELVRHHNMHQRNMTKLQL
AL