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(54) DIAGNOSIS AND TREATMENT OF CANCER USING ANTI-ITM2A ANTIBODY

DIAGNOSE UND BEHANDLUNG VON KREBS MITTELS ANTI-ITM2A-ANTIKÖRPERN

DIAGNOSTIC ET TRAITEMENT DU CANCER À L'AIDE D'UN ANTICORPS ANTI-ITM2A

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Description

Technical Field

Technical Field

[0001] The present invention relates to an antibody binding to an ITM2A protein.

Background Art

[0002] The ITM2A molecule is a type II membrane protein that is expressed in precursor cells involved in chondrogenesis or osteogenesis (Non Patent Literature 1). This protein is known to be expressed at the early stage of chondrogenesis (Non Patent Literature 2) to inhibit the chondrogenesis (Non Patent Literature 3). ITM2A is also expressed in T cells in the thymus gland (Non Patent Literature 4). The inhibition of T cell activation by an anti-ITM2A polyclonal antibody is disclosed (Patent Literature 1). Patent Literature 1 claims the treatment of T cell leukemia/lymphoma using an anti-ITM2A antibody, but does not specifically discuss the expression of ITM2A in T cell leukemia/lymphoma or the treatment of these diseases. According to the reports, the expression of ITM2A at the gene level in Ewing's sarcoma or acute myeloid leukemia has been confirmed by microarray analysis (Non Patent Literatures 5, 6, and 7). Nonetheless, it has not been specifically confirmed so far that T cell leukemia/lymphoma, Ewing's sarcoma, or acute myeloid leukemia can be treated using an anti-ITM2A antibody.

[0003] References cited herein are as shown below.

Citation List

Patent Literature

[0004] Patent Literature 1: WO2008137500

Non Patent Literature

[0005]

Non Patent Literature 1: J Biol Chem (1996) 271: 19475
Non Patent Literature 2: Biol Cell (2004) 96: 463
Non Patent Literature 3: Differentiation (2009) 78: 108
Non Patent Literature 4: J Exp Med (1999) 190: 217
Non Patent Literature 5: Cancer Res (2004) 64: 8213
Non Patent Literature 6: Cancer Res (2008) 68: 2176
Non Patent Literature 7: PLoS One (2010) 5: e9466

Summary of Invention

Technical Problem

[0006] An object of the present invention is to provide a novel antibody binding to an ITM2A protein.

Solution to Problem

[0007] The present inventors have found that ITM2A mRNA is expressed in Ewing's sarcoma having EWS-FLI1 translocation. The present inventors have prepared an anti-ITM2A monoclonal antibody and also found that ITM2A protein is expressed in Ewing's sarcoma, acute myeloid leukemia, T cell lymphoma, and T cell acute lymphocytic leukemia cell lines. The present inventors have further found that the anti-ITM2A monoclonal antibody exerts an antibody-dependent cell-mediated cytotoxicity (ADCC) activity and inhibits the growth of the Ewing's sarcoma cells, acute myeloid leukemia, and T cell lymphoma cell lines, and T cell acute lymphocytic leukemia cells in the presence of a toxin-conjugated secondary antibody. From these results, the present inventors have found that the anti-ITM2A antibody is useful in the treatment and diagnosis of cancer involving ITM2A expression, such as Ewing's sarcoma, acute myeloid leukemia, T cell lymphoma, and T cell acute lymphocytic leukemia, and consequently completed the present invention.

[0008] Specifically, the present invention provides a monoclonal antibody binding to an ITM2A protein. The present invention further provides a monoclonal antibody which binds to an ITM2A protein and has a cytotoxic activity against cells expressing the ITM2A protein. Preferably, the cytotoxic activity is an ADCC activity. The present invention also provides an anti-ITM2A monoclonal antibody conjugated with a cytotoxic substance.

[0009] The present invention further provides a pharmaceutical composition comprising the monoclonal antibody binding to an ITM2A protein as an active ingredient. The present invention further provides a cell growth inhibitor comprising the monoclonal antibody binding to an ITM2A protein as an active ingredient. The present invention further provides an anticancer agent comprising the monoclonal antibody binding to an ITM2A protein as an active ingredient.

[0010] The present invention further provides a pharmaceutical composition comprising the monoclonal antibody binding to an ITM2A protein and a pharmaceutically acceptable carrier. More specifically, the present invention provides the following [1] to [18]:

[1] A monoclonal antibody binding to a fragment of an ITM2A protein consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1, wherein the antibody has a cytotoxic activity.

[2] The monoclonal antibody of (1), wherein the monoclonal antibody is as described in any of the following (1) to (7):

(1) an antibody comprising an H chain having the amino acid sequence represented by SEQ ID NO: 9 as CDR1, the amino acid sequence represented by SEQ ID NO: 10 as CDR2, and the amino acid sequence represented by SEQ ID NO: 11 as CDR3, and an L chain having the amino acid sequence represented by SEQ ID NO: 12 as CDR1, the amino acid sequence represented by SEQ ID NO: 13 as CDR2, and the amino acid sequence represented by SEQ ID NO: 14 as CDR3;
(2) the antibody described in (1) which is a chimeric antibody;
(3) the antibody described in (1) which is a humanized antibody;
(4) the antibody described in (1) comprising the amino acid sequence of the heavy chain variable domain represented by SEQ ID NO: 32;
(5) the antibody described in (1) comprising the amino acid sequence of the light chain variable domain represented by SEQ ID NO: 34;
(6) the antibody described in (4) to (5) which is a chimeric antibody; and
(7) an antibody capable of inhibiting the binding of a second antibody to an ITM2A protein fragment consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1, wherein the second antibody is the antibody described in any of (1) to (6).

[3] The antibody according to (1) or (2), wherein the cytotoxic activity is

(a) an antibody-dependent cell-mediated cytotoxicity (ADCC) activity; or
(b) is a complement-dependent cytotoxicity (CDC) activity.

[4] The antibody according to any of (1) to (3), wherein:

(a) the antibody is conjugated with a cytotoxic substance; and/or
(b) the antibody inhibits cancer cell growth.

[5] The antibody according to (4(a)), wherein the antibody has an internalization activity.

[6] The antibody according to any of (1) to (5), wherein:

(a) the antibody has a human constant region; and/or
(b) the antibody is deficient in fucose added to its sugar chain or has a sugar chain having bisecting GlcNAc.

[7] The antibody according to (6(a)), wherein the antibody is a chimeric antibody, a humanized antibody, or a human antibody.

[8] A pharmaceutical composition comprising the antibody according to any of (1) to (7) as an active ingredient.

[9] A cell growth inhibitor comprising the antibody according to any of (1) to (7) as an active ingredient.

[10] An anticancer agent comprising the antibody according to any of (1) to (7) as an active ingredient.

[11] The antibody according to any of (1) to (7) for use in a method for treating cancer.

[12] A method for predicting the efficacy of cancer treatment by the administration of the antibody according to any of (1) to (7), comprising the step of detecting the expression level of an ITM2A in a biological sample collected from a test subject using the antibody of any one of (1) to (7), wherein the cancer is Ewing's sarcoma or blood cancer, wherein the sample is tissue or blood.

[13] The method according to (12), wherein an ITM2A protein in the sample collected from a test subject is detected.

[14] A method for determining the presence of cancer in a test subject using the antibody of any one of (1) to (7):

(A) comprising detecting an ITM2A protein in a sample collected from the test subject; or
(B) comprising the following steps:

(a) providing a sample collected from the test subject; and
(b) detecting an ITM2A protein contained in the sample of step (a) using an antibody binding to the ITM2A protein; or

(C) comprising the following steps:

(a) administering, to the test subject, a radioisotope-labeled antibody having a binding activity to an ITM2A protein; and
(b) detecting the accumulation of the radioisotope, wherein the cancer is Ewing's sarcoma or blood cancer, wherein the sample is tissues or blood.

[15] The monoclonal antibody of (6(b)), anticancer agent of (10), antibody for use in a method of (11), or the method of any one of (12) to (14), wherein the cancer whose presence is to be determined is:

(a) Ewing's sarcoma; or
(b) blood cancer.

[16] The monoclonal antibody, anticancer agent, antibody for use in a method of or method according to (15), wherein the Ewing's sarcoma has observable chromosomal translocation.
[17] The monoclonal antibody, anticancer agent, antibody for use in a method of or method according to (16), wherein the chromosomal translocation is t(11;22) (q24;q12).
[18] The monoclonal antibody, anticancer agent, antibody for use in a method of or method according to (15), wherein the blood cancer is any of T cell leukemia, T cell lymphoma, acute myeloid leukemia, B cell tumor, and multiple myeloma.

Brief Description of Drawings

[0011]

[Figure 1] Figure 1 shows the expression profile of ITM2A mRNA in normal tissues, Ewing's sarcoma cell lines, Ewing's sarcoma tissues, and blood cancer cell lines obtained using Human Exon 1.0 ST Array. Figure 1(A) shows the expression profile of ITM2A mRNA in various normal tissues. Figure 1(B) shows the expression profile of ITM2A mRNA in blood cancer cell lines, Ewing's sarcoma cell lines, and Ewing's sarcoma tissues.
[Figure 2] Figure 2 shows the binding activity of an isolated anti-ITM2A antibody to GST-ITM2A-L and GST-ITM2A-S obtained using ELISA assay. Figure 2(A) shows the binding activity of the anti-ITM2A antibody to GST-ITM2A-L. Figure 2(B) shows the binding activity of the anti-ITM2A antibody to GST-ITM2A-S.
[Figure 3] Figure 3 shows results of examining binding domains of isolated anti-ITM2A antibodies obtained using FACS. Figure 3(A) shows the binding activity of the anti-ITM2A antibodies to ITM2A-expressing CHO cells. Figure 3(B) shows the binding activity of the anti-ITM2A antibodies to ITM2A-furin-expressing CHO cells. Figure 3(C) shows the binding activity of the anti-ITM2A antibodies to CHO cells. Figure 3(D) shows the binding activity of an anti-HA antibody to ITM2A- or ITM2A-furin-expressing CHO cells.
[Figure 4] Figure 4 shows results of examining interspecies cross reactivity of the isolated anti-ITM2A antibodies. Figure 4(A) shows the binding activity of the anti-ITM2A antibodies to human ITM2A-expressing CHO cells. Figure 4(B) shows the binding activity of the anti-ITM2A antibodies to mouse ITM2A-expressing CHO cells.
[Figure 5] Figure 5 shows results of evaluating the binding activity of the isolated anti-ITM2A antibodies to ITM2A by Western blot. Lane A represents a whole cell lysate of ITM2A-expressing CHO cells. Lane B represents a whole cell lysate of ITM2A-furin-expressing CHO cells. Lane C represents a whole cell lysate of CHO cells. Figure 5(A) shows the binding activity of the antibody BE5-1. Figure 5(B) shows the binding activity of the antibody BE6-1. Figure 5(C) shows the binding activity of the antibody BE7-1-1. Figure 5(D) shows the binding activity of the antibody BE13-1. Figure 5(E) shows the binding activity of the anti-HA antibody.
[Figure 6] Figure 6 shows the expression of ITM2A in human cancer cell lines examined using FACS. Figure 6(A) shows the expression of ITM2A in the Ewing's sarcoma cell line A-673. Figure 6(B) shows the expression of ITM2A in the Ewing's sarcoma cell line RD-ES. Figure 6(C) shows the expression of ITM2A in the Ewing's sarcoma cell

line SK-ES-1. Figure 6(D) shows the expression of ITM2A in the Ewing's sarcoma cell line SK-N-MC. Figure 6(E) shows the expression of ITM2A in the T cell acute lymphocytic leukemia cell line CCRF-CEM. Figure 6(F) shows the expression of ITM2A in the T cell acute lymphocytic leukemia cell line Jurkat. Figure 6(G) shows the expression of ITM2A in the T cell acute lymphocytic leukemia cell line MOLT4. Figure 6(H) shows the expression of ITM2A in the T cell lymphoma cell line HuT78. Figure 6(1) shows the expression of ITM2A in the acute myeloid leukemia cell line KG-1a. Figure 6(J) shows the expression of ITM2A in the acute myeloid leukemia cell line TF-1a.

[Figure 7] Figure 7 shows an ADCC activity exerted by an isolated anti-ITM2A antibody against various cancer cell lines. Figure 7(A) shows the ADCC activity against the Ewing's sarcoma cell line A-673. Figure 7(B) shows the ADCC activity against the Ewing's sarcoma cell line SK-N-MC. Figure 7(C) shows the ADCC activity against the T cell acute lymphocytic leukemia cell line CCRF-CEM. Figure 7(D) shows the ADCC activity against the acute myeloid leukemia cell line KG-1a.

[Figure 8] Figure 8 shows a cytotoxic activity exerted by an isolated anti-ITM2A antibody against various cancer cell lines in the presence of a toxin-conjugated secondary antibody. Figure 8(A) shows the cytotoxic activity against the Ewing's sarcoma cell line A-673. Figure 8(B) shows the cytotoxic activity against the T cell acute lymphocytic leukemia cell line CCRF-CEM. Figure 8(C) shows the cytotoxic activity against the T cell lymphoma cell line HuT78.

[Figure 9] Figure 9 shows results of evaluating the expression of EWS-FLI1 fusion genes and ITM2A in clinical Ewing's sarcoma samples by PCR. Figure 9(A) shows the expression of EWS-FLI1 fusion genes in clinical Ewing's sarcoma samples. Figure 9(B) shows the expression of ITM2A in clinical Ewing's sarcoma samples.

Description of Embodiments

ITM2A

[0012] In the present invention, ITM2A is a type II membrane protein. The amino acid sequence of human ITM2A and a gene sequence encoding this amino acid sequence are disclosed in NCBI Accession Nos. NP_004858.1 (SEQ ID NO: 1) and NM_004867.4 (SEQ ID NO: 2), respectively. An ITM2A used in the present invention may be a splicing variant or a variant (or a mutant). In the present invention, the ITM2A protein is meant to include both of the full-length protein and its fragment. A monoclonal antibody of the invention though binds to a fragment of ITM2A consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1 and has a cytotoxic activity. The extracellular region of the ITM2A protein corresponds to positions 75 to 263 in the amino acid sequence of SEQ ID NO: 1.

Preparation of anti-ITM2A antibody

[0013] The present invention provides a monoclonal antibody binding to a fragment of an ITM2A protein consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1, wherein the antibody has a cytotoxic activity.

[0014] The monoclonal anti-ITM2A antibody used in the present invention needs only to bind to the ITM2A protein and is not limited by its origin, type, shape, etc. Specifically, an antibody known in the art can be used, such as a non-human animal antibody (e.g., a mouse, rat, or camel antibody), a human antibody, a chimeric antibody, or a humanized antibody. In the present invention, the antibody of the invention is a monoclonal antibody. The binding of the antibody to the ITM2A protein is preferably specific binding. The monoclonal anti-ITM2A antibody used in the present invention is an antibody that recognizes human ITM2A. In particular, the monoclonal antibody of the invention binds to a fragment of ITM2A consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1 and has a cytotoxic activity. In such a case, an antibody that specifically recognizes human ITM2A can be used. Alternatively, an antibody that simultaneously recognizes human ITM2A and non-human animal-derived ITM2A (e.g., mouse ITM2A) can also be preferably used.

[0015] The monoclonal anti-ITM2A antibody used in the present invention can be obtained using means known in the art. The monoclonal anti-ITM2A antibody used in the present invention is particularly preferably a mammal-derived monoclonal antibody. The mammal-derived monoclonal antibody encompasses, for example, those produced by hybridomas and those produced by hosts transformed with expression vectors containing an antibody gene by a genetic engineering approach.

[0016] Basically, monoclonal antibody-producing hybridomas can be prepared according to a technique known in the art as follows: first, animals are immunized with an ITM2A protein used as a sensitizing antigen according to a usual immunization method. Immunocytes obtained from the immunized animals can be fused with parental cells known in the art by a usual cell fusion method to obtain hybridomas. These hybridomas can be further screened for cells producing the antibody of interest by a usual screening method to select hybridomas producing anti-ITM2A antibodies.

[0017] Specifically, a monoclonal antibody is prepared, for example, as follows: first, ITM2A gene can be expressed to obtain ITM2A protein used as a sensitizing antigen to obtain antibodies. The nucleotide sequence of the ITM2A gene is disclosed in, for example, NCBI Accession No. NM_004867.4 (SEQ ID NO: 2). Specifically, an ITM2A-encoding gene sequence is inserted into an expression vector known in the art, with which appropriate host cells are then transformed. Then, the human ITM2A protein of interest can be purified from the host cells or from a culture supernatant thereof by a method known in the art. Also, purified natural ITM2A protein can be used similarly. Alternatively, fusion proteins comprising the desired partial polypeptide of the ITM2A protein fused with a different polypeptide may be used as immunogens, as used in the present invention. For example, antibody Fc fragments, peptide tags, and the like can be used for producing the fusion proteins serving as immunogens. Two or more genes respectively encoding the desired polypeptide fragments are fused in frame, and the fusion gene can be inserted into expression vectors to prepare expression vectors for the fusion proteins. The method for preparing the fusion proteins is described in Molecular Cloning 2nd ed. (Sambrook, J. et al., Molecular Cloning 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. Press, 1989).

[0018] An ITM2A protein thus purified can be used as a sensitizing antigen for the immunization of a mammal. A partial peptide of ITM2A may also be used as a sensitizing antigen. For example, the following peptides can be used as a sensitizing antigen:

a peptide obtained by chemical synthesis on the basis of the amino acid sequence of human ITM2A,
a peptide obtained by the incorporation of a portion of the ITM2A gene into an expression vector, followed by gene expression, and
a peptide obtained by the degradation of the ITM2A protein with a proteolytic enzyme.

[0019] The region and size of the partial peptide of ITM2A used are not limited provided that the antibody binds to a fragment of ITM2A consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1 and has a cytotoxic activity. A preferable region can be selected from the amino acid sequence constituting the extracellular domain of ITM2A (positions 75 to 263 in the amino acid sequence of SEQ ID NO: 2). The number of amino acids constituting the peptide serving as a sensitizing antigen is preferably at least 3 or more, for example, 5 or more or 6 or more. More specifically, peptides of 6 to 263 residues, preferably 7 to 200 residues, more preferably 8 to 100, 8 to 50, or 10 to 30 residues, can be used as sensitizing antigens.

[0020] The mammal to be immunized with the sensitizing antigen is not particularly limited. For obtaining the monoclonal antibody by the cell fusion method, the animal to be immunized is preferably selected in consideration of compatibility with the parental cells used in cell fusion. In general, a rodent is preferable as the animal to be immunized. Specifically, mouse, rat, hamster, or rabbit can be used as the animal to be immunized. In addition, monkey or the like may be used as the animal to be immunized.

[0021] The animal can be immunized with the sensitizing antigen according to a method known in the art. For example, a general method can involve immunizing the mammal with the sensitizing antigen by intraperitoneal or subcutaneous injection. Specifically, the sensitizing antigen is administered to the mammal several times at 4-to 21-day intervals. The sensitizing antigen is diluted with PBS (phosphate-buffered saline), saline, or the like at an appropriate dilution ratio and used in the immunization. The sensitizing antigen may be administered together with an adjuvant. For example, the antigen is mixed with a Freund's complete adjuvant and emulsified, and the resulting emulsion can be used as the sensitizing antigens. Also, an appropriate carrier can be used in the immunization with the sensitizing antigens. Particularly, in the case of using partial peptides having a small molecular weight as the sensitizing antigen, the sensitizing antigen peptide bound with a carrier protein such as albumin or keyhole limpet hemocyanin can be preferably used in the immunization.

[0022] On the other hand, the monoclonal antibody can also be obtained by DNA immunization. The DNA immunization is an immunostimulation method involving: immunizing an animal by the administration of vector DNA that has been constructed in a form capable of expressing an antigenic protein-encoding gene (e.g., the gene represented by SEQ ID NO: 2) in the immunized animal; and allowing the immunized animal to express the immunizing antigen *in vivo.* The DNA immunization can be expected to be superior to general immunization methods using the administration of a protein antigen as follows:

- the DNA immunization can provide immunostimulation with membrane protein (e.g., ITM2A) with its structure maintained; and
- the DNA immunization eliminates the need of purifying an immunizing antigen.

[0023] The DNA immunization, however, is difficult to combine with immunostimulation means such as an adjuvant. The amino acid sequence of ITM2A is highly homologous among species. The amino acid sequence of human-derived ITM2A represented by SEQ ID NO: 1 has identity of 99%, 98%, 96%, 94%, 94%, and 90% to, for example, the amino acid sequences of rabbit (*Oryctolagus cuniculus*)-, horse-, mouse-, giant panda-, rat-, and pig-derived ITM2A proteins, respectively. In light of such structural identity, it is an unexpected consequence that the monoclonal antibody binding to ITM2A was obtained by the DNA immunization and the administration of protein antigens involving immunostimulation means such as adjuvant.

[0024] In order to obtain the monoclonal antibody of the present invention by the DNA immunization, an animal is first immunized by the administration of a DNA expressing an ITM2A protein. An ITM2A-encoding DNA can be synthesized by a method known in the art such as PCR. The obtained DNA is inserted into an appropriate expression vector, which is then administered to an animal. For example, a commercially available expression vector such as pcDNA3.1 can be used as the expression vectors. Also, a method generally used can be used for administering the vectors to the animals. For example, gold particles with the expression vector adsorbed thereon can be inserted into cells using a gene gun to perform the DNA immunization.

[0025] A rise in the amount of the desired antibody is confirmed in the serum of the mammals thus immunized. Then, immunocytes are collected from the mammal and subjected to cell fusion. Particularly, spleen cells can be used as preferable immunocytes.

[0026] Mammalian myeloma cells are used in the cell fusion with the immunocytes. The myeloma cells preferably have an appropriate selection marker for screening. The selection marker refers to a character that can survive (or cannot survive) under particular culture conditions. For example, hypoxanthine-guanine phosphoribosyltransferase deficiency (hereinafter, abbreviated to HGPRT deficiency) or thymidine kinase deficiency (hereinafter, abbreviated to TK deficiency) is known in the art as the selection marker. Cells having the HGPRT or TK deficiency is sensitive to hypoxanthine-aminopterin-thymidine (hereinafter, abbreviated to HAT-sensitive). The HAT-sensitive cells are killed in a HAT selective medium because the cells fail to synthesize DNA. By contrast, these cells, when fused with normal cells, grow even in the HAT selective medium because the fused cells can continue DNA synthesis by use of the salvage pathway of the normal cells.

[0027] The cells having the HGPRT or TK deficiency can be selected in a medium containing 6-thioguanine or 8-azaguanine (hereinafter, abbreviated to 8AG) for the HGPRT deficiency or 5'-bromodeoxyuridine for the TK deficiency. The normal cells are killed by incorporating these pyrimidine analogs into their DNAs. By contrast, the cells deficient in these enzymes can survive in the selective medium because the cells cannot incorporate the pyrimidine analogs therein. A selection marker based on an index of 2-deoxystreptamine antibiotic (gentamicin analog) resistance brought about by a neomycin resistance gene is called G418 resistance. Various myeloma cells suitable for the cell fusion are known in the art. For example, the following myeloma cells can be used in the production of the monoclonal antibody according to the present invention:

P3 (P3x63Ag8.653) (J. Immunol.(1979) 123, 1548-1550),
P3x63Ag8U.1 (Current Topics in Microbiology and Immunology (1978) 81, 1-7),
NS-1 (Kohler. G. and Milstein, C. Eur. J. Immunol. (1976) 6, 511-519),
MPC-11 (Margulies. D.H. et al., Cell (1976) 8, 405-415),
SP2/0 (Shulman, M. et al., Nature (1978) 276, 269-270),
FO (de St. Groth, S. F. et al., J. Immunol. Methods (1980) 35, 1-21),
S194 (Trowbridge, I. S. J. Exp. Med. (1978) 148, 313-323),
R210 (Galfre, G. et al., Nature (1979) 277, 131-133), etc.

[0028] Basically, the cell fusion of the immunocytes with the myeloma cells is performed according to a method known in the art, for example, the method of Kohler and Milstein et al. (Kohler. G. and Milstein, C., Methods Enzymol. (1981) 73, 3-46).

[0029] More specifically, the cell fusion can be carried out, for example, in a usual nutrient culture medium in the presence of a cell fusion promoter. For example, polyethylene glycol (PEG) or hemagglutinating virus of Japan (HVJ) can be used as the fusion promoter. In addition, an auxiliary such as dimethyl sulfoxide is preferably added thereto, if desired, for enhancing fusion efficiency.

[0030] The ratio between the immunocytes and the myeloma cells used can be arbitrarily set. For example, the amount of the immunocytes is preferably set to 1 to 10 times that of the myeloma cells. For example, an RPMI1640 or MEM culture medium suitable for the growth of the myeloma cell line as well as a usual culture medium used in this kind of cell culture can be used as the culture medium in the cell fusion. A solution supplemented with serum (e.g., fetal calf serum (FCS)) can be further added to the culture medium.

[0031] In the procedures of the cell fusion, the immunocytes and the myeloma cells are well mixed in the predetermined amounts in the culture medium and mixed with a PEG solution preheated to approximately 37°C to form the fusion cells (hybridomas) of interest. In the procedures of the cell fusion, for example, PEG having an average molecular weight on the order of 1000 to 6000 can usually be added at a concentration of 30 to 60% (w/v) to the cell suspension containing the immunocytes and the myeloma cells. Subsequently, the appropriate culture medium exemplified above is sequentially added to the cell suspension, and its supernatant is removed by centrifugation. This removal procedure is repeated to remove the cell fusion agents or the like unfavorable for hybridoma growth.

[0032] The hybridomas thus obtained can be grown in a selective medium appropriate for the selection marker of the myeloma cells used in the cell fusion. For example, the cells having the HGPRT or TK deficiency can be selected by culture in a HAT medium (culture medium containing hypoxanthine, aminopterin, and thymidine). Specifically, when HAT-sensitive myeloma cells are used in the cell fusion, only cells successfully fused with normal cells can be grown selectively in the HAT culture medium. The culture using the HAT culture medium is continued for a time long enough to kill cells (non-fused cells) other than the hybridomas of interest. Specifically, the culture can generally be performed for a few days to a few weeks to select the hybridomas of interest. Subsequently, hybridomas producing the antibody of interest are screened for and cloned as single clones by a usual limiting dilution method. Alternatively, the antibody that recognizes ITM2A may be prepared by a method described in International Publication No. WO2003104453.

[0033] The screening for the antibody of interest and the cloning as single clones thereof can be preferably carried out by a screening method based on antigen-antibody reaction known in the art. For example, a carrier (e.g., beads made of polystyrene or the like) or a commercially available 96-well microtiter plate bound with antigens are reacted with the culture supernatant of the hybridomas. Subsequently, the carrier is washed and then reacted with enzyme-labeled secondary antibodies or the like. When the culture supernatant contains the antibody of interest reactive with the sensitizing antigen, the secondary antibodies bind to the carrier via this antibody. The secondary antibodies bound with the carrier can be finally detected to determine the presence of the antibody of interest in the culture supernatant. As described above, the hybridomas producing the desired antibody capable of binding to the antigen can be cloned by a limiting dilution method or the like. In this screening and cloning as single clones, in addition to the ITM2A protein used in the immunization, an ITM2A protein substantially identical thereto can be preferably used as an antigen. As an example of the ITM2A protein substantially identical thereto, cell lines expressing ITM2A, the extracellular domain of ITM2A, or oligopeptides consisting of a partial amino acid sequence constituting the domain can be used as an antigen.

[0034] In addition to the method for obtaining the hybridomas by immunizing non-human animal with the antigen, human lymphocytes may be sensitized with the antigen to obtain the desired antibody. Specifically, the human lymphocytes are first sensitized with an ITM2A protein *in vitro.* Subsequently, the sensitized lymphocytes are fused with appropriate fusion partners. For example, human-derived myeloma cells capable of dividing throughout their lives can be used as the fusion partners (Japanese Patent Publication No. 1-59878). For example, human myeloma cells such as U266 can be used as the fusion partners. The anti-ITM2A antibody obtained by this method is a human antibody having a binding activity to the ITM2A protein.

[0035] The anti-ITM2A human antibody can also be obtained by administering the antigen ITM2A protein to transgenic animals having all repertoires of human antibody genes or by immunizing the animals with a DNA that has been constructed so as to express ITM2A in the animals. Antibody-producing cells from the immunized animals can acquire immortalizing characters by cell fusion with appropriate fusion partners or infection with Epstein-Barr virus. From the immortalized cells thus obtained, human antibodies against the ITM2A protein can be isolated (WO1994025585, WO1993012227, WO1992003918, and WO1994002602). The immortalized antibody-producing cells can be further cloned as cells producing antibodies having the reaction specificity of interest. In the case of immunizing the transgenic animals, the immune systems of the animals recognize human ITM2A as foreigners. Thus, the human antibodies against human ITM2A can be easily obtained.

[0036] The monoclonal antibody-producing hybridomas thus prepared can be subcultured in a usual medium. The hybridomas can also be stored over a long period in liquid nitrogen.

[0037] The hybridomas can be cultured according to a usual method. The monoclonal antibody of interest can be obtained from the culture supernatant thereof. Alternatively, the hybridomas may be administered to a mammal compatible therewith and grown, and the monoclonal antibody can be obtained from the ascitic fluids thereof. The former method is suitable for obtaining highly pure antibodies.

[0038] In the present invention, an antibody encoded by an antibody gene cloned from an antibody-producing cell may also be used. The cloned antibody gene incorporated in an appropriate vector is expressed as an antibody encoded thereby in a host by the introduction of the vector into the host. Methods for the antibody gene isolation, the introduction into vector, and the transformation of host cells have already been established (e.g., Vandamme, A. M. et al., Eur. J. Biochem. (1990) 192, 767-775).

[0039] For example, cDNAs encoding the variable regions (V regions) of the anti-ITM2A antibody can be obtained from the anti-ITM2A antibody-producing hybridoma cells. In order to obtain the cDNAs, usually, the total RNA is first extracted from the hybridoma. For example, the following methods can be used for the total RNA extraction from the cells:

guanidine ultracentrifugation method (Chirgwin, J. M. et al., Biochemistry (1979) 18, 5294-5299), and
AGPC method (Chomczynski, P. et al., Anal. Biochem. (1987) 162, 156-159).

[0040] From the extracted total RNA, mRNA can be purified using mRNA Purification Kit (manufactured by GE Healthcare Bio-Sciences Corp.) or the like. Alternatively, a kit for directly extracting total mRNA from cells is also commercially available, such as QuickPrep mRNA Purification Kit (manufactured by GE Healthcare Bio-Sciences Corp.). The total mRNA may be obtained from the hybridoma using such a kit. From the mRNA thus obtained, antibody V region-encoding cDNAs can be synthesized using reverse transcriptase. Arbitrary 15- to 30-base sequences selected from sequences common to mouse antibody genes can be used as primers for cDNA synthesis. Specifically, the antibody V region-encoding cDNAs can be obtained using primers having a DNA sequence represented by any of SEQ ID NOs: 97 to 100. The cDNAs can be synthesized using reverse transcriptase such as AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (manufactured by Seikagaku Corp.). Alternatively, 5'-Ampli FINDER RACE Kit (manufactured by Clontech Laboratories, Inc.) and 5'-RACE PCR (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA (1988) 85, 8998-9002; and Belyavsky, A. et al., Nucleic Acids Res. (1989) 17, 2919-2932) may be appropriately used for the cDNA synthesis and amplification. In the course of such cDNA synthesis, appropriate restriction sites as described later can be introduced into both ends of the cDNAs.

[0041] Also, a cDNA library may be appropriately used for obtaining the antibody variable region-encoding genes. First, cDNAs are synthesized with mRNA extracted from the antibody-producing cells as template to obtain a cDNA library. A commercially available kit can be appropriately used in the cDNA library synthesis. In actuality, mRNA from only a small number of cells are obtained in a very small amount. Therefore, the direct purification of the mRNA generally results in low mRNA yields. Thus, the mRNA are usually purified from a mRNA preparation supplemented with carrier RNA shown to be free from the antibody genes. Alternatively, RNA may be extracted in a given amount. In such a case, antibody variable region-encoding mRNAs can be efficiently extracted from RNA obtained only from the antibody-producing cells without the addition of carrier RNA. The addition of the carrier RNA may be unnecessary for RNA extraction from, for example, 10 or more or 30 or more, preferably 50 or more antibody-producing cells.

[0042] The antibody genes can be amplified by PCR using the obtained cDNA library as template. A plurality of primers for the PCR amplification of the antibody genes are known in the art. For example, primers for human antibody gene amplification can be preferably designed on the basis of the disclosure of the paper (J. Mol. Biol. (1991) 222, 581-597) or the like. These primers have their respective nucleotide sequences differing on an immunoglobulin subclass basis. Thus, when cDNA library whose subclass is unknown is used as template, PCR is carried out using primers in consideration of every possibility.

[0043] From the PCR products thus obtained, the desired cDNA fragments are purified. Subsequently, the purified cDNA fragments are ligated with a vector DNA. The recombinant vectors thus prepared are introduced into E. *coli* or the like. A colony of E. *coli* transformed with the recombinant vectors is selected. A recombinant vector can be isolated from the E. *coli* that has formed the colony. Then, the cDNA inserted in the isolated recombinant vector can be sequenced by a method known in the art, for example, a dideoxynucleotide chain termination method.

[0044] Specifically, primers capable of amplifying genes respectively encoding γ1 to y5 heavy chains and κ and λ light chains can be used, for example, for the purpose of obtaining human IgG-encoding genes. Primers annealing to a portion corresponding to the hinge region are generally used as 3' primers for amplifying IgG variable region genes. On the other hand, primers appropriate for each subclass are used as 5' primers.

[0045] The PCR products obtained by amplification using the primers for gene amplification corresponding to these heavy and light chain subclasses are synthesized as their respective independent gene library. The library thus synthesized can be combined to reshape immunoglobulins comprising the heavy and light chains in combination. The immunoglobulins thus reshaped can be screened for the antibody of interest with their binding activities to ITM2A as an index.

[0046] For example, for obtaining the antibody against ITM2A, more preferably, the antibody specifically binds to ITM2A. The antibody binding to ITM2A can be screened for, for example, by the following steps:

(1) contacting antibodies comprising V regions encoded by the obtained cDNAs, with ITM2A;
(2) detecting a complex of an ITM2A-bound antibody; and
(3) selecting the antibody binding to ITM2A.

[0047] The complex of an ITM2A-bound antibody (antigen-antibody complex) is detected by a method known in the art. Specifically, a test antibody is contacted with ITM2A immobilized on a carrier. Next, a labeled antibody that recognizes the antibody is contacted therewith. When the labeled antibody remaining on the carrier after washing of the carrier is detected, the binding of the test antibody to ITM2A can be demonstrated. An enzymatically active protein such as peroxidase or β-galactosidase, or a fluorescent material such as FITC can be appropriately used in the antibody labeling. Also, fixed preparations of ITM2A-expressing cells can be appropriately used for evaluating the binding activity of the antibody.

[0048] Panning using phage vectors may be used as a method for screening for the antibody with its binding activity as an index. When the antibody genes are obtained as libraries of heavy and light chain subclasses as described above, a screening method using phage vectors is advantageous. Genes respectively encoding heavy and light chain variable regions can be linked via an appropriate linker sequence to prepare genes encoding single chain Fv (scFv) molecules in which the heavy and light variable regions of the antibody are arranged on one chain. The scFv-encoding genes can be inserted to phage vectors to obtain phages expressing scFvs on their surface. The phages thus obtained are contacted with the antigen of interest. Then, antigen-bound phages are recovered. In this way, DNAs encoding scFvs having a binding activity to the desired antigen can be recovered. This procedure can be repeated, if necessary, to concentrate scFvs having the desired binding activity to the antigen.

[0049] In the present invention, the polynucleotide encoding the antibody may be a polynucleotide encoding the full-length antibody or may be a polynucleotide encoding a portion of the antibody. The term "a portion of the antibody" refers to an arbitrary portion of the antibody molecule. Hereinafter, the term "antibody fragment" is also used to represent a portion of the antibody. The antibody fragment according to the present invention is preferably an antibody fragment comprising a complementarity determining region (CDR) of the antibody. An antibody fragment comprising heavy and light chain complementarity determining regions (CDRs) is also preferable. More preferably, the antibody fragment of the present invention is an antibody fragment comprising three CDRs of a heavy chain variable region or/and a light chain variable region.

[0050] After obtainment of each cDNA encoding the V region of the desired anti-ITM2A antibody, the cDNA is digested with restriction enzymes that recognize the restriction sites inserted in both ends of the cDNA. The restriction enzymes are preferably restriction enzymes that can recognize and digest a nucleotide sequence unlikely to appear in the nucleotide sequences constituting the antibody genes. For inserting one copy of the digested fragment in the correct direction in an expression vector, restriction sites that provide cohesive ends are preferably inserted in the ends of the cDNA. The anti-ITM2A antibody V region-encoding cDNAs thus digested can be inserted to appropriate expression vectors to obtain antibody expression vectors. In this case, antibody constant region (C region)-encoding genes and the V region-encoding genes can be fused in frame to obtain chimeric antibodies. In this context, the chimeric antibodies refer to antibodies comprising constant and variable regions of different origins. Thus, heterogeneous (e.g., mouse-human) chimeric antibodies as well as human-human homogeneous chimeric antibodies are also encompassed by the chimeric antibody according to the present invention. The V region genes may be inserted in frame to expression vectors preliminarily having constant region-encoding DNA inserts to construct chimeric antibody expression vectors.

[0051] Specifically, for example, recognition sequences for restriction enzymes that digest the ends of the restriction sites inserted in both ends of the V region gene can be located on the 5' side of each expression vector carrying the DNA encoding the desired antibody constant region (C region). This expression vector and a vector having an insert of the V region gene are digested with the same combination of restriction enzymes. The resulting expression vector and V region gene are fused in frame to construct a chimeric antibody expression vector.

[0052] In order to produce the anti-ITM2A antibody used in the present invention, the antibody genes can be inserted into an expression vector such that these genes are expressed under the control of expression control regions. The expression control regions for antibody expression encompass, for example, enhancers and promoters. Subsequently, appropriate host cells can be transformed with these expression vectors to obtain recombinant cells expressing anti-ITM2A antibody-encoding DNAs.

[0053] For the antibody gene expression, the antibody heavy chain (H chain)- and light chain (L chain)-encoding DNAs can be incorporated separately in different expression vectors. The same host cell can be co-transfected with the H chain- and L chain-incorporated vectors and thereby allowed to express antibody molecules comprising the H and L chains. Alternatively, the H chain- and L chain-encoding DNAs may be inserted to a single expression vector, with which host cells can also be transformed to express antibody molecules comprising the H and L chains (WO1994011523).

[0054] Expression systems having many combinations of hosts and expression vectors used for preparing the antibody by introducing the isolated antibody genes into appropriate hosts are known in the art. All of these expression systems can be applied to the present invention. In the case of using eukaryotic cells as the hosts, animal, plant, or fungus cells can be used. Specifically, examples of the animal cells that can be used in the present invention can include the following cells:

(1) mammalian cells such as CHO, COS, myeloma, BHK (baby hamster kidney), Hela, Vero, HEK293, Ba/F3, HL-60, Jurkat, and SK-HEP1 cells;
(2) amphibian cells such as *Xenopus* oocytes; and
(3) insect cells such as sf9, sf21, and Tn5 cells.

[0055] Alternatively, antibody gene expression systems using cells derived from the genus *Nicotiana* (e.g., *Nicotiana tabacum*) as the plant cells that can be used in the present invention are known in the art. Cultured callus cells can be appropriately used for the plant cell transformation.

[0056] The following cells can be used as the fungus cells according to the present invention:

cells derived from yeasts of the genus *Saccharomyces* (e.g., *Saccharomyces cerevisiae*) and the genus *Pichia* (e.g., *Pichia pastoris*), and
cells derived from filamentous fungi of the genus *Aspergillus* (e.g., *Aspergillus niger*).

[0057] Alternatively, antibody gene expression systems using prokaryotic cells as hosts are also known in the art as the expression systems that can be used in the present invention. In the case of using, for example, bacterial cells, cells of bacteria such as E. *coli* and *Bacillus subtilis* can be preferably used in the present invention.

[0058] In the case of using mammalian cells in the present invention, a useful promoter routinely used (regardless of the presence or absence of an enhancer), the antibody gene to be expressed, and a poly A signal to be located 3'-downstream thereof can be functionally linked so that the antibody gene is expressed. Examples of the promoter/enhancer preferably include a human cytomegalovirus immediate early promoter/enhancer.

[0059] In addition, for example, virus promoters/enhancers or mammalian cell-derived promoters/enhancers (e.g., human elongation factor 1α (HEF1α)) may be used for the antibody expression. Specific examples of the viruses whose promoter/enhancer can be used preferably include retrovirus, polyomavirus, adenovirus, and simian virus 40 (SV40).

[0060] The SV40 promoter/enhancer can be preferably used according to the method of Mulligan et al. (Nature (1979) 277, 108). Also, the HEF1α promoter/enhancer can be easily used in the desired gene expression by the method of Mizushima et al. (Nucleic Acids Res. (1990) 18, 5322).

[0061] In the case of using E. *coli* as hosts, a useful promoter routinely used, a signal sequence for antibody secretion, and the antibody gene to be expressed can be functionally linked so that the gene is expressed. Examples of the promoter used preferably include lacZ and araB promoters. The lacZ promoter can be used according to the method of Ward et al. (Nature (1989) 341, 544-546; and FASEBJ. (1992) 6, 2422-2427). Alternatively, the araB promoter can be preferably used in the gene expression of interest according to the method of Better et al. (Science (1988) 240, 1041-1043).

[0062] In the case of antibody production in E. *coli* periplasm, a pelB signal sequence (Lei, S. P. et al., J. Bacteriol. (1987) 169, 4379) can be used for antibody secretion. The antibody thus produced in the periplasm is isolated and then refolded by use of a protein denaturant such as guanidine hydrochloride of urea such that the resulting antibody has the desired binding activity.

[0063] In the case of antibody production using animal cells, the signal sequence of the heavy or light chain gene of the antibody is preferably used as a signal sequence required for the extracellular secretion of the antibody. Alternatively, the signal sequence of a secretory protein such as IL-3 or IL-6 is also preferably used.

[0064] A replication origin derived from SV40, polyomavirus, adenovirus, bovine papillomavirus (BPV), or the like can be inserted in the expression vectors. A selection marker may be further inserted in the expression vectors for increasing the copy numbers of the inserted genes in the host cells. Specifically, the following selection markers can be used:

aminoglycoside phosphotransferase (APH) gene,
thymidine kinase (TK) gene,
E. coli xanthine-guanine phosphoribosyltransferase (Ecogpt) gene,
dihydrofolate reductase (dhfr) gene, etc.

[0065] Subsequently, the host cells transformed by the introduction of these expression vectors are cultured *in vitro* or *in vivo.* In this way, the antibody of interest is produced in, for example, the cultures of the host cells. The host cells are cultured according to a method known in the art. For example, a DMEM, MEM, RPMI1640, or IMDM medium can be used and may be used in combination with a solution supplemented with serum such as fetal calf serum (FCS).

[0066] The antibody thus expressed and produced can be purified by using, alone or in appropriate combination, usual protein purification methods known in the art. For example, affinity or chromatography columns (e.g., protein A columns), filters, ultrafiltration, salting-out, and dialysis can be selected and combined appropriately to separate and purify the antibody (Antibodies A Laboratory Manual. Ed Harlow, David Lane, Cold Spring Harbor Laboratory, 1988).

[0067] In addition to the host cells, transgenic animals may be used for the recombinant antibody production. Specifically, the antibody of interest can be obtained from animals transfected with the genes encoding this antibody of interest. For example, the antibody genes can be inserted in frame into genes encoding proteins specifically produced in milk to construct fusion genes encoding the protein and the antibody. For example, goat β casein can be used as the proteins secreted into milk. DNA fragments comprising the fusion genes having the antibody gene insert are injected into goat embryos, which are in turn introduced into female goats. From milk produced by transgenic goats (or progeny thereof) brought forth by the goats that have received the embryos, the desired antibody can be obtained as a fusion protein with the milk protein. In addition, hormone is appropriately administered to the transgenic goats for increasing the amount of milk containing the desired antibody produced from the transgenic goats (Ebert, K. M. et al., Bio/Technology (1994) 12, 699-702).

[0068] Non-human animal antibody-derived C regions can be used as the C regions in the recombinant antibody of the present invention. For example, Cγ1, Cγ2a, Cγ2b, Cy3, Cμ, Cδ, Cα1, Cα2, and Cε can be used as mouse antibody H chain C regions, and Cκ and Cγ can be used as mouse antibody L chain C regions. Alternatively, an antibody of a rat, a rabbit, a goat, sheep, a camel, a monkey, or the like may be used as an antibody of an animal other than the mouse. Their sequences are known in the art. The C regions may be appropriately modified for improving the stability of the antibody itself or of its production.

[0069] In the case of administering the antibody according to the present invention to humans, a genetically recombinant antibody that has been engineered artificially can be administered, for example, for the purpose of reducing heteroantigenicity in humans. The genetically recombinant antibody encompasses, for example, chimeric antibodies and humanized antibodies. These engineered antibodies can be produced using a method known in the art.

[0070] The chimeric antibodies refer to antibodies comprising variable and constant regions of different origins linked to each other. For example, mouse-human heterogeneous chimeric antibodies are antibodies consisting of the heavy and light chain variable regions of a mouse antibody and the heavy and light chain constant regions of a human antibody. Mouse antibody variable region-encoding DNAs ligated in frame with human antibody constant region-encoding DNAs can be incorporated into expression vectors to prepare chimeric antibody-expressing recombinant vectors. Cells transformed with these vectors (recombinant cells) can be cultured to express the DNA inserts. The chimeric antibodies produced during the culture can be obtained from the cultures of the recombinant cells. Human antibody C regions are preferably used as the C regions of the chimeric antibodies and humanized antibodies.

[0071] For example, Cγ1, Cγ2, Cγ3, Cγ4, Cμ, Cδ, Cα1, Cα2, and Cε can be used as H chain C regions. Also, Cκ and Cλ can be used as L chain C regions. The amino acid sequences of these C regions and nucleotide sequences encoding these amino acid sequences are known in the art. The human antibody C regions may be appropriately modified for improving the stability of the antibody itself or of its production.

[0072] In general, the chimeric antibodies are composed of non-human animal-derived antibody V regions and human antibody-derived C regions. By contrast, the humanized antibodies are composed of non-human animal-derived antibody complementarity determining regions (CDRs), human antibody-derived framework regions (FRs), and human antibody-derived C regions. The humanized antibodies are useful as active ingredients for a therapeutic agent of the present invention, owing to their reduced immunogenicity in the human body.

[0073] Each antibody variable region is typically composed of three complementarity determining regions (CDRs) flanked by four framework regions (FRs). The CDR regions substantially determine the binding specificity of the antibody for its antigen. The CDRs have diverse amino acid sequences. On the other hand, amino acid sequences constituting the FRs are often highly analogous even among antibodies having binding specificity for different antigens. Therefore, in general, the binding specificity of a certain antibody can allegedly be transplanted to other antibodies through CDR grafting to FRs.

[0074] The humanized antibodies are also called reshaped human antibodies. Specifically, for example, humanized antibodies comprising non-human animal (e.g., mouse) antibody CDRs grafted in human antibodies are known in the art. General gene recombination approaches are also known for obtaining the humanized antibodies.

[0075] Specifically, for example, Overlap Extension PCR is known in the art as a method for grafting mouse antibody CDRs to human FRs. In the Overlap Extension PCR, a nucleotide sequence encoding each mouse antibody CDR to be grafted is added to the sequences of primers used for human antibody FR synthesis. The primers used are prepared for each of the four FRs. For grafting the mouse CDRs to the human FRs, in general, it is allegedly advantageous to select human FRs highly homologous to the FRs of the mouse antibody from which the mouse CDRs are derived, in order to maintain the CDR functions. Specifically, in general, the mouse CDRs are preferably grafted to human FRs consisting of amino acid sequences highly homologous to those of the mouse FRs adjacent to the mouse CDRs to be grafted.

[0076] As described above, the nucleotide sequences to be ligated are designed such that the sequences are ligated in frame. The human FR-encoding nucleotide sequences are individually synthesized by PCR using their respective primers. The resulting PCR products contain the mouse CDR-encoding DNA added to each human FR-encoding sequence. The mouse CDR-encoding nucleotide sequences are designed such that the nucleotide sequence in each product overlaps with another. Subsequently, the overlapping CDR portions in the PCR products synthesized with human antibody genes as templates are annealed to each other for complementary strand synthesis reaction. Through this reaction, the human FR sequences are ligated via the mouse CDR sequences.

[0077] Finally, the full-length gene of the V region comprising three CDRs and four FRs ligated is amplified by PCR using primers that respectively anneal to the 5' and 3' ends thereof and have an added recognition sequence for an appropriate restriction enzyme. The V region gene DNA thus obtained and a human antibody C region-encoding DNA can be inserted into expression vectors such that these DNAs are fused in frame to prepare vectors for human-type antibody expression. These expression vectors are introduced into hosts to establish recombinant cells. The recombinant cells are cultured for the expression of the humanized antibody-encoding DNA to produce the humanized antibodies into the cultures of the cultured cells (EP239400 and WO1996002576).

[0078] The humanized antibodies thus prepared can be evaluated for their binding activities to the antigen by qualitative or quantitative assay to thereby preferably select human antibody FRs such that these FRs allow CDRs to form a favorable antigen-binding site when ligated via the CDRs. If necessary, human antibody FR amino acid residue(s) may be substituted such that the CDRs of the resulting reshaped human antibody form an appropriate antigen-binding site. For example, the desired mutation can be introduced in the amino acid sequence of human FR by the application of the PCR method used in the mouse CDR grafting to the human FRs. Specifically, a mutation of a partial nucleotide sequence can be introduced to the primers annealing to a human FR nucleotide sequence. The human FR nucleotide sequence synthesized using such primers contains the mutation thus introduced so as to bring about the desired amino acid substitution. The variant antibodies having the substituted amino acid(s) can be evaluated for their binding activities to the antigen by the same assay as above to select variant FR sequences having the desired properties (Sato, K. et al., Cancer Res, 1993, 53, 851-856).

[0079] As described above, the method for obtaining human antibodies is also known in the art. In addition, a technique of obtaining human antibodies by panning using human antibody libraries is also known. For example, human antibody V regions are expressed as a single chain antibody (scFv) on the surface of phages by a phage display method. The gene of a phage selected with its binding activity to the antigen as an index can be analyzed to determine DNA sequences encoding the V regions of the human antibody binding to the antigen. After the determination of the DNA sequence of the antigen-binding scFv, the V region sequences fused in frame with the sequences of the desired human antibody C regions are inserted to appropriate expression vectors to prepare human antibody expression vectors. The expression vectors are introduced into the preferable expression cells as exemplified above. The expression cells are cultured for the expression of the human antibody-encoding genes to obtain the human antibodies. These methods are already known in the art (WO1992001047, WO19992020791, WO1993006213, WO1993011236, WO1993019172, WO1995001438, and WO1995015388).

[0080] In a preferable aspect, examples of the antibody used in the present invention also include an antibody having a human constant region, as described above.

[0081] The antibody of the present invention encompasses not only bivalent antibodies typified by IgG but also monovalent antibodies or polyvalent antibodies typified by IgM as long as these antibodies bind to the ITM2A protein. The polyvalent antibody of the present invention encompasses polyvalent antibodies having antigen-binding sites, all of which are the same as each other or some or all of which are different from each other. The antibody of the present invention is not limited to whole antibody molecules, and a low-molecular antibody or a modified form thereof can be preferably used as long as the antibody binds to the ITM2A protein.

[0082] The low-molecular antibody encompasses an antibody fragment deficient in a portion of the whole antibody (e.g., whole IgG). Such partial deficiency of the antibody molecule is accepted as long as the resulting antibody fragment is capable of binding to the ITM2A antigen. The antibody fragment according to the present invention preferably comprises one or both of heavy chain variable (VH) and light chain variable (VL) regions. Also, the antibody fragment according to the present invention preferably contains CDRs. The amino acid sequence of VH or VL may have substitution, deletion, addition, and/or insertion. The antibody fragment of the present invention may be deficient in a portion of one or both of VH and VL as long as the resulting antibody fragment is capable of binding to the ITM2A antigen. Alternatively, a chimerized or humanized variable region may be used. Specific examples of the antibody fragment preferably include Fab, Fab', F(ab')2, and Fv. Specific examples of the low-molecular antibody preferably include Fab, Fab', F(ab')2, Fv, scFv (single chain Fv), Diabody, sc(Fv)2 (single chain (Fv)2), and scFv-Fc. Multimers (e.g., dimmers, trimers, tetramers, and polymers) of these antibodies are also encompassed by the low-molecular antibody of the present invention.

[0083] The antibody fragment can be obtained by the enzymatic treatment of the whole antibody. For example, papain, pepsin, or plasmin is known in the art as an enzyme for forming the antibody fragment. Alternatively, genes encoding such antibody fragments may be constructed, and these genes can be introduced into expression vectors so that the antibody fragments are expressed in appropriate host cells (e.g., Co, M.S. et al., J. Immunol. (1994) 152, 2968-2976; Better, M. & Horwitz, A. H. Methods in Enzymology (1989) 178, 476-496; Plueckthun, A. & Skerra, A. Methods in Enzymology (1989) 178, 476-496; Lamoyi, E., Methods in Enzymology (1989) 121, 652-663; Rousseaux, J. et al., Methods in Enzymology (1989) 121, 663-669; and Bird, R. E. et al., TIBTECH (1991) 9, 132-137) .

[0084] Each digestive enzyme recognizes a particular amino acid sequence in the whole antibody and cleaves the whole antibody into the following antibody fragment having a particular structure:

papain digestion: F(ab)2 or Fab,
pepsin digestion: F(ab')2 or Fab', and
plasmin digestion: Facb.

[0085] The use of a genetic engineering approach for the antibody fragments thus obtained enzymatically can delete an arbitrary portion of the antibody.

[0086] Thus, the low-molecular antibody according to the present invention encompasses antibody fragments that lack an arbitrary region as long as these antibody fragments have binding affinity for ITM2A.

[0087] The Diabody refers to a bivalent antibody fragment constructed by gene fusion (e.g., Holliger P et al., Proc. Natl. Acad. Sci. USA (1993) 90, 6444-6448, EP404097, and WO1993011161). The Diabody is a dimer composed of two polypeptide chains. Usually, each of the polypeptide chains constituting the dimer comprises VL and VH linked in frame via a linker. The linker in the Diabody is generally too short to form a single chain variable region fragment having an antigen-binding site in which VL and VH on the same polypeptide chain are associated with each other. Specifically, the number of amino acid residues constituting the linker is, for example, approximately 5 residues. Therefore, VL and

VH encoded on the same polypeptide chain form a dimer by association with VH and VL, respectively, on another polypeptide chain. As a result, the Diabody has two antigen-binding sites.

[0088] The scFv is obtained by linking VH and VL of the antibody. In the scFv, VH and VL are linked via a linker, preferably, a peptide linker (Huston, J. S. et al., Proc. Natl. Acad. Sci. USA, (1988), 85, 5879-5883). VH and VL in the scFv may be derived from any of the antibodies described herein. The structure of the peptide linker that links VH and VL is not particularly limited. For example, an arbitrary single chain peptide of approximately 3 to 25 residues can be used as the linker. Specifically, for example, a peptide linker described later can be used.

[0089] VL and VH can be linked, for example, by the PCR method described above. First, of the following DNA sequences, DNAs encoding the whole or desired partial amino acid sequence are used as templates for linking VL and VH by PCR:

DNA sequences encoding the H chain or VH of the antibody, and
DNA sequences encoding the L chain or VL of the antibody.

[0090] The VL-encoding DNA and the VH-encoding DNA are separately amplified by PCR using a pair of primers respectively having both terminal partial sequences of each DNA to be amplified. Subsequently, a DNA encoding the peptide linker moiety is prepared. The peptide linker-encoding DNA can also be synthesized using PCR. Specifically, nucleotide sequences that can be linked to the amplification products of the VL and VH nucleotide sequences synthesized separately are respectively added in advance to the 5' sequences of primers used. Subsequently, PCR is performed using each DNA of [VH-encoding DNA]-[peptide linker-encoding DNA]-[VL-encoding DNA] and primers for assembly PCR.

[0091] The primers for assembly PCR consist of the combination of a primer annealing to the 5' sequence of the [VH-encoding DNA] and a primer annealing to the 3' sequence of the [VL-encoding DNA]. Specifically, the primers for assembly PCR are a primer set that allows PCR amplification of a DNA encoding the full-length sequence of the scFv to be synthesized. By contrast, the [peptide linker-encoding DNA] contains a preliminarily added nucleotide sequence that can be linked to the VH- and VL-encoding DNAs. As a result, these DNAs are linked and, further, finally prepared into a full-length scFv gene amplification product by PCR using the primers for assembly PCR. Once the scFv-encoding DNA is prepared, expression vectors containing this DNA and cells transformed with the expression vectors (recombinant cells) can be obtained according to a routine method. In addition, the resulting recombinant cells can be cultured for the expression of the scFv-encoding DNA to obtain the scFv from the cultures of the cells.

[0092] The scFv-Fc is a low-molecular antibody comprising an Fc region fused in frame to scFv comprising antibody VH and VL (Cellular & Molecular Immunology (2006) 3, 439-443). The origin of the scFv used in scFv-Fc preparation is not particularly limited, and, for example, scFv derived from IgM can be preferably used. The origin of the Fc is not particularly limited, and, for example, Fc derived from mouse IgG (mouse IgG2a, etc.) or human IgG (human IgG1, etc.) can be appropriately used. Thus, in a preferable aspect, examples of the scFv-Fc can include scFv-Fc comprising an IgM antibody scFv fragment linked to mouse IgG2a CH2 (e.g., Cy2) and CH3 (e.g., Cy3) via the hinge region (Hy) of mouse IgG2a, and scFv-Fc comprising an IgM antibody scFv fragment linked to human IgG1 CH2 and CH3 via the hinge region of human IgG1.

[0093] The sc(Fv)2 is a low-molecular antibody having a single chain polypeptide formed by two VHs and two VLs linked via linkers or the like (Hudson et al, J. Immunol. Methods (1999) 231, 177-189). The sc(Fv)2 can be prepared, for example, by linking two scFvs via a linker.

[0094] Examples of the sc(Fv)2 include an antibody wherein two VHs and two VLs are aligned as VH, VL, VH, and VL (i.e., [VH]-linker-[VL]-linker-[VH]-linker-[VL]) in this order starting at the N-terminus of the single chain polypeptide.

[0095] The order of two VHs and two VLs is not particularly limited to the arrangement described above and may be any order of arrangement. Examples thereof can also include the following arrangements:

[VL]-linker-[VH]-linker-[VH]-linker-[VL],
[VH]-linker-[VL]-linker-[VL]-linker-[VH],
[VH]-linker-[VH]-linker-[VL]-linker-[VL],
[VL]-linker-[VL]-linker-[VH]-linker-[VH], and
[VL]-linker-[VH]-linker-[VL]-linker-[VH].

[0096] For example, an arbitrary peptide linker or a synthetic compound linker (e.g., linkers disclosed in Protein Engineering (1996) 9 (3), 299-305) that can be introduced by genetic engineering can be preferably used as the linker that links the antibody variable regions. The peptide linker can be preferably used as the linker according to the present invention. The length of the peptide linker is not particularly limited and may be appropriately selected by those skilled in the art according to the purpose. The number of amino acid residues constituting the peptide linker is usually 1 to 100 amino acids, preferably 3 to 50 amino acids, more preferably 5 to 30 amino acids, particularly preferably 12 to 18 amino acids (e.g., 15 amino acids).

[0097] An arbitrary sequence can be appropriately adopted as the amino acid sequence constituting the peptide linker as long as this sequence does not inhibit the binding effect of the scFv. For example, the following amino acid sequences can be used for the peptide linker:

Ser,

Gly-Ser,

Gly-Gly-Ser,

Ser-Gly-Gly,

Gly-Gly-Gly-Ser (SEQ ID NO: 79),

Ser-Gly-Gly-Gly (SEQ ID NO: 80),
Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 81),
Ser-Gly-Gly-Gly-Gly (SEQ ID NO: 82),
Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 83),
Ser-Gly-Gly-Gly-Gly-Gly (SEQ ID NO: 84),
Gly-Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 85),
Ser-Gly-Gly-Gly-Gly-Gly-Gly (SEQ ID NO: 86),
$(\text{Gly-Gly-Gly-Gly-Ser})_n$, and
$(\text{Ser-Gly-Gly-Gly-Gly})_n$
wherein n is an integer of 1 or larger.

[0098] The amino acid sequence of the peptide linker can be appropriately selected by those skilled in the art according to the purpose. For example, the integer n that determines the length of the peptide linker is usually 1 to 5, preferably 1 to 3, more preferably 1 or 2.

[0099] Accordingly, in a particularly preferable aspect, examples of the sc(Fv)2 according to the present invention can include the following sc(Fv)2:
[VH]-peptide linker (15 amino acids)-[VL]-peptide linker (15 amino acids)-[VH]-peptide linker (15 amino acids)-[VL] .

[0100] Alternatively, the V regions may be linked using the chemically synthesized linker (chemical cross-linking agent). Cross-linking agents usually used in the crosslink of peptide compounds or the like can be preferably used in the present invention. For example, chemical cross-linking agents as shown below are known in the art. These cross-linking agents are commercially available:

N-hydroxysuccinimide (NHS),
disuccinimidyl suberate (DSS),
bis(sulfosuccinimidyl) suberate (BS3),
dithiobis(succinimidyl propionate) (DSP),
dithiobis(sulfosuccinimidyl propionate) (DTSSP),
ethylene glycol bis(succinimidyl succinate) (EGS),
ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS),
disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST),
bis[2-(succinimidoxycarbonyloxy)ethyl]sulfone (BSOCOES), and
bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl]sulfone (sulfo-BSOCOES), etc.

[0101] Three linkers are usually necessary for linking four antibody variable regions. A plurality of linkers having the same sequences may be used, and linkers having different sequences can also be preferably used. In the present invention, the low-molecular antibody is preferably Diabody or sc(Fv)2. Such a low-molecular antibody is formed by the treatment of the whole antibody with an enzyme, for example, papain or pepsin, as described above. Alternatively, such a low-molecular antibody is isolated from the cultures of appropriate host cells transfected with expression vectors having an insert of DNA encoding the antibody fragment (e.g., Co, M. S. et al., J. Immunol. (1994) 152, 2968-2976; Better, M. and Horwitz, A. H., Methods Enzymol. (1989) 178, 476-496; Pluckthun, A. and Skerra, A., Methods Enzymol. (1989) 178, 497-515; Lamoyi, E., Methods Enzymol. (1986) 121, 652-663; Rousseaux, J. et al., Methods Enzymol. (1986) 121, 663-669; and Bird, R. E. and Walker, B. W., Trends Biotechnol. (1991) 9, 132-137).

[0102] The antibody of the present invention also encompasses not only monovalent antibodies but also polyvalent antibodies. The polyvalent antibody of the present invention encompasses polyvalent antibodies having antigen-binding sites, all of which are the same as each other or some or all of which are different from each other.

[0103] Antibodies conjugated with various molecules such as polyethylene glycol (PEG) may be used as modified antibodies. Alternatively, antibodies conjugated with cytotoxic substances such as chemotherapeutic agents, toxic peptide, or radioactive chemicals may also be used as modified antibodies. Such modified antibodies (hereinafter, referred to as antibody conjugates) can be obtained by chemically modifying the obtained antibody. A method for the antibody modification has already been established in the art. The toxic peptide-conjugated modified antibodies can be obtained by allowing appropriate host cells to express fusion genes of the antibody genes linked in frame with genes encoding the toxic peptides, and then isolating the resulting fusion proteins from the cultures of the cells. As described later, the modified antibody of the present invention may be obtained in a molecular form such as a bispecific antibody designed by a gene recombination technique so as not only to recognize the ITM2A protein but also to recognize a cytotoxic substance such as a chemotherapeutic agent, a toxic peptide, or a radioactive chemical. These antibodies are also encompassed by the "antibody" according to the present invention.

[0104] Examples of the chemotherapeutic agent whose cytotoxic activity functions through the conjugation to the ITM2A antibody can include the following chemotherapeutic agents: azaribine, anastrozole, azacytidine, bleomycin, bortezomib, bryostatin-1, busulfan, camptothecin, 10-hydroxycamptothecin, carmustine, Celebrex, chlorambucil, cisplatin, irinotecan, carboplatin, cladribine, cyclophosphamide, cytarabine, dacarbazine, docetaxel, dactinomycin, daunomycin glucuronide, daunorubicin, dexamethasone, diethylstilbestrol, doxorubicin, doxorubicin glucuronide, epirubicin, ethinyl estradiol, estramustine, etoposide, etoposide glucuronide, floxuridine, fludarabine, flutamide, fluorouracil, fluoxymesterone, gemcitabine, hydroxyprogesterone caproate, hydroxyurea, idarubicin, ifosfamide, leucovorin, lomustine, maytansinoid, mechlorethamine, medroxyprogesterone acetate, megestrol acetate, melphalan, mercaptopurine, methotrexate, mitoxantrone, mithramycin, mitomycin, mitotane, phenylbutyrate, prednisone, procarbazine, paclitaxel, pentostatin, semustine, streptozocin, tamoxifen, taxanes, Taxol, testosterone propionate, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vinblastine, vinorelbine, and vincristine.

[0105] In the present invention, the chemotherapeutic agent is preferably a low-molecular chemotherapeutic agent. The low-molecular chemotherapeutic agent is unlikely to interfere with the antibody functions even after its conjugation to the antibody. In the present invention, the low-molecular chemotherapeutic agent usually has a molecular weight of 100 to 2000, preferably 200 to 1000. All of the chemotherapeutic agents exemplified herein are low-molecular chemotherapeutic agents. These chemotherapeutic agents according to the present invention encompass prodrugs that are converted *in vivo* to active chemotherapeutic agents. The prodrug activation may be enzymatic conversion or nonenzymatic conversion.

[0106] Alternatively, the antibody may be modified with the toxic peptide (toxin). Examples of the toxic peptide preferably include the followings:

diphtheria toxin A chain (Langone J.J., et al., Methods in Enzymology (1983) 93, 307-308),
Pseudomonas exotoxin (Nature Medicine (1996) 2, 350-353), ricin A chain (Fulton R.J. et al., J. Biol. Chem. (1986) 261, 5314-5319; Sivam G. et al., Cancer Res. (1987) 47, 3169-3173; Cumber A.J. et al., J. Immunol. Methods (1990) 135, 15-24; Wawrzynczak E.J. et al., Cancer Res. (1990) 50, 7519-7562; and Gheeite V. et al., J. Immunol. Methods (1991) 142, 223-230),
deglycosylated ricin A chain (Thorpe P.E. et al., Cancer Res. (1987) 47, 5924-5931),
abrin A chain (Wawrzynczak E.J. et al., Br. J. Cancer (1992) 66, 361-366; Wawrzynczak E.J., et al. Cancer Res. (1990) 50, 7519-7562; Sivam G., et al. Cancer Res. (1987) 47, 3169-3173; and Thorpe P.E. et al., Cancer Res. (1987) 47, 5924-5931),
gelonin (Sivam G. et al., Cancer Res. (1987) 47, 3169-3173; Cumber A.J. et al., J. Immunol. Methods (1990) 135, 15-24; Wawrzynczak E.J. et al. Cancer Res., (1990) 50, 7519-7562; and Bolognesi A. et al., Clin. exp. Immunol. (1992) 89, 341-346),
pokeweed anti-viral protein from seeds (PAP-s) (Bolognesi A. et al., Clin. exp. Immunol. (1992) 89, 341-346),
bryodin (Bolognesi A. et al., Clin. exp. Immunol. (1992) 89, 341-346),
saporin (Bolognesi A., et al., Clin. exp. Immunol. (1992) 89, 341-346),
momordin (Cumber A.J. et al., J. Immunol. Methods (1990) 135, 15-24; Wawrzynczak E.J. et al., Cancer Res. (1990) 50, 7519-7562; and Bolognesi A. et al., Clin. exp. Immunol. (1992) 89, 341-346),
momorcochin (Bolognesi A. et al., Clin. exp. Immunol. (1992) 89, 341-346),
dianthin 32 (Bolognesi A. et al., Clin. exp. Immunol. (1992) 89, 341-346),
dianthin 30 (Stirpe F., Barbieri L., FEBS letter (1986) 195, 1-8),
modeccin (Stirpe F., Barbieri L., FEBS letter (1986) 195, 1-8),
viscumin (Stirpe F., Barbieri L.,FEBS letter (1986) 195, 1-8),
volkensin (Stirpe F., Barbieri L., FEBS letter (1986) 195, 1-8),

dodecandrin (Stirpe F., Barbieri L., FEBS letter (1986) 195, 1-8),
tritin (Stirpe F., Barbieri L., FEBS letter (1986) 195, 1-8),
luffin (Stirpe F., Barbieri L., FEBS letter (1986) 195, 1-8), and
trichokirin (Casellas P., et al., Eur. J. Biochem. (1988) 176, 581-588; and Bolognesi A., et al., Clin. exp. Immunol., (1992) 89, 341-346).

[0107] In the present invention, the radioactive chemical refers to a chemical containing a radioisotope. The radioisotope used is not particularly limited, and any radioisotope may be used. Examples thereof can preferably include ^{32}P, ^{14}C, ^{125}I, ^{3}H, ^{131}I, ^{186}Re, and ^{188}Re.

[0108] In another aspect, one or two or more low-molecular chemotherapeutic agents and one or two or more toxic peptides can be used in combination to modify the antibody. The anti-ITM2A antibody can be conjugated to the low-molecular chemotherapeutic agent via a covalent or noncovalent bond. Such a chemotherapeutic agent-conjugated antibody is prepared by a method known in the art.

[0109] A proteinous agent or toxin can be conjugated to the antibody by a genetic engineering approach. Specifically, for example, the anti-ITM2A antibody-encoding DNAs are fused in frame with DNAs encoding the toxic peptides, and the resulting fused DNAs can be incorporated into expression vectors to construct recombinant vectors. The vectors are introduced into appropriate host cells, and the resulting transformed cells are cultured so that the DNA inserts are expressed. In this way, toxic peptide-conjugated anti-ITM2A antibodies can be obtained as fusion proteins. In the case of obtaining such antibody-fusion proteins, the proteinous agent or toxin is generally conjugated to the C-terminal side of each antibody. A peptide linker may be allowed to intervene between the antibody and the proteinous agent or toxin.

[0110] The monoclonal antibody of the present invention further encompasses bispecific antibodies. The bispecific antibodies refer to antibodies containing, in the same antibody molecule, variable regions that recognize different epitopes. The bispecific antibody will bind to a fragment of an ITM2A protein consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1, wherein the antibody has a cytotoxic activity. The bispecific antibody according to the present invention can have antigen-binding sites that recognize different epitopes on the ITM2A molecule. When such bispecific antibody molecules are to bind to ITM2A, two or more molecules of the bispecific antibody can bind to one ITM2A molecule. As a result, the bispecific antibodies having cytotoxic activities, can be expected to recruit a larger number of effector cells, resulting in stronger cytotoxic effect.

[0111] Alternatively, a bispecific antibody having antigen-binding sites, one of which binds to ITM2A and the other of which binds to a cytotoxic substance may be used in the present invention. The cytotoxic substance specifically encompasses, for example, chemotherapeutic agents, toxic peptides, and radioactive chemicals. Such a bispecific antibody binds to ITM2A-expressing cells, while capturing the cytotoxic substance. As a result, the cytotoxic substance can be allowed to directly act on the ITM2A-expressing cells. Specifically, use of the bispecific antibody that recognizes ITM2A as well as the cytotoxic substance can specifically damage tumor cells, resulting in the inhibited growth of the tumor cells.

[0112] Also, a bispecific antibody that binds to ITM2A as well as an antigen other than ITM2A expressed in tumor cells may be used in the present invention. For example, a bispecific antibody that binds to ITM2A and an antigen that is specifically expressed on the surface of target cancer cells, as with ITM2A, but is different from ITM2A, can be used.

[0113] The bispecific antibody is produced by a method known in the art. For example, two types of antibodies differing in antigen recognized thereby can be bound to prepare the bispecific antibody. Each of the antibodies bound may be a 1/2 molecule having H and L chains or may be a 1/4 molecule consisting of H chains. Alternatively, different monoclonal antibody-producing hybridomas may be fused to prepare fusion cells producing bispecific antibodies. The bispecific antibody can also be prepared by a genetic engineering approach.

[0114] The antigen binding activity of the antibody (Antibodies A Laboratory Manual. Ed Harlow, David Lane, Cold Spring Harbor Laboratory, 1988) can be determined using means known in the art. For example, ELISA (enzyme-linked immunosorbent assay), EIA (enzyme immunoassay), RIA (radioimmunoassay), flow cytometry such as FACS, or fluoroimmunoassay can be preferably used.

[0115] The monoclonal antibody of the present invention also encompasses an antibody having a modified sugar chain of the antibody of the present invention. The cytotoxic activities of antibodies are known to be enhanced by the modification of their sugar chains. For example, the following antibodies are known in the art as the antibody having a modified sugar chain:

glycosylated antibodies (WO1999054342, etc.),
antibodies deficient in fucose added to their sugar chains (WO2000061739, WO2002031140, WO2006067913, etc.), and
antibodies having a sugar chain having bisecting GlcNAc (WO2002079255, etc.).

[0116] The monoclonal antibody of the present invention used for the therapeutic purpose is an antibody having a cytotoxic activity. Examples of the cytotoxic activity according to the present invention preferably include antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activities. Another example of the antibody having a cytotoxic activity includes an antibody having both ADCC and CDC activities. In the present invention, the CDC activity refers to a cytotoxic activity mediated by the complement system. On the other hand, the ADCC activity refers to an activity of damaging target cells by Fcγ receptor-expressing cells (immunocytes, etc.) as a result of binding of the Fcγ receptor-expressing cells (immunocytes, etc.) via the Fcγ receptors to the Fc domains of antibodies specifically attached to the cell surface antigens of the target cells.

[0117] Whether or not the anti-ITM2A antibody has an ADCC activity or has a CDC activity can be determined by a method known in the art (e.g., Current protocols in Immunology, (1993) Chapter 7. Immunologic studies in Humans, Editor, John E, Coligan et al., John Wiley & Sons, Inc.) .

[0118] Specifically, effector cells, a complement solution, and target cells are first prepared.

(1) Preparation of effector cells

[0119] The spleens are excised from CBA/N mice or the like, and spleen cells are separated therefrom in an RPMI1640 medium (manufactured by Invitrogen Corp.). The spleen cells can be washed with this medium containing 10% fetal bovine serum (FBS, manufactured by HyClone Laboratories, Inc.) and then adjusted to a cell concentration of 5×10^6 cells/ml to prepare effector cells.

(2) Preparation of complement solution

[0120] Baby Rabbit Complement (manufactured by CEDARLANE Laboratories Ltd.) can be diluted 10-fold with a medium (manufactured by Invitrogen Corp.) containing 10% FBS to prepare a complement solution.

(3) Preparation of target cells

[0121] Cells expressing ITM2A proteins can be cultured at 37°C for 1 hour, together with 0.2 mCi 51Cr-sodium chromate (manufactured by GE Healthcare Bio-Sciences Corp.), in a DMEM medium containing 10% FBS to radiolabel the target cells. Cells transformed with ITM2A protein-encoding genes, Ewing's sarcoma cells, acute myeloid leukemia cells, T cell lymphoma cells, T cells lymphocytic leukemia cells, or the like can be used as the cells expressing ITM2A proteins. The cells thus radiolabeled can be washed three times with an RPMI1640 medium containing 10% FBS and adjusted to a cell concentration of 2×10^5 cells/ml to prepare the target cells.

[0122] The ADCC or CDC activity can be assayed by a method described below. For the ADCC activity assay, a U-bottom 96-well plate (manufactured by Becton, Dickinson and Company) supplemented with the target cells and the anti-ITM2A antibody (each 50 μl/well) is left standing for 15 minutes on ice. Then, 100 μl of the effector cells is added to each well of the plate, and the resulting plate is incubated for 4 hours in a CO_2 incubator. The final concentration of the antibody is adjusted to 0 or 10 μg/ml. After the incubation, the radioactivity of 100 μl of the supernatant recovered from each well is measured using a gamma counter (COBRA II AUTO-GAMMA, MODEL D5005, manufactured by Packard Instrument Company). The cytotoxic activity (%) can be calculated on the basis of the calculation expression $(A - C) / (B - C) \times 100$ using the following values obtained by such measurement:

A represents radioactivity (cpm) from each sample,
B represents radioactivity (cpm) from a sample supplemented with 1% NP-40 (manufactured by Nacalai Tesque, Inc.), and
C represents radioactivity (cpm) from a sample containing only the target cells.

[0123] For the CDC activity assay, a flat-bottomed 96-well plate (manufactured by Becton, Dickinson and Company) supplemented with the target cells and the anti-ITM2A antibody (each 50 μl/well) is left standing for 15 minutes on ice. Then, 100 μl of the complement solution is added to each well of the plate, and the resulting plate is incubated for 4 hours in a CO_2 incubator. The final concentration of the antibody is adjusted to 0 or 3 μg/ml. After the incubation, the radioactivity of 100 μl of the supernatant recovered from each well is measured using a gamma counter. The cytotoxic activity based on the CDC activity can be calculated according to a calculation expression similar to that of the ADCC activity.

[0124] In the case of assaying the cytotoxic activity of the antibody conjugate, a flat-bottomed 96-well plate (manufactured by Becton, Dickinson and Company) supplemented with the target cells and the anti-ITM2A antibody conjugate (each 50 μl/well) is left standing for 15 minutes on ice. Subsequently, the plate is incubated for 1 to 4 hours in a CO_2 incubator. The final concentration of the antibody is adjusted to 0 or 3 μg/ml. After the incubation, the radioactivity of 100 μl of the supernatant recovered from each well is measured using a gamma counter. The cytotoxic activity of the antibody conjugate can be calculated according to a calculation expression similar to that of the ADCC activity assay.

[0125] In an alternative aspect, examples of the antibody used in the present invention also preferably include an antibody having an internalization activity. In the present invention, the "antibody having an internalization activity" means an antibody that is transported into a cell (cytoplasm, vesicle, any other organelle, etc.) through its binding to ITM2A on the cell surface.

[0126] Whether or not the antibody has an internalization activity can be confirmed by a method generally known to those skilled in the art and can be confirmed by, for example, a method involving contacting labeling material-bound anti-ITM2A antibodies with ITM2A-expressing cells and confirming whether or not the labeling material is incorporated into the cells by the contact, or a method involving contacting cytotoxic substance-conjugated anti-ITM2A antibodies with ITM2A-expressing cells and confirming whether or not the death of the ITM2A-expressing cells is induced by the contact. More specifically, whether or not the antibody has an internalization activity can be confirmed by a method described in, for example, Examples below.

[0127] For example, the cytotoxic substance-conjugated antibody having an internalization activity can be used as a pharmaceutical composition such as an anticancer agent.

[0128] An antibody of the invention is a monoclonal antibody binding to a fragment of an ITM2A protein consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1, wherein the antibody has a cytotoxic activity.

[0129] In the present invention, the phrase "having an activity equivalent to that of the antibody of the present invention" means that a cytotoxic activity against ITM2A-expressing cells is equivalent to that of the antibody of the present invention. In the present invention, the phrase "having a binding activity equivalent to that of the antibody of the present invention" means that an ITM2A binding activity is equivalent to that of the antibody of the present invention.

[0130] A method for introducing a mutation to a polypeptide is one of methods well known to those skilled in the art for preparing a polypeptide functionally equivalent to a certain polypeptide. For example, those skilled in the art can appropriately introduce a mutation in the antibody of the present invention using site-directed mutagenesis (Hashimoto-Gotoh, T. et al. Gene (1995) 152, 271-275; Zoller, MJ, and Smith, M. Methods Enzymol., (1983) 100, 468-500; Kramer, W. et al. Nucleic Acids Res., (1984) 12, 9441-9456; Kramer W, and Fritz HJ Methods. Enzymol., (1987) 154, 350-367; Kunkel, TA Proc. Natl. Acad. Sci. USA., (1985) 82, 488-492; and Kunkel, Methods Enzymol., (1988) 85, 2763-2766) or the like and thereby prepare an antibody functionally equivalent to the antibody concerned. Amino acid mutations may occur in the natural world. Such an antibody that has an amino acid sequence of the antibody of the present invention with a mutation of one or more amino acid(s) and has an activity functionally equivalent to or a binding activity equivalent to that of the antibody concerned is also encompassed by the antibody of the present invention.

[0131] The number of amino acids mutated in such a variant is usually within 50 amino acids, preferably within 30 amino acids, more preferably within 15 amino acids or within 10 amino acids (e.g., within 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid(s)).

[0132] For amino acid residues to be mutated, this mutation is preferably performed conservatively between amino acids having the same side chain properties. For example, the following classification based on the properties of amino acid side chains has been established:

hydrophobic amino acids (A, I, L, M, F, P, W, Y, and V),
hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, and T),
amino acids having an aliphatic side chain (G, A, V, L, I, and P),
amino acids having a hydroxy group-containing side chain (S, T, and Y),
amino acids having a sulfur atom-containing side chain (C and M),
amino acids having a side chain containing carboxylic acid and amide (D, N, E, and Q),
amino acids having a base-containing side chain (R, K, and H), and
amino acids having an aromatic group-containing side chain (H, F, Y, and W)

(all symbols within the parentheses represent single letter codes of amino acids).

[0133] A polypeptide having an amino acid sequence modified from a certain amino acid sequence by the deletion and/or addition of one or more amino acid residue(s) and/or the substitution thereof by other amino acids is already known to maintain the biological activity of the original polypeptide (Mark, D. F. et al., Proc. Natl. Acad. Sci. USA (1984) 81, 5662-5666; Zoller, M. J. and Smith, M., Nucleic Acids Research (1982) 10, 6487-6500; Wang, A. et al., Science (1984) 224, 1431-1433; and Dalbadie-McFarland, G. et al., Proc. Natl. Acad. Sci. USA (1982) 79, 6409-6413). Specifically, when amino acids in an amino acid sequence constituting a certain polypeptide are substituted by amino acids classified in the same group thereas, it is generally said that the polypeptide is likely to maintain its activity. In the present invention, the substitution between amino acids within the same amino acid group described above is referred to as conservative substitution.

[0134] The present invention also provides an antibody binding to the same epitope as that to which the anti-ITM2A antibody disclosed herein binds. Specifically, the present invention relates to an antibody binding to the same epitope as that to which BE5-1, BE6-1, BE7-1-1, or BE13-1 binds, and use thereof. Such an antibody can be obtained, for example, by a method show below.

[0135] Whether a test antibody shares an epitope with a certain antibody can be confirmed on the basis of their competition for the same epitope. The competition between the antibodies is detected by cross-blocking assay or the like. The cross-blocking assay is preferably, for example, competitive ELISA assay.

[0136] Specifically, the cross-blocking assay involves preincubating ITM2A proteins coated on the wells of a microtiter plate in the presence or absence of a candidate competing antibody and then adding the anti-ITM2A antibody of the present invention to the wells. The amount of the anti-ITM2A antibody of the present invention bound to the ITM2A protein in each well indirectly correlates with the binding capability of the candidate competing antibody (test antibody) that competes therewith for binding to the same epitope. Specifically, correlation is confirmed such that the higher affinity of the test antibody for the same epitope results in the smaller amount of the anti-ITM2A antibody of the present invention bound to the ITM2A protein-coated well and instead, the larger amount of the test antibody bound to the ITM2A protein-coated well.

[0137] The amount of each antibody bound to the well can be easily determined by labeling the antibody in advance. For example, the amount of a biotinylated antibody can be determined using an avidin-peroxidase conjugate and an appropriate substrate. The cross-blocking assay using enzyme (e.g., peroxidase) labeling is particularly called competitive ELISA assay. The antibody may be labeled with any of other detectable or measurable labeling materials. Specifically, for example, radiolabeling or fluorescent labeling is known in the art.

[0138] Alternatively, the cross-blocking assay is preferably competitive FACS assay.

[0139] Specifically, the competitive FACS assay employs cells containing expressed ITM2A proteins instead of ITM2A proteins coated on the wells of a microtiter plate in the competitive ELISA assay. The cells containing expressed ITM2A proteins are preincubated in the presence or absence of a candidate competing antibody. Then, the biotinylated anti-ITM2A antibody of the present invention is added to the wells. Fluorescence can be detected using a streptavidin-fluorescein conjugate to determine the competition between the antibodies. The cross-blocking assay using flow cytometry is particularly called competitive FACS assay. The antibody can be preferably labeled with any of other detectable or measurable fluorescent labeling materials.

[0140] When the test antibody contains constant regions derived from an organism species different from that of the anti-ITM2A antibody of the present invention, the antibody (derived from any organism species) bound to the well can be assayed using a labeled antibody that recognizes the constant region of the antibody of the organism species. Alternatively, even in the case of detecting the binding of antibodies derived from the same organism species but differing in class, each antibody bound to the well can be assayed using an antibody specifically binding to the antibody of each class.

[0141] Provided that the candidate antibody can block the binding of the anti-ITM2A antibody by at least 20%, preferably at least 30%, more preferably at least 40%, even more preferably 50%, compared with the binding activity obtained as a result of the control test conducted in the absence of the candidate competing antibody, this candidate competing antibody is determined as an antibody that binds to substantially the same epitope as that to which the anti-ITM2A antibody of the present invention binds or as an antibody that competes therewith for the binding to the same epitope. For the epitope assay, the constant region of the antibody of the present invention may be replaced with the same constant region as that of the test antibody.

[0142] The epitope to which the anti-ITM2A antibody of the present invention binds can be appropriately determined by the method described above. Preferably, the epitope can be present in a fragment comprising the extracellular region of the ITM2A protein. The antibody of the invention binds to a fragment of ITM2A consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1 and has a cytotoxic activity.

Pharmaceutical composition

[0143] In an alternative aspect, the present invention provides a pharmaceutical composition comprising the monoclonal antibody binding to ITM2A protein as an active ingredient. The present invention also relates to a cell growth inhibitor, particularly, an anticancer agent, comprising the monoclonal antibody binding to ITM2A protein as an active ingredient. The cell growth inhibitor and the anticancer agent of the present invention are preferably administered to a subject having cancer or possibly having cancer. Hence, the invention further provides a monoclonal antibody of the invention for use as a method for treating cancer. As shown later in Examples, ITM2A is expressed at a low level in normal cells, but is overexpressed in cancer cells. Therefore, the administration of the anti-ITM2A antibody probably produces cancer cell-specific cytotoxic effect.

[0144] The anti-ITM2A antibody used in the pharmaceutical composition (e.g., the anticancer agent) of the present invention is a monoclonal anti-ITM2A antibody of the invention. For example, any of the anti-ITM2A antibodies described above can be preferably used.

[0145] In the present invention, the phrase "comprising the antibody binding to ITM2A as an active ingredient" means containing the monoclonal anti-ITM2A antibody as a main active ingredient and is not intended to limit the content of the anti-ITM2A antibody.

[0146] When the disease targeted by the pharmaceutical composition of the present invention is cancer, the targeted cancer is not particularly limited as long as the ITM2A protein is expressed in the cancer. The cancer is preferably Ewing's sarcoma or blood cancer such as T cell leukemia, T cell lymphoma, acute myeloid leukemia, B cell tumor, or multiple myeloma, particularly preferably Ewing's sarcoma. Among the Ewing's sarcomas, Ewing's sarcoma having t(11;22)(q24;q12) chromosomal translocation may be preferably targeted. Such cancer may be any of primary foci and metastatic foci.

[0147] In the present invention, a method known in the art such as FISH or PCR can be appropriately adopted as a cell damaging method or for determining whether or not Ewing's sarcoma cells whose growth is to be inhibited have t(11;22)(q24;q12) chromosomal translocation.

[0148] In order to determine whether to have the t(11;22)(q24;q12) chromosomal translocation by the FISH method, for example, a probe for EWS gene detection and a probe for FLI-1 gene detection separately labeled so as to emit different fluorescences are hybridized to test tissue samples immobilized by a method known in the art. The presence of the t(11;22)(q24;q12) chromosomal translocation can be confirmed by determining whether or not fusion signals of these fluorescences are detected as a result of the hybridization.

[0149] Alternatively, FISH using two probes for respectively detecting portions of a chromosome split by the translocation may also be appropriately adopted for determining whether to have the t(11;22)(q24;q12) chromosomal translocation. Specifically, these two probes (e.g., a set of probes for detecting the EWS gene split by the translocation or a set of probes for FLI-1 gene detection) separately labeled so as to emit different fluorescences are hybridized to test tissue samples immobilized by a method known in the art. The presence of the t(11;22)(q24;q12) chromosomal translocation can be confirmed by determining whether or not split signals of these fluorescences are detected as a result of the hybridization.

[0150] In order to determine whether to have the t(11;22)(q24;q12) chromosomal translocation by the PCR method, a set of two primers is designed such that the EWS gene and the FLI-1 gene can be detected. The primers are designed such that a fusion gene formed by the translocation is amplified as a result of PCR using the set of the primers. The presence of the t(11;22)(q24;q12) chromosomal translocation can be confirmed by detecting the PCR amplification of the fusion gene formed by the translocation.

[0151] Alternatively, a set of two primers that allow detection of fragments of a chromosome split by the translocation is designed for determining whether to have the t(11;22)(q24;q12) chromosomal translocation. For example, PCR is carried out using a set of primers for detecting the EWS gene split by the translocation or a set of primers for FLI-1 gene detection, and a test tissue sample as a template. The presence of the t(11;22)(q24;q12) chromosomal translocation can be confirmed provided that chromosomal fragments formed by PCR using a sample free from the translocation as a template are not detected in the PCR products obtained with the test tissue sample as a template.

[0152] The pharmaceutical composition of the present invention can be administered either orally or parenterally to a patient. Parenteral administration is preferable. Specific examples of such an administration method preferably include injection, transnasal, pulmonary, and transdermal administrations. Examples of the injection administration include intravenous, intramuscular, intraperitoneal, and subcutaneous injections, through which the pharmaceutical composition of the present invention can be administered systemically or locally. The administration method can be appropriately selected according to the age or symptoms of the patient. The dose of the pharmaceutical composition of the present invention can be selected from among the range of, for example, 0.0001 mg to 1000 mg per kg body weight per dosing. Alternatively, the dose in each patient may be selected from among the range of, for example, 0.001 to 100000 mg per body. However, the pharmaceutical composition of the present invention is not limited by these doses.

[0153] The pharmaceutical composition of the present invention can be formulated according to a routine method (e.g., Remington's Pharmaceutical Science, Latest edition, Mark Publishing Company, Easton, U.S.A). The pharmaceutical composition preferably used may additionally contain pharmaceutically acceptable carriers or additives. Examples of such carriers or additives include, but not limited thereto, surfactants, excipients, coloring agents, flavoring agents, preservatives, stabilizers, buffers, suspending agents, tonicity agents, binders, disintegrants, lubricants, flow promoters, and corrigents. Other carriers routinely used may be appropriately used. Specific examples of such carriers routinely used can include light anhydrous silicic acid, lactose, crystalline cellulose, mannitol, starch, carmellose calcium, carmellose sodium, hydroxypropylcellulose, hydroxypropylmethylcellulose, polyvinyl acetal diethylaminoacetate, polyvinylpyrrolidone, gelatin, medium chain fatty acid triglyceride, polyoxyethylene hydrogenated castor oil 60, white sugar, carboxymethylcellulose, corn starch, and inorganic salts.

[0154] Also disclosed is a method for damaging ITM2A-expressing cells or inhibiting the growth of ITM2A-expressing cells, comprising contacting the ITM2A-expressing cells with the antibody binding to ITM2A protein.

[0155] The antibody for use in treating cancer is a monoclonal antibody of the invention, and, for example, any of the antibodies described above may be used. The cells to which the anti-ITM2A antibody binds are not particularly limited as long as ITM2A is expressed in the cells. The ITM2A-expressing cells according to the present invention are preferably cancer cells, more preferably Ewing's sarcoma cells or cells of blood cancer such as T cell leukemia, T cell lymphoma, acute myeloid leukemia, B cell tumor, or multiple myeloma. The present invention can be applied to any of primary foci and metastatic foci of these cancers. The cancer cells are more preferably Ewing's sarcoma cells or metastatic Ewing's sarcoma cells. Among the Ewing's sarcoma cells, Ewing's sarcoma cells having t(11;22)(q24;q12) chromosomal translocation may be preferably targeted. Such Ewing's sarcoma cells having the chromosomal translocation may be located in any of primary foci and metastatic foci.

[0156] In the present invention, a method known in the art such as FISH or PCR can be appropriately adopted as a cell damaging method or for determining whether or not Ewing's sarcoma cells whose growth is to be inhibited have t(11;22)(q24;q12) chromosomal translocation.

[0157] In order to determine whether to have the t(11;22)(q24;q12) chromosomal translocation by the FISH method, for example, a probe for EWS gene detection and a probe for FLI-1 gene detection separately labeled so as to emit different fluorescences are hybridized to test tissue samples immobilized by a method known in the art. The presence of the t(11;22)(q24;q12) chromosomal translocation can be confirmed by determining whether or not adjacent or fusion signals of these fluorescences are detected as a result of the hybridization.

[0158] Alternatively, FISH using two probes for respectively detecting portions of a chromosome split by the translocation may also be appropriately adopted for determining whether to have the t(11;22)(q24;q12) chromosomal translocation. Specifically, these two probes (e.g., a set of probes for detecting the EWS gene split by the translocation or a set of probes for FLI-1 gene detection) separately labeled so as to emit different fluorescences are hybridized to test tissue samples immobilized by a method known in the art. The presence of the t(11;22)(q24;q12) chromosomal translocation can be confirmed by determining whether or not split signals of these fluorescences are detected as a result of the hybridization.

[0159] In order to determine whether to have the t(11;22)(q24;q12) chromosomal translocation by the PCR method, a set of two primers is designed such that the EWS gene and the FLI-1 gene can be detected. The primers are designed such that a fusion gene formed by the translocation is amplified as a result of PCR using the set of the primers. The presence of the t(11;22)(q24;q12) chromosomal translocation can be confirmed by detecting the PCR amplification of the fusion gene formed by the translocation.

[0160] Alternatively, a set of two primers that allow detection of fragments of a chromosome split by the translocation is designed for determining whether to have the t(11;22)(q24;q12) chromosomal translocation. For example, PCR is carried out using a set of primers for detecting the EWS gene split by the translocation or a set of primers for FLI-1 gene detection, and a test tissue sample as a template. The presence of the t(11;22)(q24;q12) chromosomal translocation can be confirmed provided that chromosomal fragments formed by PCR using a sample free from the translocation as a template are not detected in the PCR products obtained with the test tissue sample as a template.

[0161] In the present invention, the "contact" is performed, for example, by adding the antibody to cultures of ITM2A-expressing cells cultured *in vitro.* In the present invention, the "contact" is also performed by administering the antibody to non-human animals implanted with ITM2A-expressing cells in their bodies or to animals endogenously having ITM2A-expressing cancer cells.

[0162] Methods shown below are preferably used for evaluating or determining cytotoxicity caused against the ITM2A-expressing cells by the contact of the anti-ITM2A antibody. Examples of the methods for evaluating or determining the cytotoxic activity *in vitro* can include the antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activity assay methods described above. Whether or not the anti-ITM2A antibody has an ADCC activity or has a CDC activity can be determined by a method known in the art (e.g., Current protocols in Immunology, Chapter 7. Immunologic studies in Humans, Editor, John E, Coligan et al., John Wiley & Sons, Inc., (1993)). In the activity assay, a binding antibody that has an isotype identical to that of the anti-ITM2A antibody and does not have the cytotoxic activity is used as a control antibody in the same way as in the anti-ITM2A antibody. When the anti-ITM2A antibody exhibits a stronger cytotoxic activity than that of the control antibody, the anti-ITM2A antibody can be determined to have the activity.

[0163] The isotype of an antibody is defined by the sequence of the H chain constant region in the amino acid sequence of this antibody. The antibody isotype is finally determined depending on class switching caused by genetic recombination on the chromosome during the maturation of antibody-producing B cells *in vivo.* Difference in isotype is reflected by the difference between the physiological/pathological functions of antibodies. Specifically, it is known that, for example, the strength of the cytotoxic activity is influenced not only by the expression level of the antigen but also by the isotype of the antibody. Thus, for the cytotoxic activity assay described above, the antibody used as a control preferably has an isotype identical to that of the test antibody.

[0164] In order to evaluate or determine the cytotoxic activity *in vivo,* for example, ITM2A-expressing cancer cells are intradermally or subcutaneously transplanted to non-human test animals. Then, the test antibody is intravenously or intraperitoneally administered thereto on a daily basis or at a few day-intervals from the administration day or the next day. The cytotoxic activity of the test antibody can be determined by measuring tumor sizes over time. A control antibody having an isotype identical thereto is administered, as in the *in vitro* evaluation. When the test anti-ITM2A antibody-administered group exhibits a significantly smaller tumor size than that of the control antibody-administered group, the test anti-ITM2A antibody can be determined to have the cytotoxic activity. In the case of using mice as the non-human test animals, nude (nu/nu) mice can be preferably used, which are genetically deficient in the thymus gland and thus lack the functions of T lymphocytes. The use of these mice excludes the involvement of the endogenous T lymphocytes of the test animals in the evaluation or determination of the cytotoxic activities of administered antibodies.

[0165] In one aspect, the method of the present invention provides the diagnosis of cancer by detecting ITM2A protein in a test sample. In this aspect, preferably, the extracellular region of the ITM2A protein is detected. An antibody that recognizes the ITM2A protein can be preferably used in the detection of the ITM2A protein.

[0166] One specific example of the diagnosis method of the present invention can include a method for diagnosing cancer, comprising the following steps:

(a) providing a sample collected from a test subject; and
(b) detecting ITM2A protein contained in the collected sample using an antibody binding to the ITM2A protein.

[0167] In the present invention, the detection encompasses quantitative or qualitative detection. Examples of the qualitative detection can include the following assays: assay to simply determine the presence or absence of the ITM2A protein,
assay to determine the presence or absence of more than a predetermined amount of the ITM2A protein, and assay to compare the amount of the ITM2A protein with that contained in another sample (e.g., a control sample).

[0168] On the other hand, examples of the quantitative detection can include the measurement of an ITM2A protein concentration and the measurement of the amount of the ITM2A protein.

[0169] The test sample according to the present invention is not particularly limited as long as the sample possibly contains the ITM2A protein. Specifically, samples collected from living bodies such as mammals are preferable. Samples collected from humans are more preferable. Specific examples of the test sample can include blood, interstitial fluid, plasma, extravascular fluid, cerebrospinal fluid, synovial fluid, pleural fluid, serum, lymph, saliva, urine, tissues, ascitic fluid, and intraperitoneal lavage. The sample is preferably a sample obtained from the test sample, such as a preparation in which tissues or cells collected from a living body are fixed, or cell cultures.

[0170] The cancer diagnosed by the present invention is not particularly limited and may be any cancer. Specific examples thereof can include Ewing's sarcoma and blood cancer such as T cell leukemia, T cell lymphoma, acute myeloid leukemia, B cell tumor, and multiple myeloma. In the present invention, any of primary foci and metastatic foci of these cancers can be diagnosed. Among the Ewing's sarcoma cells, Ewing's sarcoma having t(11;22)(q24;q12) chromosomal translocation may be diagnosed. Such Ewing's sarcoma having the chromosomal translocation may be any of primary foci and metastatic foci.

[0171] In the present invention, the cancer is diagnosed using, as an index, the level of the ITM2A protein detected in the test sample. Specifically, when the amount of the ITM2A protein detected in the test sample is larger than that of a negative control or a healthy individual, the test subject is shown to have cancer or be highly likely to have cancer in the future. Specifically, the present invention relates to a method for diagnosing cancer, comprising the following steps:

(1) detecting the expression level of ITM2A in a biological sample collected from a test subject, and
(2) comparing the expression level of ITM2A detected in step (1) with that of a control, wherein when the expression level of ITM2A is higher than that of the control, the test subject is determined to have cancer.

[0172] Hence, the present invention provides a method for determining the presence of cancer, in a test subject using the antibody of the invention:

(A) comprising detecting an ITM2A protein in a sample collected from the test subject; or
(B) comprising the following steps:

  (a) providing a sample collected from the test subject; and
  (b) detecting an ITM2A protein contained in the sample of step (a) using an antibody binding to the ITM2A protein; or

(C) comprising the following steps:

  (a) administering, to the test subject, a radioisotope-labeled antibody having a binding activity to an ITM2A protein; and
  (b) detecting the accumulation of the radioisotope, wherein the cancer is Ewing's sarcoma or blood cancer, wherein the sample is tissues or blood.

[0173] In the present invention, the control refers to a reference sample for comparison and encompasses negative controls and biological samples of healthy individuals. The negative controls can be obtained by collecting biological samples of healthy individuals and mixing them, if necessary. The expression level of ITM2A in the control can be detected in parallel with the detection of the expression level of ITM2A in the biological sample of the test subject. Alternatively, the expression level of ITM2A in a large number of biological samples of healthy individuals may be detected in advance to statistically determine the standard expression level in the healthy individuals. Such statistically determined values are also used as control values for the expression level of ITM2A in the test biological sample. Specifically, for example, mean ± 2 x standard deviation (S.D.) or mean ± 3 x standard deviation (S.D.) can be used as the standard value. Statistically, the mean ± 2 x standard deviation (S.D.) and the mean ± 3 x standard deviation (S.D.) include values of 80% and 90% of the healthy individuals, respectively.

[0174] Alternatively, the expression level of ITM2A in the control may be set using an ROC curve. The ROC curve, or receiver operating characteristic curve, is a graph showing detection sensitivity in the ordinate and false positive rates (i.e., "1 - specificity") in the abscissa. In the present invention, the ROC curve can be obtained by plotting changes in sensitivity and false positive rate at a series of varying reference values for determining the expression level of ITM2A in the biological sample.

[0175] The "reference value" for obtaining the ROC curve is a numeric value temporarily used for statistical analysis. In general, the "reference value" for obtaining the ROC curve is serially varied within a range which can cover all selectable reference values. For example, the reference value can be varied between the minimal and maximal measured values of ITM2A in a population to be analyzed.

[0176] A standard value that can be expected to offer the desired detection sensitivity and precision can be selected on the basis of the obtained ROC curve. The standard value statistically set on the basis of the ROC curve or the like is also called a cut-off value. In a method for detecting cancer on the basis of the cut-off value, step (2) described above comprises comparing the expression level of ITM2A detected in step (1), with the cut-off value. Then, when the expression level of ITM2A detected in step (1) is higher than the cut-off value, cancer is detected in the test subject.

[0177] In the present invention, the expression level of ITM2A can be determined by an arbitrary method. Specifically, the expression level of ITM2A can be determined by evaluating the amount of the ITM2A protein and the biological activity of the ITM2A protein. The amount of the ITM2A protein can be determined by the method as described herein.

[0178] An arbitrary animal species expressing the ITM2A protein can be selected as the test subject. For example, many non-human mammalian individuals such as rhesus macaque (*Macaca mulatta*) (ENSMMUG00000003564), common marmoset (*Callithrix jacchus*) (ENSCJAG00000009591), Sumatran orangutan (*Pongo abelii*) (LOC100431628), rabbit (*Oryctolagus cuniculus*) (ENSOCUG00000008651), horse (*Equus caballus*) (ENSECAG00000011335), mouse (Mus *musculus*) (ENSMUSG00000031239), giant panda (*Ailuropoda melanoleuca*) (LOC100476516), rat (*Rattus norvegicus*) (ENSRNOG00000002365), pig (*Sus scrofa*) (ENSSSCG00000012448), and chicken (*Gallus gallus*) (ENSGALG00000004107) are known to express the ITM2A protein. Thus, these animals are encompassed by the test subject according to the present invention. The test subject is particularly preferably a human. When a non-human animal is used as the test subject, the ITM2A protein of the animal species is detected.

[0179] The anti-ITM2A antibody may be used for detecting the ITM2A protein of a non-human animal species. In such a case, an anti-ITM2A antibody binding to only the ITM2A protein of the animal species may be used. Alternatively, an anti-ITM2A antibody capable of binding to not only the ITM2A protein derived from the animal species but also the ITM2A protein derived from another animal species, i.e., having so-called cross reactivity, can also be preferably used. The anti-ITM2A antibody may be further used for detecting the human ITM2A protein. In such a case, an anti-ITM2A antibody binding to only human ITM2A as well as an anti-ITM2A antibody capable of binding to both of human ITM2A and the ITM2A protein derived from another animal species can be preferably used.

[0180] A method for detecting the ITM2A protein contained in the test sample is not particularly limited and is preferably an immunological detection method using the anti-ITM2A antibody as exemplified below:

radioimmunoassay (RIA),
enzyme immunoassay (EIA),
fluoroimmunoassay (FIA),
luminescent immunoassay (LIA),
immunoprecipitation (IP),
turbidimetric immunoassay (TIA),
Western blot (WB),
immunohistochemical (IHC) method, and
single radial immunodiffusion (SRID).

[0181] Among these approaches, the immunohistochemical (IHC) method is a preferable immunological assay method for diagnosing cancer, comprising the step of detecting the ITM2A protein on sections in which tissues or cells obtained from a patient having cancer are fixed. The immunological methods described above, such as the immunohistochemical (IHC) method, are generally known to those skilled in the art.

[0182] Specifically, since ITM2A is a membrane protein specifically overexpressed in cancer cells, cancer cells or cancer tissues can be detected using the anti-ITM2A antibody. Cancer cells contained in cells or tissues collected from living bodies can be detected by the immunohistological analysis.

[0183] Cancer tissues can be detected *in vivo* by a noninvasive method using the anti-ITM2A antibody. Specifically, the present invention relates to a method for detecting cancer, comprising the following steps

(a) administering, to the test subject, a radioisotope-labeled antibody having a binding activity to an ITM2A protein; and
(b)detecting the accumulation of the radioisotope,

wherein the cancer is Ewing's sarcoma or blood cancer, wherein the sample is tissues or blood. The anti-ITM2A antibody can be detectably labeled for tracing the anti-ITM2A antibody administered into the living body. For example, the *in vivo* behavior of the antibody labeled with a fluorescent or luminescent material or a radioisotope can be traced. The fluorescent or luminescent material-labeled anti-ITM2 antibody can be observed using an endoscope or peritoneoscope. The localization of the anti-ITM2A antibody can be imaged by tracing the radioactivity of the radioisotope. In the present invention, the *in vivo* localization of the anti-ITM2A antibody represents the presence of cancer cells.

[0184] A positron-emitting nuclide can be used as the radioisotope for labeling the anti-ITM2A antibody used for the purpose of detecting cancer *in vivo.* For example, the antibody can be labeled with a positron-emitting nuclide such as ^{18}F, ^{55}CO, ^{64}Cu, ^{66}Ga, ^{68}Ga, ^{76}Br, ^{89}Zr, and ^{124}I. A method known in the art (Acta Oncol. 32, 825-830, 1993) can be used in the labeling of the anti-ITM2A antibody with these positron-emitting nuclides.

[0185] The anti-ITM2A antibody labeled with the positron-emitting nuclide is administered to humans or animals. Then, radiation emitted by the radionuclide is measured *ex vivo* using PET (positron emission tomograph). The measurement results are converted to images by a computed tomographic approach. The PET apparatus is intended to noninvasively obtain data about *in vivo* pharmacokinetics or the like. The PET can quantitatively image radiation intensity indicated by signal intensity. By such use of the PET, antigen molecules highly expressed in particular cancer can be detected without collecting samples from patients. Specifically, in the present invention, the ITM2A protein highly expressed in Ewing's sarcoma or blood cancer such as T cell leukemia, T cell lymphoma, acute myeloid leukemia, B cell tumor, or multiple myeloma can be detected. In the present invention, the ITM2A protein expressed in Ewing's sarcoma having t(11;22)(q24;q12) chromosomal translocation, among the Ewing's sarcoma cells, can be detected. The Ewing's sarcoma, the acute myeloid leukemia, the B cell tumor, the multiple myeloma, or the Ewing's sarcoma having t(11;22)(q24;q12) chromosomal translocation may be any of primary foci and metastatic foci. The anti-ITM2A antibody may be radiolabeled with a short-life nuclide using a positron-emitting nuclide such as ^{11}C, ^{13}N, ^{15}O, ^{18}F, or ^{45}Ti, in addition to the nuclides described above.

[0186] Research and development have been pursued as to, for example, techniques of producing short-life nuclides using a medical cyclotron and the nuclides described above or producing short-life radiolabeling compounds. The anti-ITM2A antibody can be labeled with various radioisotopes by these techniques. The anti-ITM2A antibody administered to patients accumulates in primary foci and metastatic foci according to the specificity of the anti-ITM2A antibody for pathological tissues at each site. When the anti-ITM2A antibody is labeled with the positron-emitting nuclide, its radioactivity can be detected to detect the presence of the primary foci and the metastatic foci based on the localization of the radioactivity. An active value of gamma radiation or positron emission of 25 to 4000 keV can be appropriately used for the diagnostic use. Moreover, therapeutic effect can also be expected by selecting an appropriate nuclide and administering the selected nuclide in larger amounts. A nuclide that provides a value of gamma radiation or positron emission of 70 to 700 keV can be used for obtaining anticancer effect attributed to radiation.

[0187] Also disclosed is a method for selecting a test subject to receive the pharmaceutical composition comprising the antibody binding to ITM2A protein as an active ingredient, or a test subject applicable to a method for damaging ITM2A-expressing cells or inhibiting the growth of ITM2A-expressing cells by contacting the ITM2A-expressing cells with the antibody binding to ITM2A protein. In a further alternative aspect, the present invention provides a method for predicting the efficacy of cancer treatment using the anti-ITM2A antibody of the present invention. In particular, the present invention provides a method for predicting the efficacy of cancer treatment by the administration of the antibody according to the invention, comprising the step of detecting the expression level of an ITM2A in a biological sample collected from a test subject using the antibody of the invention, wherein the cancer is Ewing's sarcoma or blood cancer, wherein the sample is tissue or blood.

[0188] As described above, a test subject containing the ITM2A protein expressed in Ewing's sarcoma cells or cells of blood cancer such as T cell leukemia, T cell lymphoma, acute myeloid leukemia, B cell tumor, or multiple myeloma *in vivo* is preferably selected as a test subject to receive the antibody binding to ITM2A protein or the pharmaceutical composition comprising this antibody as an active ingredient, or as a test subject for which the efficacy of treatment by the administration thereof is predicted. The present invention is not limited by whether or not these tumors or cancers are primary foci or metastatic foci. A test subject having *in vivo* Ewing's sarcoma having t(11;22) (q24;q12) chromosomal translocation, among the Ewing's sarcomas, may be selected as a preferable subject. Such Ewing's sarcoma having the chromosomal translocation may be any of primary foci and metastatic foci.

[0189] The cell damaging method or the method for determining whether or not Ewing's sarcoma cells whose growth is to be inhibited have t(11;22)(q24;q12) chromosomal translocation according to the present invention is as described above.

[0190] Also disclosed is a diagnostic drug or kit for cancer diagnosis, comprising a reagent for detecting ITM2A protein in a test sample. The diagnostic drug comprises at least the anti-ITM2A antibody.

[0191] The reagent for cancer diagnosis can be used as a kit for cancer diagnosis, in combination with other factors used in ITM2A detection. Specifically, also disclosed is a kit for cancer diagnosis which comprises: an antibody binding to ITM2A; and a reagent for detecting the binding of the antibody to ITM2A and may further comprise a control sample consisting of a biological sample containing ITM2A. A manual for instruction of assay procedures may be further included in the kit.

[0192] An aspect represented by the expression "comprising" used herein encompasses an aspect represented by the expression "essentially consisting of" and an aspect represented by the expression "consisting of".

Examples

[0193] Hereinafter, the present invention will be described further specifically with reference to Examples. However, the technical scope of the present invention is not limited by these Examples.

[Example 1] Expression analysis of ITM2A mRNA

[0194] The expression of ITM2A mRNA was assayed in clinical Ewing's sarcoma samples, Ewing's sarcoma cell lines, blood cancer cell lines, and normal tissues using Human Exon 1.0 ST Array (Affymetrix, Inc.). The expression analysis employed 1 μg of total RNAs from each sample shown in Figure 1. The analysis was conducted according to a method described in GeneChip Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix, Inc.). The data was digitized using Exon Array Computational Tool software (Affymetrix, Inc.). The total RNAs of normal tissues used in the analysis were normal tissues-derived total RNAs purchased from Clontech Laboratories, Inc., Ambion, Inc., Stratagene Corp., Cell Applications, Inc., Panomics, Inc., CHEMICON International, Inc., and BioChain Institute, Inc. Total RNAs were prepared from the tumor sites and normal sites of clinical cancer tissues (sampled after informed consent was obtained) and from cancer cell lines using Trizol (Invitrogen Corp.) or Isogen (Nippon Gene Co., Ltd.) according to methods included in these products. A mean of numeric values obtained with ITM2A core probe sets (probe set IDs: 4013550, 4013551, 4013552, 4013553, 4013554, 4013557, 4013559, 4013560, 4013561, 4013564, and 4013565) was estimated as expression data.

[0195] As a result of the expression analysis, the expression level of ITM2A mRNA was around 1000 counts at the maximum in normal tissues, but was 2000 to 7000 counts in cell lines or clinical samples of Ewing's sarcoma, demonstrating the expression of ITM2A in Ewing's sarcoma (Figure 1). Also, the expression of ITM2A was confirmed at a level around 2000 in an acute myeloid leukemia cell line KG-1 or HL60, a B cell tumor cell line IM9, and a multiple myeloma cell line KMS-12-BM. These results showed that ITM2A was able to serve as a therapeutic target and a diagnostic marker for Ewing's sarcoma or blood cancer such as T cell leukemia, T cell lymphoma, acute myeloid leukemia, B cell tumor, or multiple myeloma.

[Example 2] Preparation of monoclonal antibody against ITM2A

(2-1) Cloning of ITM2A gene

[0196] cDNAs were prepared using SuperScript III Reverse Transcriptase (Invitrogen Corp.) with total RNAs prepared from a cancer cell line IM9 using Trizol as a template. A nucleotide sequence encoding ITM2A was amplified by PCR using the cDNAs as a template, a forward primer (SEQ ID NO: 43), and a reverse primer (SEQ ID NO: 44). This PCR employed PrimeSTAR GXL DNA Polymerase (Takara Bio Inc.) and was performed by 30 repetitive reaction cycles each involving 98°C for 10 seconds, 55°C for 15 seconds, and 68°C for 1 minute. The amplification products formed from the PCR were cloned into pCR2.1-TOPO vectors (Invitrogen Corp.) to obtain pCR2.1_ITM2A. The inserted sequence of pCR2.1_ITM2A was sequenced to confirm that the inserted sequence was the same as a sequence registered under RefSeq Accession No. NM_004867.4.

(2-2) Preparation of expression vector for DNA immunization

[0197] A nucleotide sequence encoding the extracellular region of ITM2A (predicted to be Tyr75-Glu263 as a result of analysis according to http://www.uniprot.org/) was cloned into expression vectors (pMCN2i) for mammal cells. The vector pMCN2i allows induction of expression of a gene insert under the control of mouse CMV promoter (GenBank Accession No. U68299) and contains a neomycin resistance gene incorporated therein. The signal sequence used was the signal sequence of mouse interleukin 3. First, the ITM2A extracellular region-encoding nucleotide sequence was amplified by PCR using pCR2.1_ITM2A as a template, a forward primer (SEQ ID NO: 45) having SfiI site, and a reverse primer (SEQ ID NO: 46) having NotI site. The amplification products formed from the PCR were cloned into pCR2.1-TOPO vectors. SfiI/NotI-digested fragments of the plasmids obtained as a result of the cloning were cloned into the SfiI-NotI sites of pMCN2i_mIL3ss-mIgG2aFc vectors to obtain plasmids (pMCN2i_mIL3ss-ITM2Aoutside). The pMCN2i_mIL3ss-mIgG2aFc vectors contained the mouse interleukin 3 signal sequence cloned in the EcoRI-SfiI site and the mouse IgG2a antibody Fc region gene cloned in the CpoI-NotI site. The nucleotide sequence from start codon to stop codon in the inserted sequence comprising the ITM2A extracellular region-encoding nucleotide sequence in pMCN2i_mIL3ss-ITM2Aoutside is shown in SEQ ID NO: 47, and an amino acid sequence encoded thereby is shown in SEQ ID NO: 48.

(2-3) Preparation of protein for immunization

[0198] A fusion protein (ITM2A-Fc) of the ITM2A extracellular region (Tyr75-Glu263) and the Fc region of mouse IgG2a was prepared. First, the ITM2A extracellular region-encoding nucleotide sequence was amplified by PCR using pCR2.1_ITM2A as a template, a forward primer (SEQ ID NO: 49) having SfiI site, and a reverse primer (SEQ ID NO: 50) having CpoI site, and cloned into pCR2.1-TOPO vectors. SfiI/CpoI-digested fragments of the plasmids obtained as a result of the cloning were cloned into the SfiI-CpoI sites of pMCN2i_mIL3ss-mIgG2aFc vectors to obtain plasmids pMCN2i_mIL3ss-ITM2Aoutside-Fc. The nucleotide sequence from start codon to stop codon in the inserted sequence comprising the ITM2A-Fc-encoding nucleotide sequence in pMCN2i_mIL3ss-ITM2Aoutside-Fc is shown in SEQ ID NO: 51, and an amino acid sequence encoded thereby is shown in SEQ ID NO: 52.

[0199] Next, pMCN2i_mIL3ss-ITM2Aoutside-Fc digested with PvuI was transduced into a CHO cell line DG44 (Invitrogen Corp.) by electroporation. Transductants were screened for with Geneticin (500 μg/mL) to establish a CHO cell line constantly secreting ITM2A-Fc. The cells were cultured using a CHO-S-SFM II medium (Invitrogen Corp.) supplemented with Geneticin (500 μg/mL), HT Supplement (Invitrogen Corp.), and penicillin/streptomycin (Invitrogen Corp.) as a culture medium. ITM2A-Fc proteins were purified from the culture supernatant of the cells thus established. First, the culture supernatant was applied to a HiTrap rProtein A FF column (GE Healthcare Bio-Sciences Corp.). The column was washed with a binding buffer (20 mM sodium phosphate, pH 7.0), followed by antibody elution with an eluting buffer (0.1 M glycine-HCl, pH 2.7). The buffer solution of the eluate neutralized with a neutralizing buffer (1 M Tris-HC1, pH 9.0) was replaced with PBS using a PD-10 column (GE Healthcare Bio-Sciences Corp.). The protein concentration was measured using DC Protein Assay Kit I (Bio-Rad Laboratories, Inc.).

(2-4) Preparation of cell line forced to express ITM2A

[0200] A nucleotide sequence encoding C-terminally HA-tagged ITM2A was cloned into pMCN2i vectors. First, the ITM2A-encoding nucleotide sequence was amplified by PCR using pCR2.1_ITM2A as a template, a forward primer (SEQ ID NO: 53) having EcoRI site, and a reverse primer (SEQ ID NO: 54) having NotI site and an HA tag sequence, and cloned into pCR2.1-TOPO vectors. EcoRI/NotI-digested fragments of the plasmids obtained as a result of the cloning were cloned into the EcoRI-NotI sites of pMCN2i vectors to obtain plasmids pMCN2i_ITM2A-HA. The nucleotide sequence from start codon to stop codon in the inserted sequence comprising the ITM2A-encoding nucleotide sequence in pMCN2i_ITM2A-HA is shown in SEQ ID NO: 55, and an amino acid sequence encoded thereby is shown in SEQ ID NO: 56. pMCN2i_ITM2A-HA digested with PvuI was transduced into DG44 cells by electroporation. Transductants were screened for with Geneticin (500 μg/mL) to establish a CHO cell line constantly expressing C-terminally HA-tagged ITM2A (ITM2A_CHO).

(2-5) Preparation of anti-ITM2A monoclonal antibody

[0201] A Balb/c mouse (female, 8 weeks old, Charles River Laboratories Japan Inc.) was subjected to DNA immunization seven times (days 0, 7, 11, 14, 17, 21, and 24) using Helios Gene Gun (Bio-Rad Laboratories, Inc.). The DNA immunization employed pMCN2i_mIL3ss-ITM2Aoutside. Following the DNA immunization, 50 μg of the ITM2A-Fc proteins mixed with a Freund's incomplete adjuvant (BD Diagnostics) was subcutaneously injected to the mouse (days 49, 91, 99, and 107). At day 115, 50 μg of the ITM2A-Fc proteins was administered to the tail vein without being mixed with an adjuvant. Three days thereafter, the spleen was excised and used as a starting material to prepare hybridomas. First, the excised spleen cells were mixed with a mouse myeloma cell line P3-X63Ag8U1 (P3U1, ATCC) at a ratio of 2:1. PEG1500 (Roche Diagnostics K.K.) was gradually added to the mixed solution to perform cell fusion. An RPMI1640 medium (Invitrogen Corp.) supplemented with penicillin/streptomycin was added to the mixed solution, and the mixture was centrifuged, followed by the removal of the supernatant to remove PEG1500 from the mixed solution. Next, the cells were suspended in a HAT medium (RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, 1 x HAT Media Supplement (Sigma-Aldrich Corp.), and 0.5 x BM-Condimed H1 Hybridoma Cloning Supplement (Roche Diagnostics K.K.)), and the resulting cell suspension was inoculated at a concentration of 1 x 10^5 P3U1 cells/well to eight 96-well plates. The plates were incubated at 37°C for 8 days in a 5% CO2 incubator, followed by screening using the culture supernatant in each well. The screening was performed by assaying binding to the ITM2A_CHO cells and the parent CHO cells using a flow cytometer (FACSCalibur, Becton, Dickinson and Company). Clones producing antibodies specifically binding to the ITM2A_CHO cells were selected and cloned as single clones by the limiting dilution method to isolate hybridomas producing antibodies binding to ITM2A. From these experiments, anti-ITM2A monoclonal antibodies BE5-1, BE6-1, BE7-1-1, and BE13-1 were established. These antibodies were isotyped using Isostrip (Roche Diagnostics K.K.) and consequently, all determined to be mouse IgG1κ.

[0202] The established hybridomas of BE5-1, BE6-1, BE7-1-1, and BE13-1 were each cultured in a HAT medium supplemented with Ultra Low IgG FBS (Invitrogen Corp.) instead of FBS. From each culture supernatant, each anti-ITM2A antibody (BE5-1, BE6-1, BE7-1-1, and BE13-1) was purified using a HiTrap Protein G HP column (GE Healthcare Bio-Sciences Corp.). The concentration of the purified antibody was measured using DC Protein Assay Kit I.

[Example 3] Analysis on epitope for anti-ITM2A monoclonal antibody by ELISA

(3-1) Preparation of partial ITM2A protein

[0203] The ITM2A extracellular region (Tyr75-Glu263) or a portion thereof (Tyr75-Lys182) was expressed as a GST-fusion protein in *E. coli* (Tyr75-Glu263: GST-ITM2A-L, and Tyr75-Lys182: GST-ITM2A-S). The fusion protein was C-terminally His-tagged. First, a nucleotide sequence encoding ITM2A (Tyr75-Glu263) or ITM2A (Tyr75-Lys182) was amplified by PCR using pCR2.1_ITM2A as a template, a forward primer (SEQ ID NO: 57) having EcoRI site, and a reverse primer (SEQ ID NO: 58 or 59) having SalI site and a His tag sequence, and cloned into pCR2.1-TOPO vectors. EcoRI/SalI-digested fragments of the plasmids obtained as a result of the cloning were cloned into the EcoRI-SalI sites of pGEX6P-1 vectors (GE Healthcare Bio-Sciences Corp.) to obtain plasmids pGEX_GST-ITM2A-L and pGEX_GST-ITM2A-S, respectively. The nucleotide sequence from start codon to stop codon in pGEX_GST-ITM2A-L is shown in SEQ ID NO: 60, and an amino acid sequence encoded thereby is shown in SEQ ID NO: 61. The nucleotide sequence from start codon to stop codon in the inserted sequence comprising the ITM2A-encoding nucleotide sequence in pGEX_GST-ITM2A-S is shown in SEQ ID NO: 62, and an amino acid sequence encoded thereby is shown in SEQ ID NO: 63.

[0204] BL21 (DE3) Competent Cells (Takara Bio Inc.) were transformed with pGEX_GST-ITM2A-L or pGEX_GST-ITM2A-S, and each transformant was induced to express GST-ITM2A-L or GST-ITM2A-S using isopropyl-thiogalactopyranoside. After washing with B-PER (Thermo Fisher Scientific K.K.), cell pellets were solubilized with a solubilizing buffer (8 M urea, 50 mM Tris-HCl (pH 8.0), and 300 mM NaCl). Cell extracts prepared with a solubilizing buffer supplemented with 10 mM imidazole were applied to a HisTrap HP column (GE Healthcare Bio-Sciences Corp.). The column was washed with a solubilizing buffer supplemented with 40 mM imidazole, followed by the elution of GST-ITM2A-L and GST-ITM2A-S using a solubilizing buffer supplemented with 500 mM imidazole. The protein concentrations of GST-ITM2A-L and GST-ITM2A-S were calculated on the basis of absorbance at 280 nm.

(3-2) Analysis on epitope for anti-ITM2A monoclonal antibody by ELISA

[0205] Each anti-ITM2A monoclonal antibody prepared in Example 2 was evaluated for its binding to GST-ITM2A-L and GST-ITM2A-S by ELISA. First, 100 μL each of GST-ITM2A-L and GST-ITM2A-S solutions having a concentration of 3 μg/mL was added to each well of a 96-well plate for ELISA (Nunc-Immuno Plate, Thermo Fisher Scientific K.K.) to coat the well with GST-ITM2A-L or GST-ITM2A-S. The coated well was blocked with a buffer containing 1% bovine serum albumin. Then, 100 μL each of solutions of the antibodies BE5-1, BE6-1, BE7-1-1, and BE13-1 diluted with the same buffer as above was added to each well. The plate was incubated at room temperature for 1 hour. The positive control used was an anti-His antibody (mouse IgG1, Santa Cruz Biotechnology, Inc.). The negative control used was mouse IgG1 (BD Pharmingen). Each antibody was diluted into 8 dilutions at a common ratio of 3.16 from a concentration of 1 μg/mL. After reaction with a secondary antibody (alkaline phosphatase-goat anti-mouse IgG (Gamma), Invitrogen Corp.), a substrate (phosphatase substrate, Sigma-Aldrich Corp.) was added to each well. Color developed by the reaction solution in each well was determined by the measurement of absorbance at 405 nm to 655 nm.

[0206] As a result, all the antibodies BE5-1, BE6-1, BE7-1-1, and BE13-1 bound to GST-ITM2A-L, whereas only the antibodies BE5-1 and BE6-1 bound to GST-ITM2A-S (Figure 2). This suggested that the antibodies BE5-1 and BE6-1 recognized ITM2A Tyr75-Lys182 and the antibodies BE7-1-1 and BE13-1 recognized ITM2A Leu183-Glu263.

[Example 4] Analysis on epitope for anti-ITM2A monoclonal antibody by FACS

(4-1) Preparation of cell line forced to express C-terminally truncated ITM2A

[0207] The extracellular region of ITM2A contains a consensus sequence (Arg226-Leu227-Arg228-Arg229) that is cleaved by furin. Thus, a cell line expressing a protein (ITM2A-furin) comprising the sequence from Met1 to Leu227 of ITM2A and an HA tag added downstream thereof was prepared. First, a nucleotide sequence encoding ITM2A (Met1-Leu227) was amplified by PCR using pCR2.1_ITM2A as a template, a forward primer (SEQ ID NO: 53) having EcoRI site, and a reverse primer (SEQ ID NO: 64) having NotI site and an HA tag sequence, and cloned into pCR2.1-TOPO vectors. EcoRI/NotI-digested fragments of the plasmids obtained as a result of the cloning were cloned into the EcoRI-NotI sites of pMCN2i vectors to obtain plasmids pMCN2i_ITM2A-furin-HA. The nucleotide sequence from start codon to stop codon in the inserted sequence comprising the ITM2A-furin-encoding nucleotide sequence in pMCN2i_ITM2A-furin-HA is shown in SEQ ID NO: 65, and an amino acid sequence encoded thereby is shown in SEQ ID NO: 66. pMCN2i_ITM2A-furin-HA digested with PvuI was transduced into DG44 cells by electroporation. Transductants were screened for with Geneticin (500 μg/mL) to establish a CHO cell line (ITM2A-furin_CHO) constantly expressing C-terminally HA-tagged ITM2A-furin.

(4-2) Analysis on epitope for anti-ITM2A monoclonal antibody by FACS

[0208] Each anti-ITM2A monoclonal antibody prepared in Example 2 was assayed for its binding to ITM2A-furin by flow cytometry (FACS). The cells used were ITM2A-furin_CHO prepared in the paragraph (4-1), ITM2A_CHO prepared in the paragraph (2-4), and host cells CHO. These cells were separately suspended in PBS supplemented with 0.2% bovine serum albumin and 0.1% NaN3 (FACS buffer). To each cell suspension, the antibody BE5-1, BE6-1, BE7-1-1, or BE13-1, mouse IgG1, or an anti-HA antibody (clone HA-7, mouse IgG1, Sigma-Aldrich Corp.) was added, and the resulting cell suspension was incubated for 1 hour on ice. Each antibody was diluted into 6 dilutions at a common ratio of 5 from a concentration of 10 μg/mL. After washing of the cells with a FACS buffer, an FITC-labeled anti-mouse IgG antibody (Goat F(ab')2 Fragment Anti-mouse IgG (Fcy)-FITC, Beckman Coulter, Inc.) was added thereto as a secondary antibody. The resulting cell suspension was incubated for 1 hour on ice. The cells were washed with a FACS buffer, then suspended in a FACS buffer supplemented with 10 μg/mL propidium iodide (PI, Sigma-Aldrich Corp.), and subjected to assay using a flow cytometer. The assay data was analyzed using CELLQuest software (Becton, Dickinson and Company) to evaluate a PI-negative live cell population.

[0209] All the antibodies BE5-1, BE6-1, BE7-1-1, and BE13-1 bound to ITM2A_CHO, whereas only the antibodies BE5-1 and BE6-1 bound to ITM2A-furin_CHO (Figures 3A and 3B). This suggested that the antibodies BE5-1 and BE6-1 recognized ITM2A Tyr75-Leu227 and the antibodies BE7-1-1 and BE13-1 recognized Arg228-Glu263. None of the antibodies BE5-1, BE6-1, BE7-1-1, and BE13-1 (each 10 μg/mL) bound to host CHO cells (Figure 3C). The anti-HA antibody (10 μg/mL) was used to confirm the expression of ITM2A or ITM2A-furin in each cell line (Figure 3D).

[Example 5] Analysis on binding of anti-ITM2A monoclonal antibody to mouse ITM2A

(5-1) Cloning of mouse ITM2A gene

[0210] A nucleotide sequence encoding mouse ITM2A was amplified by PCR using mouse brain cDNA (Mouse MTC Panel I, Clontech Laboratories, Inc.) as a template, a forward primer (SEQ ID NO: 67), and a reverse primer (SEQ ID NO: 68), and cloned into pCR2.1-TOPO vectors to obtain pCR2.1_mITM2A. This PCR employed KOD Plus (Toyobo Co., Ltd.) and was performed by denaturation at 94°C for 2 minutes followed by 30 repetitive reaction cycles each involving 98°C for 10 seconds, 59°C for 30 seconds, and 68°C for 1 minute. The inserted sequence in pCR2.1_mITM2A was sequenced to confirm that the inserted sequence was the same as a sequence registered under RefSeq Accession No. NM_008409.

(5-2) Preparation of cell line forced to express mouse ITM2A

[0211] A nucleotide sequence encoding C-terminally HA-tagged mouse ITM2A was cloned into pMCN2i vectors. First, the mouse ITM2A-encoding nucleotide sequence was amplified by PCR using pCR2.1_mITM2A as a template, a forward primer (SEQ ID NO: 69) having EcoRI site, and a reverse primer (SEQ ID NO: 70) having NotI site and an HA tag sequence. The amplified fragments were digested with EcoRI and NotI and cloned into the EcoRI-NotI sites of pMCN2i vectors to obtain plasmids pMCN2i_mITM2A-HA. The nucleotide sequence from start codon to stop codon in the inserted sequence comprising the mouse ITM2A-encoding nucleotide sequence in pMCN2i_mITM2A-HA is shown in SEQ ID NO: 71, and an amino acid sequence encoded thereby is shown in SEQ ID NO: 72. pMCN2i_mITM2A-HA digested with PvuI was transduced into DG44 cells by electroporation. Transductants were screened for with Geneticin (500 μg/mL) to establish a CHO cell line (mITM2A_CHO) constantly expressing C-terminally HA-tagged mouse ITM2A.

(5-3) Analysis on binding of anti-ITM2A monoclonal antibody to mouse ITM2A

[0212] Each anti-ITM2A monoclonal antibody prepared in Example 2 was evaluated for its binding to mouse ITM2A by FACS. The cells used were ITM2A_CHO prepared in the paragraph (2-4), and mITM2A_CHO prepared in the paragraph (5-2). The binding was detected using FACS in the same way as the procedures described in the paragraph 4-2.
[0213] The antibodies BE5-1, BE7-1-1, and BE13-1 bound to mITM2A_CHO, whereas the antibody BE6-1 did not bound thereto (Figure 4). This suggested that the antibodies BE5-1, BE7-1-1, and BE13-1 cross-reacted with mouse ITM2A whereas the antibody BE6-1 did not cross-react therewith.

[Example 6] Evaluation of anti-ITM2A monoclonal antibody for its binding activity to ITM2A using Western blot

[0214] Each anti-ITM2A monoclonal antibody prepared in Example 2 was evaluated for whether its binding to ITM2A was detectable using Western blot. First, 1 x 10^7 cells each of ITM2A_CHO prepared in the paragraph (2-4), ITM2A-furin_CHO prepared in the paragraph (4-1), and host CHO cells were washed with PBS and then lysed using 1 mL of a lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and Protease Inhibitor Cocktail (Sigma-Aldrich Corp.)) to obtain whole cell lysates. Each lysate and 2 x Sample Buffer (Sigma-Aldrich Corp.) mixed in equal amounts were heat-treated. Then, 5 μL of each lysate was subjected to SDS-PAGE. After the electrophoresis, proteins, etc. contained in SDS-PAGE gel were transferred to a PVDF membrane (Immobilon-P, Millipore Corp.), which was then incubated at room temperature for 1 hour with 10 μg/mL of the antibody BE5-1, BE6-1, BE7-1-1, or BE13-1, or an anti-HA antibody (clone F-7, Santa Cruz Biotechnology, Inc.). The membrane was incubated at room temperature for 1 hour with an HRP-labeled anti-mouse IgG antibody (GE Healthcare Bio-Sciences Corp.) used as a secondary antibody. Finally, light emitted using ECL Western Blotting Detection Reagents (GE Healthcare Bio-Sciences Corp.) was exposed onto an X-ray film to detect a band representing an antigen-antibody complex.
[0215] The Western blot under these conditions showed that only the antibody BE6-1 was able to form an antigen-antibody reaction complex with the antigen ITM2A (Figure 5) .

[Example 7] Determination of gene sequence encoding variable region of anti-ITM2A monoclonal antibody

[0216] The gene sequences of variable regions of each anti-ITM2A monoclonal antibody prepared in Example 2 were determined. Total RNAs were prepared from hybridomas (1 x 10^6 cells) producing each antibody using RNeasy Mini Kit (Qiagen N.V.). Next, cDNAs were synthesized using Smarter Race cDNA Amplification Kit (Clontech Laboratories, Inc.) with the RNAs as a template. The primers used were a primer (SEQ ID NO: 73) complementary to a nucleotide sequence encoding the heavy chain constant region of a mouse IgG1κ antibody and a primer (SEQ ID NO: 74) complementary to a nucleotide sequence encoding the light chain constant region thereof. The amplification products cloned into pCR2.1-TOPO vectors were sequenced. The variable region sequences of each antibody are summarized in Table 1, and the variable region CDR sequences thereof are summarized in Table 2.

[Table 1]

[0217]

Table 1 Sequence of variable region of ITM2A antibody

Antibody		SEQ ID NO (nucleotide sequence)	SEQ ID NO (amino acid sequence)
BE5-1	Heavy chain variable region	27	28
	Light chain variable region	29	30

(continued)

Antibody		SEQ ID NO (nucleotide sequence)	SEQ ID NO (amino acid sequence)
BE6-1	Heavy chain variable region	31	32
	Light chain variable region	33	34
BE7-1-1	Heavy chain variable region	35	36
	Light chain variable region	37	38
BE13-1	Heavy chain variable region	39	40
	Light chain variable region	41	42

[Table 2]

[0218]

Table 2 Amino acid sequence of variable region CDR of ITM2A antibody

Antibody			SEQ ID NO (amino acid sequence)
BE5-1	Heavy chain	CDR1	3
		CDR2	4
		CDR3	5
	Light chain	CDR1	6
		CDR2	7
		CDR3	8
BE6-1	Heavy chain	CDR1	9
		CDR2	10
		CDR3	11
	Light chain	CDR1	12
		CDR2	13
		CDR3	14
BE7-1-1	Heavy chain	CDR1	15
		CDR2	16
		CDR3	17
	Light chain	CDR1	18
		CDR2	19
		CDR3	20
BE13-1	Heavy chain	CDR1	21
		CDR2	22
		CDR3	23
	Light chain	CDR1	24
		CDR2	25
		CDR3	26

[Example 8] Analysis on expression of ITM2A in human cancer cell line and evaluation of anti-ITM2A monoclonal antibody for its ADCC activity and cell growth inhibitory activity

(8-1) Analysis on expression of ITM2A in human cancer cell line

[0219] The expression of ITM2A was assayed in human cancer cell lines by FACS. The antibody used was the antibody BE6-1 having a concentration of 10 μg/mL. The expression of ITM2A was assayed in the same way as the procedures described in Example 4. The secondary antibody used was an FITC-labeled anti-mouse Ig antibody (goat F(ab')2) included in Qifi-Kit (Dako). The negative control used was mouse IgG1 having a concentration of 10 μg/mL. The assay results demonstrated that ITM2A was expressed on the cells of Ewing's sarcoma cell lines (A-673, RD-ES, SK-ES-1, and SK-N-MC), T cell acute lymphocytic leukemia cell lines (CCRF-CEM, Jurkat, and MOLT-4), a T cell lymphoma cell line (HuT78), and acute myeloid leukemia cell lines (KG-1a and TF-1a) (Figure 6).

(8-2) Study on ADCC activity of anti-ITM2A monoclonal antibody

[0220] The antibody-dependent cell-mediated cytotoxicity (ADCC) activity of the antibody BE6-1 was assayed. Human cancer cell lines CCRF-CEM and KG-1a were separately cultured for 1 hour in the presence of chromium-51 (GE Healthcare Bio-Sciences Corp.) and then washed three times with an RPMI1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (hereinafter, referred to as an RPMI medium). Each cell suspension of 1×10^5 cells/mL was prepared using the RPMI medium. The cell suspension was added at a concentration of 100 μL/well to a 96-well plate. Next, the antibody BE6-1 or mouse IgG1 was diluted with the RPMI medium and added thereto at a concentration of 50 μL/well. The final concentration of the antibody BE6-1 was adjusted to 10, 2, 0.4, 0.08, and 0.016 μg/mL. The final concentration of the mouse IgG1 was adjusted to 10 μg/mL. The plate was left standing at room temperature for 15 minutes. Then, a cell suspension of effector cells adjusted to 1×10^6 cells/mL with the RPMI medium was added thereto at a concentration of 50 μL/well. The effector cells used were NK-92 cells (ATCC) constantly expressing chimeric proteins comprising the extracellular region of mouse Fcγ receptor III (RefSeq Accession No. NM_010188) fused in frame with the transmembrane and intracellular regions of human Fcε receptor I-gamma (RefSeq Accession No. NM_004106) (WO2008093688). The plate was incubated at 37°C for 4 hours in a 5% CO2 incubator. Then, 100 μL/well of the culture supernatant was recovered, and the radioactivity (cpm) of the culture supernatant was measured using a gamma counter (1480 WIZARD 3", Wallac). The measurement value was applied to the following expression to calculate the rate (%) of specific chromium release:

$$\text{Rate (\%) of specific chromium release} = (A - C) \times 100 / (B - C).$$

[0221] In the expression, A represents the radioactivity in each well; B represents a mean of radioactivity values of wells containing cells lysed with 1% (final concentration) Nonidet P-40; and C represents a mean of radioactivity values of wells supplemented with only target cells. The experiment was triplicated to calculate a mean of the rates of specific chromium release and standard deviation.

[0222] Human cancer cell lines A-673 and SK-N-MC were inoculated at each concentration of 5×10^3 cells/well to a plate (Cellbind surface 96-well cell culture plate (Corning Inc.)). The plate was incubated at 37°C for 4 days in a 5% CO2 incubator. After addition of chromium-51 to each well, the plate was further incubated for 1 hour. Each well was carefully washed with a medium so as not to dissociate the cells. Then, a medium was added thereto at a concentration of 50 μL/well. Next, the antibody BE6-1 or mouse IgG1 was added thereto at a concentration of 50 μL/well. The final concentration of the antibody BE6-1 was adjusted to 10, 2, 0.4, 0.08, and 0.016 μg/mL. The final concentration of the mouse IgG1 was adjusted to 10 μg/mL. The plate was left standing at room temperature for 15 minutes. Then, effector cells adjusted to 8×10^5 cells/mL with a medium were added thereto at a concentration of 100 μL/well. The rate of specific chromium release was calculated according to the expression described above. All the media used were a DMEM medium (Invitrogen Corp.) supplemented with 10% fetal bovine serum and penicillin/streptomycin.

[0223] The antibody BE6-1 induced an ADCC activity against the cells A-673, SK-N-MC, CCRF-CEM, and KG-1a in a concentration-dependent manner (Figure 7).

(8-3) Study on cell growth inhibitory activity of anti-ITM2A monoclonal antibody

[0224] The cell growth inhibitory activity of the antibody BE6-1 was assayed in the presence of a toxin-conjugated secondary antibody. The toxin-conjugated secondary antibody used was a saporin-labeled anti-mouse IgG antibody

(Mab-Zap, Advanced Targeting Systems). A human cancer cell line CCRF-CEM was inoculated at a concentration of 6 x 10^3 cells/well to a 96-well plate. The antibody BE6-1 (500, 100, 20, and 4 ng/mL) or mouse IgG1 (500 ng/mL) was added to each well. Mab-Zap was added at a concentration of 500 ng/mL to each well. The plate was incubated for 3 days. Then, cell growth in each well was assayed using WST-8 (Cell Count Reagent SF, Nacalai Tesque, Inc.). The experiment was triplicated to calculate a mean and standard deviation with cell grown in a well supplemented with only a medium as 0% and cell growth in a well supplemented with only cells as 100%. The medium used was an RPMI1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin.

[0225] A human cancer cell line HuT78 was inoculated at a concentration of 1 x 10^4 cells/well to a 96-well plate. The antibody BE6-1 (2500, 500, 100, and 20 ng/mL) or mouse IgG1 (2500 ng/mL) was added to each well. Mab-Zap was added at a concentration of 2500 ng/mL to each well. The plate was incubated for 4 days. Then, cell growth in each well was assayed using WST-8. The medium used was an IMDM medium (Invitrogen Corp.) supplemented with 20% fetal bovine serum and penicillin/streptomycin.

[0226] A human cancer cell line A-673 was inoculated at a concentration of 3 x 10^3 cells/well to a 96-well plate. The plate was incubated for 1 day. Then, the antibody BE6-1 (1000, 200, 40, and 8 ng/mL) or mouse IgG1 (1000 ng/mL) was added to each well of the plate. Mab-Zap was added at a concentration of 1000 ng/mL to each well. The plate was incubated for 3 days. Then, cell growth in each well was assayed using WST-8. The medium used was a DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin.

[0227] The antibody BE6-1 inhibited cell growth of each cell line in a concentration-dependent manner in the presence of the toxin-conjugated secondary antibody (Figure 8). This suggested that the anti-ITM2A monoclonal antibody directly conjugated with toxin was also able to inhibit the growth of cancer cells.

[Example 9] Correlation between expression of EWS-FLI1 fusion gene and ITM2A

(9-1) Expression analysis of EWS-FLI1 fusion gene in clinical Ewing's sarcoma sample

[0228] 85% of Ewing's sarcoma cases are known to have observable t(11;22)(q24;q12) chromosomal translocation and the expression of a fusion gene (EWS-FLI1) comprising the 5' end of the EWS gene fused with the 3' end of the FLI-1 gene (Cancer Lett (2007) 254, 1-10). Thus, 13 clinical Ewing's sarcoma samples including the samples used in the expression analysis of Example 1 (ews_2, ews_3, ews_4, ews_5, ews_6, ews_7, ews_8, ews_9, ews_10, ews_11, ews_12, ews_13, and ews_15) were analyzed for the expression of the EWS-FLI1 fusion gene by PCR. First, cDNAs were synthesized from the RNAs of each clinical Ewing's sarcoma sample using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corp.). Next, the EWS-FLI1 fusion gene was amplified by PCR using the cDNAs as a template, a forward primer (SEQ ID NO: 75), and a reverse primer (SEQ ID NO: 76). This amplification employed KOD Plus Version 2 (Toyobo Co., Ltd.) and was performed by thermal denaturation at 94°C for 2 minutes followed by 30 repetitive cycles each involving 98°C for 10 seconds and 68°C for 1.5 minutes. The primer sequences used were the same as those described in the literature (N Engl J Med (1994) 331, 294-9). A nucleotide sequence encoding β-actin was amplified as a control using primers included in the kit (SuperScript III First-Strand Synthesis System for RT-PCR). The amplification reaction using PCR employed KOD Plus Version 2 and was performed by thermal denaturation at 94°C for 2 minutes followed by 25 repetitive cycles each involving 98°C for 10 seconds, 58°C for 30 seconds, and 68°C for 30 seconds. The positive control used was a Ewing's sarcoma cell line SK-ES-1 expressing the EWS-FLI1 fusion gene. The negative control used was a lymphoma cell line NK-92.

[0229] The PCR results demonstrated the expression of the EWS-FLI1 fusion gene in 9 (ews_4, ews_5, ews_7, ews_8, ews_9, ews_11, ews_12, ews_13, and ews_15) out of the 13 clinical Ewing's sarcoma samples (Figure 9A).

(9-2) Analysis on expression of ITM2A in clinical Ewing's sarcoma sample

[0230] The expression of the ITM2A gene was analyzed in clinical Ewing's sarcoma samples by PCR. The ITM2A gene was amplified by PCR using the cDNAs synthesized in the paragraph (9-1) as a template, a forward primer (SEQ ID NO: 77), and a reverse primer (SEQ ID NO: 78). This amplification employed KOD Plus Version 2 and was performed by thermal denaturation at 94°C for 2 minutes followed by 25 repetitive cycles each involving 98°C for 10 seconds, 65°C for 30 seconds, and 68°C for 30 seconds. The positive control used was pCR2.1_ITM2A prepared in the paragraph (2-1). The negative control used was a lymphoma cell line NK-92.

[0231] As a result of the PCR, ITM2A was expressed in the 9 cases expressing the EWS-FLI1 fusion gene, demonstrating high correlation between their expression (Figure 9B).

SEQUENCE LISTING

[0232]

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<120> ITM2A
<130> PCG-9035WO
<150> JP 2011-092488
<151> 2011-04-18
<160> 86
<170> PatentIn version 3.1
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            20                  25                  30
Ile Leu Thr Gly Lys Glu Leu Arg Val Ala Thr Gln Glu Lys Glu Gly
        35                  40                  45
Ser Ser Gly Arg Cys Met Leu Thr Leu Leu Gly Leu Ser Phe Ile Leu
    50                  55                  60
Ala Gly Leu Ile Val Gly Gly Ala Cys Ile Tyr Lys Tyr Phe Met Pro
65                  70                  75                  80
Lys Ser Thr Ile Tyr Arg Gly Glu Met Cys Phe Phe Asp Ser Glu Asp
                85                  90                  95
Pro Ala Asn Ser Leu Arg Gly Gly Glu Pro Asn Phe Leu Pro Val Thr
            100                 105                 110
Glu Glu Ala Asp Ile Arg Glu Asp Asp Asn Ile Ala Ile Ile Asp Val
        115                 120                 125
Pro Val Pro Ser Phe Ser Asp Ser Asp Pro Ala Ala Ile Ile His Asp
    130                 135                 140
Phe Glu Lys Gly Met Thr Ala Tyr Leu Asp Leu Leu Leu Gly Asn Cys
145                 150                 155                 160
Tyr Leu Met Pro Leu Asn Thr Ser Ile Val Met Pro Pro Lys Asn Leu
                165                 170                 175
Val Glu Leu Phe Gly Lys Leu Ala Ser Gly Arg Tyr Leu Pro Gln Thr
            180                 185                 190
Tyr Val Val Arg Glu Asp Leu Val Ala Val Glu Glu Ile Arg Asp Val
        195                 200                 205
Ser Asn Leu Gly Ile Phe Ile Tyr Gln Leu Cys Asn Asn Arg Lys Ser
    210                 215                 220
Phe Arg Leu Arg Arg Arg Asp Leu Leu Leu Gly Phe Asn Lys Arg Ala
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gttgccaccc aggaaaaaga gggctcctct gggagatgta tgcttactct cttaggcctt    180
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gctgtggagg aaattcgtga tgttagtaac cttggcatct ttatttacca actttgcaat    660
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1               5                   10                  15
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1 5

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1               5                   10                  15
Gly
```

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1 5

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1 5 10 15
Gly

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ccagggaaga ggctggagtg gattgctgta attacaggca aatctgataa ttatggagca    180
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Ser Leu Lys Leu Ser Cys Val Thr Ser Gly Phe Thr Phe Ser Asp Tyr
            20                  25                  30
Arg Met His Trp Leu Arg Gln Ser Pro Gly Lys Arg Leu Glu Trp Ile
        35                  40                  45
Ala Val Ile Thr Gly Lys Ser Asp Asn Tyr Gly Ala Ser Tyr Ala Glu
    50                  55                  60
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser
65                  70                  75                  80
Val Tyr Leu Gln Met Asn Arg Leu Arg Glu Glu Asp Thr Ala Thr Tyr
                85                  90                  95
Tyr Cys Ser Arg Arg Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val
            100                 105                 110
Ser Ser
```

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ttcctgcaga ggccaggcca gtctcctcag ctcctgatat atcggatgtc caaccttgcc    180
ccaggagtcc cagacaggtt cagtggcagt gggtcaggaa ctgctttcac actgagaatc    240
agtagagtgg aggctgagga tgtgggtgtt tattactgta tgcaacatct cgaatatcct    300
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<213> Mus musculus
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Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
            20                  25                  30
Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
        35                  40                  45
Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Pro Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85                  90                  95
Leu Glu Tyr Pro Phe Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys
            100                 105                 110
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aaccagaagt tcaagggcaa ggccacattg actgtagaca agtcctccat cacagcctac    240
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```

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1               5                   10                  15
Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr Glu Tyr
            20                  25                  30
Thr Met His Trp Val Lys Gln Ser His Gly Lys Ser Leu Glu Trp Ile
        35                  40                  45
Gly Gly Ile Asn Pro Asn Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60
Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ile Thr Ala Tyr
65                  70                  75                  80
Met Glu Leu Arg Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Arg Gly Pro Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
            100                 105                 110
Ser Thr
```

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tacctgcaga agccaggcca gtctccaaag ttcctgatct acaaagtttc caaccgattt    180
tttggggtcc cagacaggtt cagtggcagt ggatcaggga cagatttcac actcaagatc    240

agcagagtgg aggctgagga tctgggagtt tatttctgct ctcaaactac acattttcca    300
ttcacgttcg gctcggggac aaagttggaa ataaaa                              336
```

<210> 34
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Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30
Asn Gly Asn Thr Tyr Phe His Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45
Pro Lys Phe Leu Ile Tyr Lys Val Ser Asn Arg Phe Phe Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Thr
                85                  90                  95
Thr His Phe Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110
```

<210> 35
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cctggacaag gccttgagtg gatcggagag attgatcctt ctgatagcta taatgactac    180
aatcaaaagt tcaagggcaa ggccacattg actgtagaca aatcctccag cacagcctac    240
atgcagctca gcagcctgac atctgaggac tctgcggtct attactgtgc aagatggggg    300
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```

<210> 36
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<400> 36

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Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30
Trp Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45
Gly Glu Ile Asp Pro Ser Asp Ser Tyr Asn Asp Tyr Asn Gln Lys Phe
    50                  55                  60
Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Arg Trp Gly Glu Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val
            100                 105                 110
Ser Ser
```

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ttgttacaga ggccaggcca gtctccaaag cgcctaatct atctggtgtc taaactggac     180
tctggagtcc ctgacaggtt cactggcagt ggatcaggga cagatttcac actgaaaatc     240
agcagagtgg aggctgagga tttgggagtt tattattgct ggcaaggtac acattttcct     300
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Gln Pro Ala Ser Ile Ser Cys Lys Ser Ser Gln Ser Leu Leu Asp Ser
            20                  25                  30
Asp Gly Lys Thr Tyr Leu Asn Trp Leu Leu Gln Arg Pro Gly Gln Ser
        35                  40                  45
Pro Lys Arg Leu Ile Tyr Leu Val Ser Lys Leu Asp Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Trp Gln Gly
                85                  90                  95
Thr His Phe Pro Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110
```

<210> 39
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catggaaaga gccttgagtg gattggagat attaatccta acaatggtgg tcttagttac    180
aaccagaagt tcaagggcaa ggccacattg actgtagaca aatcctccag cacagcctac    240
atgcagctcg ccagcctgac atctgaggac tctgcagtct attactgtgc aatatggtcc    300
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            20                  25                  30
Tyr Met Lys Trp Val Lys Gln Ser His Gly Lys Ser Leu Glu Trp Ile
        35                  40                  45
Gly Asp Ile Asn Pro Asn Asn Gly Gly Leu Ser Tyr Asn Gln Lys Phe
    50                  55                  60
Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80
Met Gln Leu Ala Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Ile Trp Ser Gly Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
            100                 105                 110
Ser Ala
```

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ttgttacaga ggccaggcca gtctccaaag cgcctaatct atctggtgtc taaactggac    180
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```

<210> 42
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<213> Mus musculus
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1               5                   10                  15
Gln Pro Ala Ser Ile Ser Cys Lys Ser Ser Gln Ser Leu Leu Asp Ser
            20                  25                  30
Asp Gly Lys Thr Tyr Leu Asn Trp Leu Leu Gln Arg Pro Gly Gln Ser
        35                  40                  45
Pro Lys Arg Leu Ile Tyr Leu Val Ser Lys Leu Asp Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Trp Gln Gly
                85                  90                  95
Thr His Phe Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110
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<211> 27
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<213> Artificial Sequence
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<223> PCR primer
<400> 44
ttactcttga cagatcttgg tctcaac 27
<210> 45
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<212> DNA
<213> Artificial Sequence
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<223> PCR primer
<400> 45
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<210> 46
<211> 30
<212> DNA
<213> Artificial Sequence
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<223> PCR primer
<400> 46
gcggccgctt actcttgaca gatcttggtc 30
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<212> DNA
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47 pMCN2i_mIL3ss-ITM2Aoutside insert
<400> 47

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gcccagccgg ccatggcgta caagtacttc atgcccaaga gcaccattta ccgtggagag     120
atgtgctttt ttgattctga ggatcctgca aattcccttc gtggaggaga gcctaacttc     180
ctgcctgtga ctgaggaggc tgacattcgt gaggatgaca acattgcaat cattgatgtg     240
cctgtcccca gtttctctga tagtgaccct gcagcaatta ttcatgactt tgaaaaggga     300
atgactgctt acctggactt gttgctgggg aactgctatc tgatgcccct caatacttct     360
attgttatgc ctccaaaaaa tctggtagag ctctttggca aactggcgag tggcagatat     420
ctgcctcaaa cttatgtggt tcgagaagac ctagttgctg tggaggaaat tcgtgatgtt     480
agtaaccttg gcatctttat ttaccaactt tgcaataaca gaaagtcctt ccgccttcgt     540
cgcagagacc tcttgctggg tttcaacaaa cgtgccattg ataaatgctg gaagattaga     600
cacttcccca acgaatttat tgttgagacc aagatctgtc aagagtaa                  648
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<210> 48
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<212> PRT
<213> Artificial Sequence
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<223> pMCN2i_mIL3ss-ITM2Aoutside insert
<400> 48

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Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu
1               5                   10                  15
Leu Leu Met Leu Ala Gln Pro Ala Met Ala Tyr Lys Tyr Phe Met Pro
            20                  25                  30
Lys Ser Thr Ile Tyr Arg Gly Glu Met Cys Phe Phe Asp Ser Glu Asp
        35                  40                  45
Pro Ala Asn Ser Leu Arg Gly Gly Glu Pro Asn Phe Leu Pro Val Thr
    50                  55                  60
Glu Glu Ala Asp Ile Arg Glu Asp Asp Asn Ile Ala Ile Ile Asp Val
65                  70                  75                  80
Pro Val Pro Ser Phe Ser Asp Ser Asp Pro Ala Ala Ile Ile His Asp
                85                  90                  95
Phe Glu Lys Gly Met Thr Ala Tyr Leu Asp Leu Leu Leu Gly Asn Cys
            100                 105                 110
Tyr Leu Met Pro Leu Asn Thr Ser Ile Val Met Pro Pro Lys Asn Leu
        115                 120                 125
Val Glu Leu Phe Gly Lys Leu Ala Ser Gly Arg Tyr Leu Pro Gln Thr
    130                 135                 140
Tyr Val Val Arg Glu Asp Leu Val Ala Val Glu Glu Ile Arg Asp Val
145                 150                 155                 160
Ser Asn Leu Gly Ile Phe Ile Tyr Gln Leu Cys Asn Asn Arg Lys Ser
                165                 170                 175
Phe Arg Leu Arg Arg Arg Asp Leu Leu Leu Gly Phe Asn Lys Arg Ala
            180                 185                 190
Ile Asp Lys Cys Trp Lys Ile Arg His Phe Pro Asn Glu Phe Ile Val
        195                 200                 205
Glu Thr Lys Ile Cys Gln Glu
    210                 215
```

<210> 49
<211> 42
<212> DNA
<213> Artificial Sequence
<220>
<223> PCR primer
<400> 49
ctggcccagc cggccatggc gtacaagtac ttcatgccca ag 42
<210> 50
<211> 38

<212> DNA
<213> Artificial Sequence
<220>
<223> PCR primer
<400> 50
gtcggtccgc gaggttcctc ttgacagatc ttggtctc 38
<210> 51
<211> 1347
<212> DNA
<213> Artificial Sequence
<220>
<223> pMCN2i_mIL3ss-ITM2Aoutside-Fc insert
<400> 51

```
atggttcttg ccagctctac caccagcatc cacaccatgc tgctcctgct cctgatgctg     60
gcccagccgg ccatggcgta caagtacttc atgcccaaga gcaccattta ccgtggagag    120
atgtgctttt ttgattctga ggatcctgca aattcccttc gtggaggaga gcctaacttc    180
ctgcctgtga ctgaggaggc tgacattcgt gaggatgaca acattgcaat cattgatgtg    240
cctgtcccca gtttctctga tagtgaccct gcagcaatta ttcatgactt tgaaaaggga    300
atgactgctt acctggactt gttgctgggg aactgctatc tgatgcccct caatacttct    360
attgttatgc ctccaaaaaa tctggtagag ctctttggca aactggcgag tggcagatat    420
ctgcctcaaa cttatgtggt tcgagaagac ctagttgctg tggaggaaat tcgtgatgtt    480
agtaaccttg gcatctttat ttaccaactt tgcaataaca gaaagtcctt ccgccttcgt    540
cgcagagacc tcttgctggg tttcaacaaa cgtgccattg ataaatgctg gaagattaga    600
cacttcccca acgaatttat tgttgagacc aagatctgtc aagaggaacc tcgcggaccg    660
acaatcaagc cctgtcctcc atgcaaatgc ccagcaccta acctcttggg tggaccatcc    720
gtcttcatct tccctccaaa gatcaaggat gtactcatga tctccctgag ccccatagtc    780
acatgtgtgg tggtggatgt gagcgaggat gacccagatg tccagatcag ctggtttgtg    840
aacaacgtgg aagtacacac agctcagaca caaacccata gagaggatta caacagtact    900
ctccgggtgg tcagtgccct ccccatccag caccaggact ggatgagtgg caaggagttc    960
aaatgcaagg tcaacaacaa agacctgcca gcgcccatcg agagaaccat ctcaaaaccc   1020
aaagggtcag taagagctcc acaggtatat gtcttgcctc caccagaaga agagatgact   1080
aagaaacagg tcactctgac ctgcatggtc acagacttca tgcctgaaga catttacgtg   1140
gagtggacca acaacgggaa aacagagcta aactacaaga acactgaacc agtcctggac   1200
tctgatggtt cttacttcat gtacagcaag ctgagagtgg aaaagaagaa ctgggtggaa   1260
agaaatagct actcctgttc agtggtccac gagggtctgc acaatcacca cacgactaag   1320
agcttctccc ggactccggg taaatga                                        1347
```

<210> 52
<211> 448
<212> PRT
<213> Artificial Sequence
<220>
<223> pMCN2i_mIL3ss-ITM2Aoutside-Fc insert
<400> 52

```
Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu
1               5                   10                  15
Leu Leu Met Leu Ala Gln Pro Ala Met Ala Tyr Lys Tyr Phe Met Pro
            20                  25                  30
Lys Ser Thr Ile Tyr Arg Gly Glu Met Cys Phe Phe Asp Ser Glu Asp
        35                  40                  45
Pro Ala Asn Ser Leu Arg Gly Gly Glu Pro Asn Phe Leu Pro Val Thr
    50                  55                  60
Glu Glu Ala Asp Ile Arg Glu Asp Asp Asn Ile Ala Ile Ile Asp Val
65                  70                  75                  80
Pro Val Pro Ser Phe Ser Asp Ser Asp Pro Ala Ala Ile Ile His Asp
                85                  90                  95
```

```
Phe Glu Lys Gly Met Thr Ala Tyr Leu Asp Leu Leu Leu Gly Asn Cys
            100                 105                 110
Tyr Leu Met Pro Leu Asn Thr Ser Ile Val Met Pro Pro Lys Asn Leu
        115                 120                 125
Val Glu Leu Phe Gly Lys Leu Ala Ser Gly Arg Tyr Leu Pro Gln Thr
    130                 135                 140
Tyr Val Val Arg Glu Asp Leu Val Ala Val Glu Glu Ile Arg Asp Val
145                 150                 155                 160
Ser Asn Leu Gly Ile Phe Ile Tyr Gln Leu Cys Asn Asn Arg Lys Ser
                165                 170                 175
Phe Arg Leu Arg Arg Arg Asp Leu Leu Leu Gly Phe Asn Lys Arg Ala
            180                 185                 190
Ile Asp Lys Cys Trp Lys Ile Arg His Phe Pro Asn Glu Phe Ile Val
        195                 200                 205
Glu Thr Lys Ile Cys Gln Glu Glu Pro Arg Gly Pro Thr Ile Lys Pro
    210                 215                 220
Cys Pro Pro Cys Lys Cys Pro Ala Pro Asn Leu Leu Gly Gly Pro Ser
225                 230                 235                 240
Val Phe Ile Phe Pro Pro Lys Ile Lys Asp Val Leu Met Ile Ser Leu
                245                 250                 255
Ser Pro Ile Val Thr Cys Val Val Val Asp Val Ser Glu Asp Asp Pro
            260                 265                 270
Asp Val Gln Ile Ser Trp Phe Val Asn Asn Val Glu Val His Thr Ala
        275                 280                 285
Gln Thr Gln Thr His Arg Glu Asp Tyr Asn Ser Thr Leu Arg Val Val
    290                 295                 300
Ser Ala Leu Pro Ile Gln His Gln Asp Trp Met Ser Gly Lys Glu Phe
305                 310                 315                 320
Lys Cys Lys Val Asn Asn Lys Asp Leu Pro Ala Pro Ile Glu Arg Thr
                325                 330                 335
Ile Ser Lys Pro Lys Gly Ser Val Arg Ala Pro Gln Val Tyr Val Leu
            340                 345                 350
Pro Pro Pro Glu Glu Glu Met Thr Lys Lys Gln Val Thr Leu Thr Cys
        355                 360                 365
Met Val Thr Asp Phe Met Pro Glu Asp Ile Tyr Val Glu Trp Thr Asn
    370                 375                 380
Asn Gly Lys Thr Glu Leu Asn Tyr Lys Asn Thr Glu Pro Val Leu Asp
385                 390                 395                 400
Ser Asp Gly Ser Tyr Phe Met Tyr Ser Lys Leu Arg Val Glu Lys Lys
                405                 410                 415
Asn Trp Val Glu Arg Asn Ser Tyr Ser Cys Ser Val Val His Glu Gly
            420                 425                 430
Leu His Asn His His Thr Thr Lys Ser Phe Ser Arg Thr Pro Gly Lys
        435                 440                 445
```

<210> 53
<211> 35
<212> DNA
<213> Artificial Sequence
<220>
<223> PCR primer
<400> 53
gaattcacca tggtgaaaat cgccttcaat acccc 35
<210> 54
<211> 62
<212> DNA
<213> Artificial Sequence
<220>
<223> PCR primer
<400> 54

gcggccgctt aagcgtaatc tggaacatcg tatgggtact cttgacagat cttggtctca 60
ac 62

<210> 55
<211> 819
<212> DNA
<213> Artificial Sequence
<220>
<223> pMCN2i_ITM2A-HA insert
<400> 55

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atggtgaaaa tcgccttcaa tacccctacc gccgtgcaaa aggaggaggc gcggcaagac     60
gtggaggccc tcctgagccg cacggtcaga actcagatac tgaccggcaa ggagctccga    120
gttgccaccc aggaaaaaga gggctcctct gggagatgta tgcttactct cttaggcctt    180
tcattcatct tggcaggact tattgttggt ggagcctgca tttacaagta cttcatgccc    240
aagagcacca tttaccgtgg agagatgtgc tttttttgatt ctgaggatcc tgcaaattcc    300
cttcgtggag gagagcctaa cttcctgcct gtgactgagg aggctgacat tcgtgaggat    360
gacaacattg caatcattga tgtgcctgtc cccagtttct ctgatagtga ccctgcagca    420
attattcatg actttgaaaa gggaatgact gcttacctgg acttgttgct ggggaactgc    480
tatctgatgc ccctcaatac ttctattgtt atgcctccaa aaaatctggt agagctcttt    540
ggcaaactgg cgagtggcag atatctgcct caaacttatg tggttcgaga agacctagtt    600
gctgtggagg aaattcgtga tgttagtaac cttggcatct ttatttacca actttgcaat    660
aacagaaagt ccttccgcct tcgtcgcaga gacctcttgc tgggtttcaa caaacgtgcc    720
attgataaat gctggaagat tagacacttc cccaacgaat ttattgttga gaccaagatc    780
tgtcaagagt acccatacga tgttccagat tacgcttaa                           819
```

<210> 56
<211> 272
<212> PRT
<213> Artificial Sequence
<220>
<223> pMCN2i_ITM2A-HA insert
<400> 56

```
Met Val Lys Ile Ala Phe Asn Thr Pro Thr Ala Val Gln Lys Glu Glu
1               5                   10                  15
Ala Arg Gln Asp Val Glu Ala Leu Leu Ser Arg Thr Val Arg Thr Gln
            20                  25                  30
Ile Leu Thr Gly Lys Glu Leu Arg Val Ala Thr Gln Glu Lys Glu Gly
        35                  40                  45
Ser Ser Gly Arg Cys Met Leu Thr Leu Leu Gly Leu Ser Phe Ile Leu
    50                  55                  60
Ala Gly Leu Ile Val Gly Gly Ala Cys Ile Tyr Lys Tyr Phe Met Pro
65                  70                  75                  80
Lys Ser Thr Ile Tyr Arg Gly Glu Met Cys Phe Phe Asp Ser Glu Asp
                85                  90                  95
Pro Ala Asn Ser Leu Arg Gly Gly Glu Pro Asn Phe Leu Pro Val Thr
            100                 105                 110
Glu Glu Ala Asp Ile Arg Glu Asp Asp Asn Ile Ala Ile Ile Asp Val
        115                 120                 125
Pro Val Pro Ser Phe Ser Asp Ser Asp Pro Ala Ala Ile Ile His Asp
    130                 135                 140
Phe Glu Lys Gly Met Thr Ala Tyr Leu Asp Leu Leu Leu Gly Asn Cys
145                 150                 155                 160
Tyr Leu Met Pro Leu Asn Thr Ser Ile Val Met Pro Pro Lys Asn Leu
                165                 170                 175
Val Glu Leu Phe Gly Lys Leu Ala Ser Gly Arg Tyr Leu Pro Gln Thr
            180                 185                 190
Tyr Val Val Arg Glu Asp Leu Val Ala Val Glu Glu Ile Arg Asp Val
        195                 200                 205
Ser Asn Leu Gly Ile Phe Ile Tyr Gln Leu Cys Asn Asn Arg Lys Ser
    210                 215                 220
Phe Arg Leu Arg Arg Arg Asp Leu Leu Leu Gly Phe Asn Lys Arg Ala
225                 230                 235                 240
Ile Asp Lys Cys Trp Lys Ile Arg His Phe Pro Asn Glu Phe Ile Val
                245                 250                 255
Glu Thr Lys Ile Cys Gln Glu Tyr Pro Tyr Asp Val Pro Asp Tyr Ala

          260                 265                 270
```

<210> 57
<211> 27
<212> DNA
<213> Artificial Sequence
<220>
<223> PCR primer
<400> 57
gaattctaca agtacttcat gcccaag 27
<210> 58
<211> 48
<212> DNA
<213> Artificial Sequence
<220>
<223> PCR primer
<400> 58
gtcgactcag tggtggtggt ggtggtgctc ttgacagatc ttggtctc 48
<210> 59
<211> 48
<212> DNA
<213> Artificial Sequence
<220>
<223> PCR primer

<400> 59
gtcgactcag tggtggtggt ggtggtgttt gccaaagagc tctaccag 48
<210> 60
<211> 1290
<212> DNA
<213> Artificial Sequence
<220>
<223> pGEX_GST-ITM2A-L insert
<400> 60

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atgtccccta tactaggtta ttggaaaatt aagggccttg tgcaacccac tcgacttctt     60
ttggaatatc ttgaagaaaa atatgaagag catttgtatg agcgcgatga aggtgataaa    120
tggcgaaaca aaaagtttga attgggtttg gagtttccca atcttcctta ttatattgat    180
ggtgatgtta aattaacaca gtctatggcc atcatacgtt atatagctga caagcacaac    240
atgttgggtg gttgtccaaa agagcgtgca gagatttcaa tgcttgaagg agcggttttg    300
gatattagat acggtgtttc gagaattgca tatagtaaag actttgaaac tctcaaagtt    360
gattttctta gcaagctacc tgaaatgctg aaaatgttcg aagatcgttt atgtcataaa    420
acatatttaa atggtgatca tgtaacccat cctgacttca tgttgtatga cgctcttgat    480
gttgttttat acatggaccc aatgtgcctg gatgcgttcc caaaattagt ttgttttaaa    540
aaacgtattg aagctatccc acaaattgat aagtacttga aatccagcaa gtatatagca    600
tggcctttgc agggctggca agccacgttt ggtggtggcg accatcctcc aaaatcggat    660
ctggaagttc tgttccaggg gcccctggga tccccggaat tctacaagta cttcatgccc    720
aagagcacca tttaccgtgg agagatgtgc tttttgatt ctgaggatcc tgcaaattcc    780
cttcgtggag gagagcctaa cttcctgcct gtgactgagg aggctgacat tcgtgaggat    840
gacaacattg caatcattga tgtgcctgtc cccagtttct ctgatagtga ccctgcagca    900
attattcatg actttgaaaa gggaatgact gcttacctgg acttgttgct ggggaactgc    960
tatctgatgc ccctcaatac ttctattgtt atgcctccaa aaaatctggt agagctcttt   1020
ggcaaactgg cgagtggcag atatctgcct caaacttatg tggttcgaga agacctagtt   1080
gctgtggagg aaattcgtga tgttagtaac cttggcatct ttatttacca actttgcaat   1140
aacagaaagt ccttccgcct tcgtcgcaga gacctcttgc tgggtttcaa caaacgtgcc   1200
attgataaat gctggaagat tagacacttc cccaacgaat ttattgttga gaccaagatc   1260
tgtcaagagc accaccacca ccaccactga                                    1290
```

<210> 61
<211> 429
<212> PRT
<213> Artificial Sequence
<220>
<223> pGEX_GST-ITM2A-L insert
<400> 61

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Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile Lys Gly Leu Val Gln Pro
1               5                   10                  15
Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu
            20                  25                  30
Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu
        35                  40                  45
Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys
    50                  55                  60
Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn
65                  70                  75                  80
Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu
                85                  90                  95
Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser
            100                 105                 110
Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu
        115                 120                 125
Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr Leu Asn
    130                 135                 140
Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp
145                 150                 155                 160
Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu
                165                 170                 175
Val Cys Phe Lys Lys Arg Ile Glu Ala Ile Pro Gln Ile Asp Lys Tyr
            180                 185                 190
Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu Gln Gly Trp Gln Ala
        195                 200                 205
Thr Phe Gly Gly Gly Asp His Pro Pro Lys Ser Asp Leu Glu Val Leu
    210                 215                 220
Phe Gln Gly Pro Leu Gly Ser Pro Glu Phe Tyr Lys Tyr Phe Met Pro
225                 230                 235                 240
Lys Ser Thr Ile Tyr Arg Gly Glu Met Cys Phe Phe Asp Ser Glu Asp
                245                 250                 255
Pro Ala Asn Ser Leu Arg Gly Gly Glu Pro Asn Phe Leu Pro Val Thr
            260                 265                 270
Glu Glu Ala Asp Ile Arg Glu Asp Asp Asn Ile Ala Ile Ile Asp Val
        275                 280                 285
Pro Val Pro Ser Phe Ser Asp Ser Asp Pro Ala Ala Ile Ile His Asp
    290                 295                 300
Phe Glu Lys Gly Met Thr Ala Tyr Leu Asp Leu Leu Leu Gly Asn Cys
305                 310                 315                 320
Tyr Leu Met Pro Leu Asn Thr Ser Ile Val Met Pro Pro Lys Asn Leu
                325                 330                 335
Val Glu Leu Phe Gly Lys Leu Ala Ser Gly Arg Tyr Leu Pro Gln Thr
            340                 345                 350
Tyr Val Val Arg Glu Asp Leu Val Ala Val Glu Glu Ile Arg Asp Val
        355                 360                 365
Ser Asn Leu Gly Ile Phe Ile Tyr Gln Leu Cys Asn Asn Arg Lys Ser
    370                 375                 380
Phe Arg Leu Arg Arg Arg Asp Leu Leu Leu Gly Phe Asn Lys Arg Ala
385                 390                 395                 400
Ile Asp Lys Cys Trp Lys Ile Arg His Phe Pro Asn Glu Phe Ile Val
                405                 410                 415
Glu Thr Lys Ile Cys Gln Glu His His His His His His
            420                 425
```

<210> 62
<211> 1047
<212> DNA
<213> Artificial Sequence
<220>
<223> pGEX_GST-ITM2A-S insert

<400> 62

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atgtccccta tactaggtta ttggaaaatt aagggccttg tgcaacccac tcgacttctt     60
ttggaatatc ttgaagaaaa atatgaagag catttgtatg agcgcgatga aggtgataaa    120
tggcgaaaca aaaagtttga attgggtttg gagtttccca atcttcctta ttatattgat    180
ggtgatgtta aattaacaca gtctatggcc atcatacgtt atatagctga caagcacaac    240
atgttgggtg gttgtccaaa agagcgtgca gagatttcaa tgcttgaagg agcggttttg    300
gatattagat acggtgtttc gagaattgca tatagtaaag actttgaaac tctcaaagtt    360
gattttctta gcaagctacc tgaaatgctg aaaatgttcg aagatcgttt atgtcataaa    420
acatatttaa atggtgatca tgtaacccat cctgacttca tgttgtatga cgctcttgat    480
gttgttttat acatggaccc aatgtgcctg gatgcgttcc caaaattagt ttgttttaaa    540
aaacgtattg aagctatccc acaaattgat aagtacttga aatccagcaa gtatatagca    600
tggcctttgc agggctggca agccacgttt ggtggtggcg accatcctcc aaaatcggat    660
ctggaagttc tgttccaggg gcccctggga tccccggaat tctacaagta cttcatgccc    720
aagagcacca tttaccgtgg agagatgtgc ttttttgatt ctgaggatcc tgcaaattcc    780
cttcgtggag gagagcctaa cttcctgcct gtgactgagg aggctgacat tcgtgaggat    840
gacaacattg caatcattga tgtgcctgtc cccagtttct ctgatagtga ccctgcagca    900
attattcatg actttgaaaa gggaatgact gcttacctgg acttgttgct ggggaactgc    960
tatctgatgc ccctcaatac ttctattgtt atgcctccaa aaaatctggt agagctcttt   1020
ggcaaacacc accaccacca ccactga                                        1047
```

<210> 63
<211> 348
<212> PRT
<213> Artificial Sequence
<220>
<223> pGEX_GST-ITM2A-S insert
<400> 63

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Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile Lys Gly Leu Val Gln Pro
1               5                   10                  15
Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu
            20                  25                  30
Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu
        35                  40                  45
Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys
    50                  55                  60
Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn
65                  70                  75                  80
Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu
                85                  90                  95
Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser
            100                 105                 110
Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu
        115                 120                 125
Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr Leu Asn
    130                 135                 140
Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp
145                 150                 155                 160
Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu
                165                 170                 175
Val Cys Phe Lys Lys Arg Ile Glu Ala Ile Pro Gln Ile Asp Lys Tyr
            180                 185                 190
Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu Gln Gly Trp Gln Ala
        195                 200                 205
Thr Phe Gly Gly Gly Asp His Pro Pro Lys Ser Asp Leu Glu Val Leu
    210                 215                 220
Phe Gln Gly Pro Leu Gly Ser Pro Glu Phe Tyr Lys Tyr Phe Met Pro
225                 230                 235                 240
Lys Ser Thr Ile Tyr Arg Gly Glu Met Cys Phe Phe Asp Ser Glu Asp
                245                 250                 255
Pro Ala Asn Ser Leu Arg Gly Gly Glu Pro Asn Phe Leu Pro Val Thr
            260                 265                 270
Glu Glu Ala Asp Ile Arg Glu Asp Asp Asn Ile Ala Ile Ile Asp Val

        275                 280                 285
Pro Val Pro Ser Phe Ser Asp Ser Asp Pro Ala Ala Ile Ile His Asp
    290                 295                 300
Phe Glu Lys Gly Met Thr Ala Tyr Leu Asp Leu Leu Leu Gly Asn Cys
305                 310                 315                 320
Tyr Leu Met Pro Leu Asn Thr Ser Ile Val Met Pro Pro Lys Asn Leu
                325                 330                 335
Val Glu Leu Phe Gly Lys His His His His His His
            340                 345
```

<210> 64
<211> 64
<212> DNA
<213> Artificial Sequence
<220>
<223> PCR primer
<400> 64

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gcggccgctt aagcgtaatc tggaacatcg tatgggtaaa ggcggaagga ctttctgtta      60
ttgc                                                                  64
```

<210> 65
<211> 711

<212> DNA
<213> Artificial Sequence
<220>
<223> pMCN2i_ITM2A-furin-HA insert
<400> 65

```
atggtgaaaa tcgccttcaa tacccctacc gccgtgcaaa aggaggaggc gcggcaagac     60
gtggaggccc tcctgagccg cacggtcaga actcagatac tgaccggcaa ggagctccga    120
gttgccaccc aggaaaaaga gggctcctct gggagatgta tgcttactct cttaggcctt    180
tcattcatct tggcaggact tattgttggt ggagcctgca tttacaagta cttcatgccc    240
aagagcacca tttaccgtgg agagatgtgc ttttttgatt ctgaggatcc tgcaaattcc    300
cttcgtggag gagagcctaa cttcctgcct gtgactgagg aggctgacat tcgtgaggat    360
gacaacattg caatcattga tgtgcctgtc cccagtttct ctgatagtga ccctgcagca    420
attattcatg actttgaaaa gggaatgact gcttacctgg acttgttgct ggggaactgc    480
tatctgatgc ccctcaatac ttctattgtt atgcctccaa aaaatctggt agagctcttt    540
ggcaaactgg cgagtggcag atatctgcct caaacttatg tggttcgaga agacctagtt    600
gctgtggagg aaattcgtga tgttagtaac cttggcatct ttatttacca actttgcaat    660
aacagaaagt ccttccgcct ttacccatac gatgttccag attacgctta a             711
```

<210> 66
<211> 236
<212> PRT
<213> Artificial Sequence
<220>
<223> pMCN2i_ITM2A-furin-HA insert
<400> 66

```
Met Val Lys Ile Ala Phe Asn Thr Pro Thr Ala Val Gln Lys Glu Glu
1               5                   10                  15
Ala Arg Gln Asp Val Glu Ala Leu Leu Ser Arg Thr Val Arg Thr Gln
            20                  25                  30
Ile Leu Thr Gly Lys Glu Leu Arg Val Ala Thr Gln Glu Lys Glu Gly
        35                  40                  45
Ser Ser Gly Arg Cys Met Leu Thr Leu Leu Gly Leu Ser Phe Ile Leu
    50                  55                  60
Ala Gly Leu Ile Val Gly Gly Ala Cys Ile Tyr Lys Tyr Phe Met Pro
65                  70                  75                  80
Lys Ser Thr Ile Tyr Arg Gly Glu Met Cys Phe Phe Asp Ser Glu Asp
                85                  90                  95
Pro Ala Asn Ser Leu Arg Gly Gly Glu Pro Asn Phe Leu Pro Val Thr
            100                 105                 110
Glu Glu Ala Asp Ile Arg Glu Asp Asp Asn Ile Ala Ile Ile Asp Val
        115                 120                 125

Pro Val Pro Ser Phe Ser Asp Ser Asp Pro Ala Ala Ile Ile His Asp
    130                 135                 140
Phe Glu Lys Gly Met Thr Ala Tyr Leu Asp Leu Leu Leu Gly Asn Cys
145                 150                 155                 160
Tyr Leu Met Pro Leu Asn Thr Ser Ile Val Met Pro Pro Lys Asn Leu
                165                 170                 175
Val Glu Leu Phe Gly Lys Leu Ala Ser Gly Arg Tyr Leu Pro Gln Thr
            180                 185                 190
Tyr Val Val Arg Glu Asp Leu Val Ala Val Glu Glu Ile Arg Asp Val
        195                 200                 205
Ser Asn Leu Gly Ile Phe Ile Tyr Gln Leu Cys Asn Asn Arg Lys Ser
    210                 215                 220
Phe Arg Leu Tyr Pro Tyr Asp Val Pro Asp Tyr Ala
225                 230                 235
```

<210> 67
<211> 29
<212> DNA
<213> Artificial Sequence
<220>
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Claims

1. A monoclonal antibody binding to a fragment of an ITM2A protein consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1, wherein the antibody has a cytotoxic activity.

2. The monoclonal antibody of claim 1, wherein the monoclonal antibody is as described in any of the following (1) to (7) :

   (1) an antibody comprising an H chain having the amino acid sequence represented by SEQ ID NO: 9 as CDR1, the amino acid sequence represented by SEQ ID NO: 10 as CDR2, and the amino acid sequence represented by SEQ ID NO: 11 as CDR3, and an L chain having the amino acid sequence represented by SEQ ID NO: 12 as CDR1, the amino acid sequence represented by SEQ ID NO: 13 as CDR2, and the amino acid sequence represented by SEQ ID NO: 14 as CDR3;
   (2) the antibody described in (1) which is a chimeric antibody;
   (3) the antibody described in (1) which is a humanized antibody;
   (4) the antibody described in (1) comprising the amino acid sequence of the heavy chain variable domain represented by SEQ ID NO: 32;
   (5) the antibody described in (1) comprising the amino acid sequence of the light chain variable domain represented by SEQ ID NO: 34;
   (6) the antibody described in (4) to (5) which is a chimeric antibody; and
   (7) an antibody capable of inhibiting the binding of a second antibody to an ITM2A protein fragment consisting of amino acids 75 to 227 in the amino acid sequence represented by SEQ ID NO: 1, wherein the second antibody is the antibody described in any of (1) to (6).

3. The antibody according to claim 1 or 2, wherein the cytotoxic activity is

   (a) an antibody-dependent cell-mediated cytotoxicity (ADCC) activity; or
   (b) is a complement-dependent cytotoxicity (CDC) activity.

4. The antibody according to any of claims 1 to 3, wherein:

   (a) the antibody is conjugated with a cytotoxic substance; and/or
   (b) the antibody inhibits cancer cell growth.

5. The antibody according to claim 4(a), wherein the antibody has an internalization activity.

6. The antibody according to any of claims 1 to 5, wherein:

   (a) the antibody has a human constant region; and/or
   (b) the antibody is deficient in fucose added to its sugar chain or has a sugar chain having bisecting GlcNAc.

7. The antibody according to claim 6 (a), wherein the antibody is a chimeric antibody, a humanized antibody, or a human antibody.

8. A pharmaceutical composition comprising the antibody according to any of claims 1 to 7 as an active ingredient.

9. A cell growth inhibitor comprising the antibody according to any of claims 1 to 7 as an active ingredient.

10. An anticancer agent comprising the antibody according to any of claims 1 to 7 as an active ingredient.

11. The antibody according to any of claims 1 to 7 for use in a method for treating cancer.

12. A method for predicting the efficacy of cancer treatment by the administration of the antibody according to any of claims 1 to 7, comprising the step of detecting the expression level of an ITM2A in a biological sample collected from a test subject using the antibody of any one of claims 1 to 7, wherein the cancer is Ewing's sarcoma or blood cancer, wherein the sample is tissue or blood.

13. The method according to claim 12, wherein an ITM2A protein in the sample collected from a test subject is detected.

14. A method for determining the presence of cancer in a test subject using the antibody of any one of claims 1 to 7:

(A) comprising detecting an ITM2A protein in a sample collected from the test subject; or
(B) comprising the following steps:

(a) providing a sample collected from the test subject; and
(b) detecting an ITM2A protein contained in the sample of step (a) using an antibody binding to the ITM2A protein; or

(C) comprising the following steps:

(a) administering, to the test subject, a radioisotope-labeled antibody having a binding activity to an ITM2A protein; and
(b) detecting the accumulation of the radioisotope,

wherein the cancer is Ewing's sarcoma or blood cancer, wherein the sample is tissues or blood.

15. The monoclonal antibody of claim 6(b), anticancer agent of claim 10, antibody for use in a method of claim 11, or the method of any one of claims 12 to 14, wherein the cancer whose presence is to be determined is:

(a) Ewing's sarcoma; or
(b) blood cancer.

16. The monoclonal antibody, anticancer agent, antibody for use in a method of or method according to claim 15, wherein the Ewing's sarcoma has observable chromosomal translocation.

17. The monoclonal antibody, anticancer agent, antibody for use in a method of or method according to claim 16, wherein the chromosomal translocation is t(11;22) (q24;q12).

18. The monoclonal antibody, anticancer agent, antibody for use in a method of or method according to claim 15, wherein the blood cancer is any of T cell leukemia, T cell lymphoma, acute myeloid leukemia, B cell tumor, and multiple myeloma.

Patentansprüche

1. Monoklonaler Antikörper, der an ein Fragment eines ITM2A-Proteins bindet, das aus Aminosäuren 75 bis 227 in der durch SEQ ID NO: 1 dargestellten Aminosäuresequenz bindet, wobei der Antikörper zytotoxische Aktivität aufweist.

2. Monoklonaler Antikörper nach Anspruch 1, wobei der monoklonale Antikörper wie unter einem der folgenden (1) bis (7) beschrieben ist:

(1) ein Antikörper, der eine H-Kette mit der durch SEQ ID NO: 9 dargestellten Aminosäuresequenz als CDR1, der durch SEQ ID NO: 10 dargestellten Aminosäuresequenz als CDR2 und der durch SEQ ID NO: 11 dargestellten Aminosäuresequenz als CDR3 und eine L-Kette mit der durch SEQ ID NO: 12 dargestellten Aminosäuresequenz als CDR1, der durch SEQ ID NO: 13 dargestellten Aminosäuresequenz als CDR2 und der durch SEQ ID NO: 14 dargestellten Aminosäuresequenz als CDR3 umfasst;
(2) der unter (1) beschriebene Antikörper, der ein chimärer Antikörper ist;
(3) der unter (1) beschriebene Antikörper, der ein humanisierter Antikörper ist;
(4) der unter (1) beschriebene Antikörper, der die Aminosäuresequenz der variablen Domäne der schweren Kette umfasst, die durch SEQ ID NO: 32 dargestellt ist;
(5) der unter (1) beschriebene Antikörper, der die Aminosäuresequenz der variablen Domäne der leichten Kette umfasst, die durch SEQ ID NO: 34 dargestellt ist;
(6) der unter (4) bis (5) beschriebene Antikörper, der ein chimärer Antikörper ist; und
(7) ein Antikörper, der in der Lage ist, die Bindung eines zweiten Antikörpers an ein ITM2A-Proteinfragment zu hemmen, das aus den Aminosäuren 75 bis 227 in der durch SEQ ID NO: 1 dargestellten Aminosäuresequenz besteht, wobei der zweite Antikörper unter beliebigen von (1) bis (6) beschrieben ist.

3. Antikörper nach Anspruch 1 oder 2, wobei die zytotoxische Aktivität ist:

(a) eine Aktivität der antikörperabhängigen zellvermittelten Zytotoxizität (ADCC); oder
(b) eine Aktivität der komplementabhängigen Zytotoxizität (CDC).

4. Antikörper nach einem der Ansprüche 1 bis 3, wobei:

(a) der Antikörper mit einer zytotoxischen Substanz konjugiert ist; und/oder
(b) der Antikörper Krebszellwachstum hemmt.

5. Antikörper nach Anspruch 4(a), wobei der Antikörper eine Intemalisierungsaktivität aufweist.

6. Antikörper nach einem der Ansprüche 1 bis 5, wobei:

(a) der Antikörper eine humane konstante Region aufweist; und/oder
(b) der Antikörper einen Mangel an zu seiner Zuckerkette addierter Fucose aufweist oder eine Zuckerkette aufweist, die GlcNAc halbiert.

7. Antikörper nach Anspruch 6(a), wobei der Antikörper ein chimärer Antikörper, ein humanisierter Antikörper oder ein humaner Antikörper ist.

8. Pharmazeutische Zusammensetzung, die den Antikörper nach einem der Ansprüche 1 bis 7 als Wirkstoff umfasst.

9. Zellwachstumshemmer, der den Antikörper nach einem der Ansprüche 1 bis 7 als Wirkstoff umfasst.

10. Antikrebsmittel, das den Antikörper nach einem der Ansprüche 1 bis 7 als Wirkstoff umfasst.

11. Antikörper nach einem der Ansprüche 1 bis 7 zur Verwendung in einem Verfahren zur Krebsbehandlung.

12. Verfahren zum Prognostizieren der Wirksamkeit einer Krebsbehandlung durch Verabreichen des Antikörpers nach einem der Ansprüche 1 bis 7, das den Schritt des Nachweisens der Expressionshöhe eines ITM2A in einer biologischen Probe, die einem Testindividuum entnommen wurde, unter Verwendung des Antikörpers nach einem der Ansprüche 1 bis 7 umfasst, wobei der Krebs Ewing-Sarkom oder Blutkrebs ist, wobei die Probe Gewebe oder Blut ist.

13. Verfahren nach Anspruch 12, wobei ein ITM2A-Protein in der einem Testindividuum entnommenen Probe nachgewiesen wird.

14. Verfahren zum Ermitteln des Vorhandenseins von Krebs bei einem Testindividuum unter Verwendung des Antikörpers nach einem der Ansprüche 1 bis 7:

(A) das das Nachweisen eines ITM2A-Proteins in einer dem Testindividuum entnommenen Probe umfasst; oder
(B) das die folgenden Schritte umfasst:

(a) Bereitstellen einer dem Testindividuum entnommenen Probe; und
(b) Nachweisen eines ITM2A-Proteins, das in der Probe von Schritt (a) enthalten ist, unter Verwendung eines an das ITM2A-Protein bindenden Antikörpers; oder

(C) das die folgenden Schritte umfasst:

(a) Verabreichen eines radioisotopmarkierten Antikörpers mit einer Bindungsaffinität für ein ITM2A-Protein an das Testindividuum; und
(b) Nachweisen der Ansammlung des Radioisotops,

wobei der Krebs Ewing-Sarkom oder Blutkrebs ist, wobei die Probe Gewebe oder Blut ist.

15. Monoklonaler Antikörper nach Anspruch 6(b), Antikrebsmittel nach Anspruch 10, Antikörper zur Verwendung in einem Verfahren nach Anspruch 11 oder Verfahren nach einem der Ansprüche 12 bis 14, wobei der Krebs, dessen Vorhandensein zu ermitteln ist, ist:

(a) Ewing-Sarkom; oder
(b) Blutkrebs.

16. Monoklonaler Antikörper, Antikrebsmittel, Antikörper zur Verwendung in einem Verfahren nach oder Verfahren nach Anspruch 15, wobei das Ewing-Sarkom eine beobachtbare chromosomale Translokation aufweist.

17. Monoklonaler Antikörper, Antikrebsmittel, Antikörper zur Verwendung in einem Verfahren nach oder Verfahren nach Anspruch 16, wobei die chromosomale Translokation t(11;22) (q24;q12) ist.

18. Monoklonaler Antikörper, Antikrebsmittel, Antikörper zur Verwendung in einem Verfahren nach oder Verfahren nach Anspruch 15, wobei der Blutkrebs beliebiges von T-Zell-Leukämie, T-Zell-Lymphom, akuter myeloischer Leukämie, B-Zell-Tumor und multiplem Myelom ist.

Revendications

1. Anticorps monoclonal se liant à un fragment d'une protéine ITM2A constitué des acides aminés 75 à 227 dans la séquence d'acides aminés représentée par SEQ ID n° : 1, dans lequel l'anticorps présente une activité cytotoxique.

2. Anticorps monoclonal selon la revendication 1, dans lequel l'anticorps monoclonal est tel que décrit dans l'une quelconque des parties (1) à (7) suivantes :

(1) un anticorps comprenant une chaîne H possédant la séquence d'acides aminés représentée par SEQ ID n° : 9 en tant que CDR1, la séquence d'acides aminés représentée par SEQ ID n° : 10 en tant que CDR2, et la séquence d'acides aminés représentée par SEQ ID n° : 11 en tant que CDR3, et une chaîne L possédant la séquence d'acides aminés représentée par SEQ ID n° : 12 en tant que CDR1, la séquence d'acides aminés représentée par SEQ ID n° : 13 en tant que CDR2, et la séquence d'acides aminés représentée par SEQ ID n° : 14 en tant que CDR3 ;
(2) l'anticorps décrit dans (1) qui est un anticorps chimérique ;
(3) l'anticorps décrit dans (1) qui est un anticorps chimérique ;
(4) l'anticorps décrit dans (1) comprenant la séquence d'acides aminés du domaine variable des chaînes lourdes représentée par SEQ ID n° : 32 ;
(5) l'anticorps décrit dans (1) comprenant la séquence d'acides aminés du domaine variable des chaînes légères représentée par SEQ ID n° : 34 ;
(6) l'anticorps décrit dans (4) à (5) qui est un anticorps chimérique ; et
(7) un anticorps capable d'inhiber la liaison d'un second anticorps à un fragment de protéine ITM2A constitué d'acides aminés 75 à 227 dans la séquence d'acides aminés représentée par SEQ ID n° : 1, dans lequel le second anticorps est l'anticorps décrit dans l'un quelconque de (1) à (6).

3. Anticorps selon la revendication 1 ou 2, dans lequel l'activité cytotoxique est

(a) une activité de cytotoxicité à médiation cellulaire dépendante des anticorps (ADCC) ; ou
(b) est une activité de cytotoxicité dépendante du complément (CDC).

4. Anticorps selon l'une quelconque des revendications 1 à 3, dans lequel :

(a) l'anticorps est conjugué avec une substance cytotoxique ; et/ou
(b) l'anticorps inhibe la croissance cellulaire cancéreuse.

5. Anticorps selon la revendication 4(a), dans lequel l'anticorps présente une activité d'internalisation.

6. Anticorps selon l'une quelconque des revendications 1 à 5, dans lequel :

(a) l'anticorps possède une région constante humaine ; et/ou
(b) l'anticorps est déficient en fucose ajouté à sa chaîne de sucres ou possède une chaîne de sucres possédant une GlcNAc bissectrice.

7. Anticorps selon la revendication 6 (a), dans lequel l'anticorps est un anticorps chimérique, un anticorps chimérique, ou un anticorps humain.

8. Composition pharmaceutique comprenant l'anticorps selon l'une quelconque des revendications 1 à 7 en tant qu'ingrédient actif.

9. Inhibiteur de croissance cellulaire comprenant l'anticorps selon l'une quelconque des revendications 1 à 7 en tant qu'ingrédient actif.

10. Agent anticancéreux comprenant l'anticorps selon l'une quelconque des revendications 1 à 7 en tant qu'ingrédient actif.

11. Anticorps selon l'une quelconque des revendications 1 à 7 pour l'utilisation dans un procédé pour traiter le cancer.

12. Procédé pour prédire l'efficacité de traitement de cancer par l'administration de l'anticorps selon l'une quelconque des revendications 1 à 7, comprenant l'étape de la détection du niveau d'expression d'une ITM2A dans un échantillon biologique prélevé chez un sujet de test en utilisant l'anticorps de l'une quelconque des revendications 1 à 7, dans lequel le cancer est le sarcome d'Ewing ou le cancer du sang, dans lequel l'échantillon est un tissu ou du sang.

13. Procédé selon la revendication 12, dans lequel une protéine ITM2A dans l'échantillon prélevé chez un sujet de test est détectée.

14. Procédé pour déterminer la présence de cancer chez un sujet de test en utilisant l'anticorps de l'une quelconque des revendications 1 à 7 :

(A) comprenant la détection d'une protéine ITM2A dans un échantillon prélevé chez le sujet de test ; ou
(B) comprenant les étapes suivantes :

(a) la fourniture d'un échantillon collecté chez le sujet de test ; et
(b) la détection d'une protéine ITM2A contenue dans l'échantillon de l'étape (a) en utilisant un anticorps se liant à la protéine ITM2A ; ou

(C) comprenant les étapes suivantes :

(a) l'administration, au sujet de test, d'un anticorps étiqueté par radio-isotope possédant une activité de liaison à une protéine ITM2A ; et
(b) la détection de l'accumulation du radio-isotope,

dans lequel le cancer est le sarcome d'Ewing ou le cancer du sang, dans lequel l'échantillon est un tissu ou du sang.

15. Anticorps monoclonal selon la revendication 6(b), agent anticancéreux selon la revendication 10, anticorps pour l'utilisation dans un procédé selon la revendication 11, ou procédé selon l'une quelconque des revendications 12 à 14, dans lequel le cancer dont la présence doit être déterminée est :

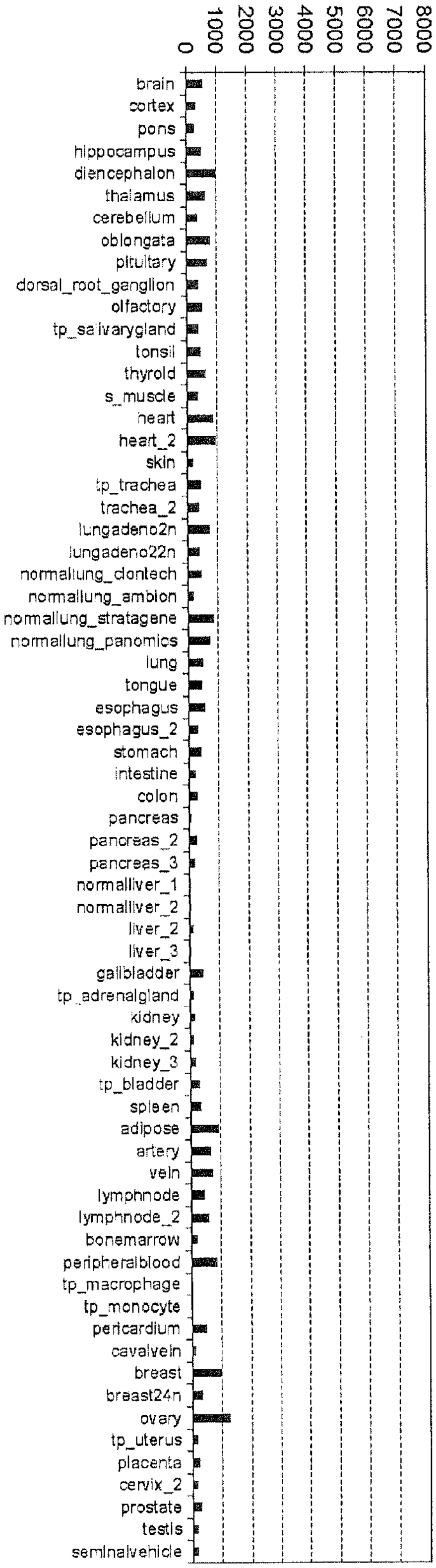
(a) le sarcome d'Ewing ; ou
(b) le cancer du sang.

16. Anticorps monoclonal, agent anticancéreux, anticorps pour l'utilisation dans un procédé de ou procédé selon la revendication 15, dans lequel le sarcome d'Ewing présente une translocation chromosomique observable.

17. Anticorps monoclonal, agent anticancéreux, anticorps pour l'utilisation dans un procédé de ou procédé selon la revendication 16, dans lequel la translocation chromosomique est t(11 ; 2) (q24 ; q12).

18. Anticorps monoclonal, agent anticancéreux, anticorps pour l'utilisation dans un procédé de ou procédé selon la revendication 15, dans lequel le cancer du sang est l'un quelconque de leucémie à cellules T, lymphome à cellules T, leucémie myéloïde aiguë, tumeur à cellules B, et myélome multiple.

FIG.1A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pituitary
dorsal_root_ganglion
olfactory
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tonsil
thyroid
s_muscle
heart
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skin
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normallung_clontech
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normallung_panomics
lung
tongue
esophagus
esophagus_2
stomach
intestine
colon
pancreas
pancreas_2
pancreas_3
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spleen
adipose
artery
vein
lymphnode
lymphnode_2
bonemarrow
peripheralblood
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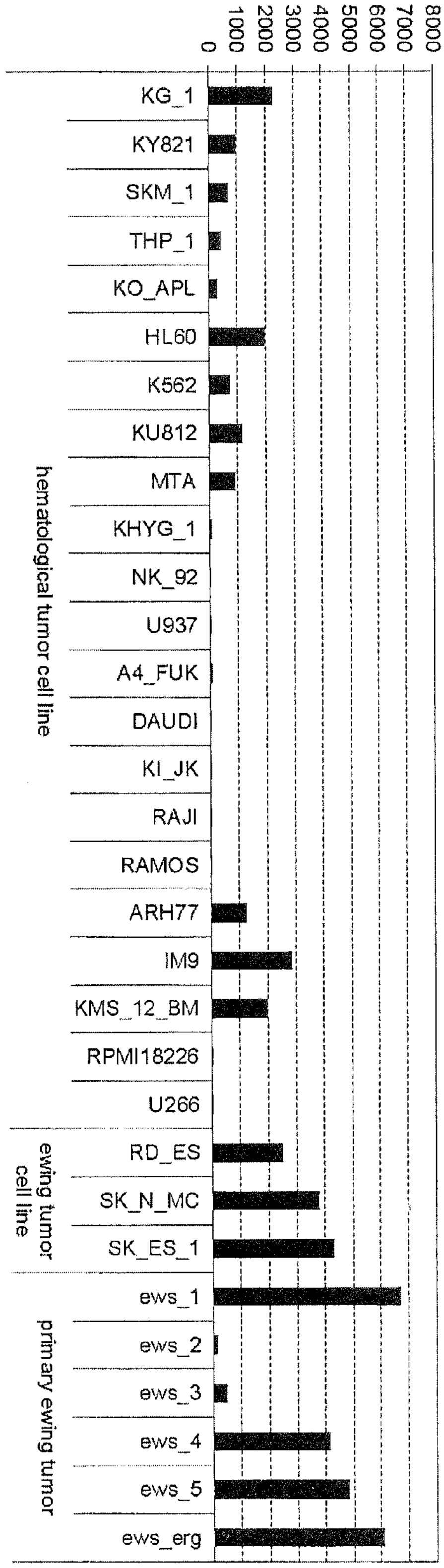
FIG.1B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NK_92
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A4_FUK
DAUDI
KI_JK
RAJI
RAMOS
ARH77
IM9
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U266
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RD_ES
SK_N_MC
SK_ES_1
primary ewing tumor
ews_1
ews_2
ews_3
ews_4
ews_5
ews_erg

FIG.2A

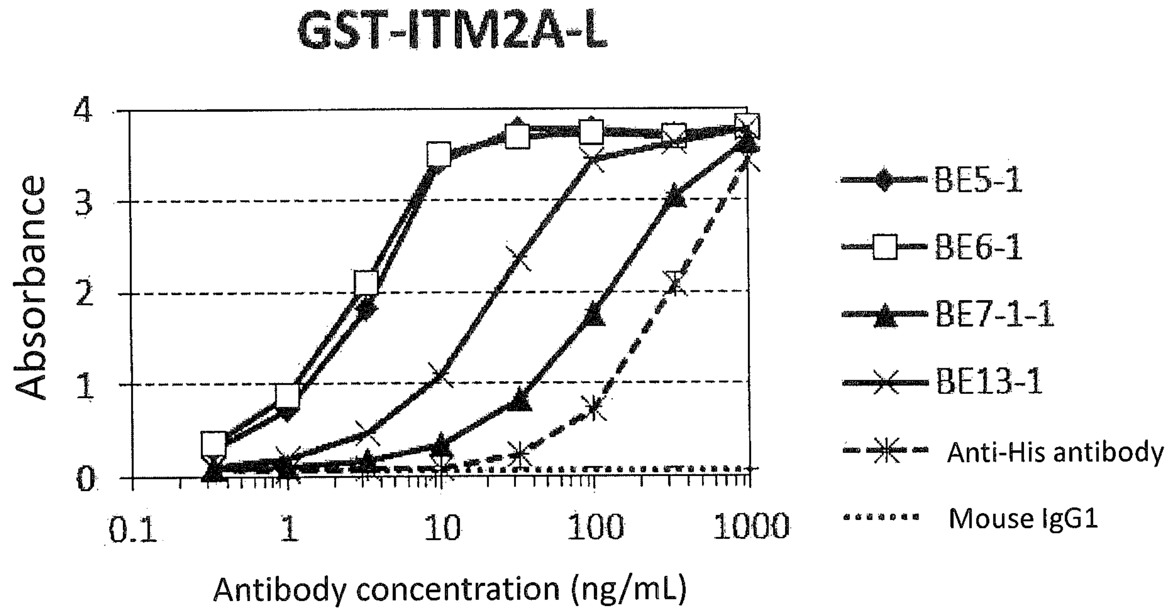

GST-ITM2A-L
Absorbance
4
3
2
1
0
0.1
1
10
100
1000
Antibody concentration (ng/mL)
BE5-1
BE6-1
BE7-1-1
BE13-1
Anti-His antibody
Mouse IgG1

FIG.2B

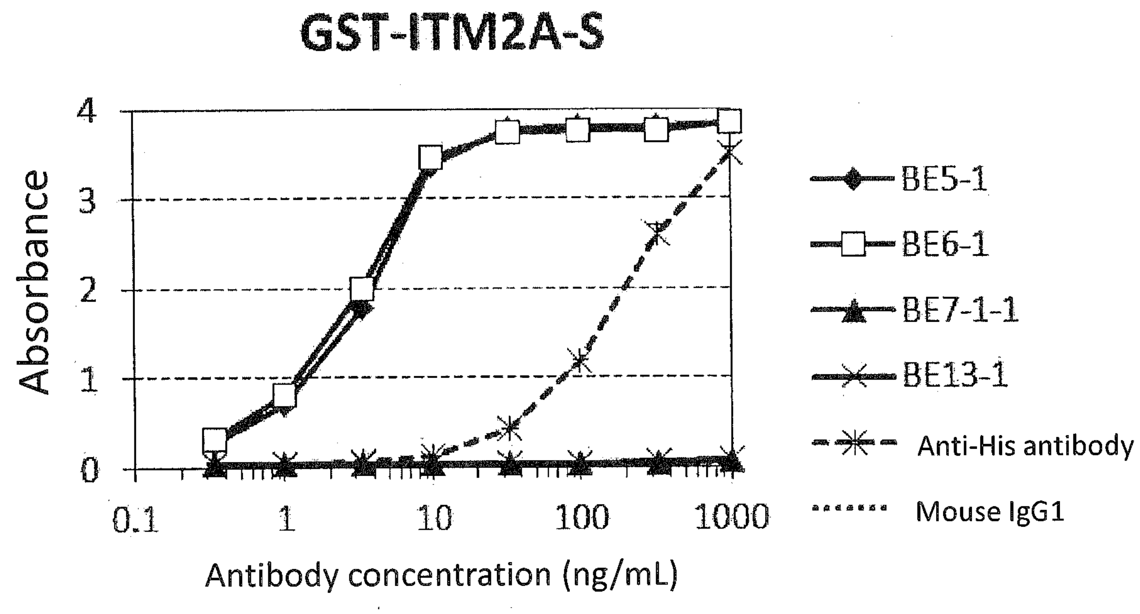

GST-ITM2A-S
Absorbance
4
3
2
1
0
0.1
1
10
100
1000
Antibody concentration (ng/mL)
BE5-1
BE6-1
BE7-1-1
BE13-1
Anti-His antibody
Mouse IgG1

FIG.3A

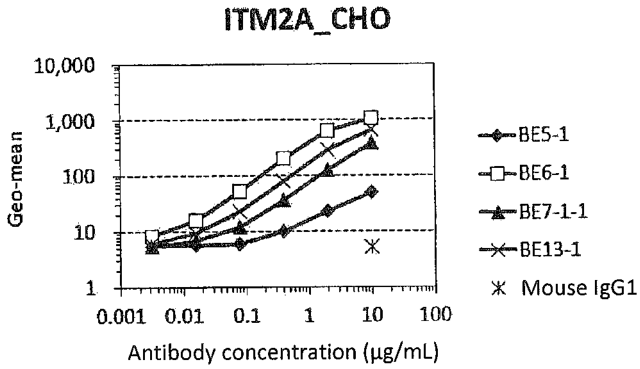


FIG.3B

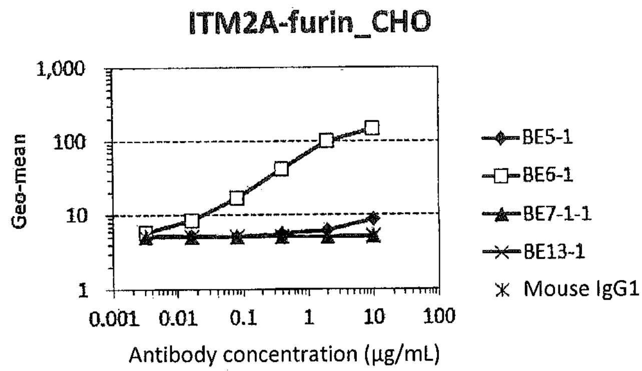


FIG.3C

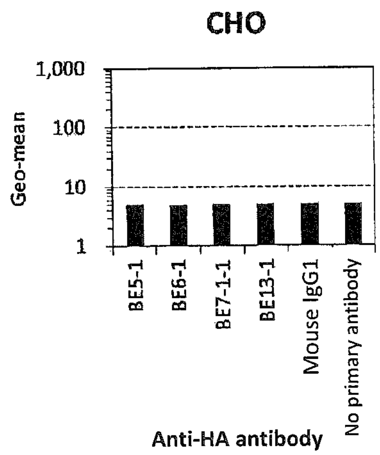


FIG.3D

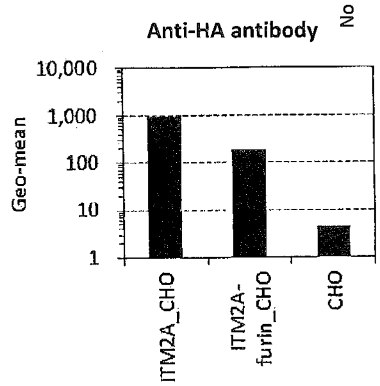


FIG.4A

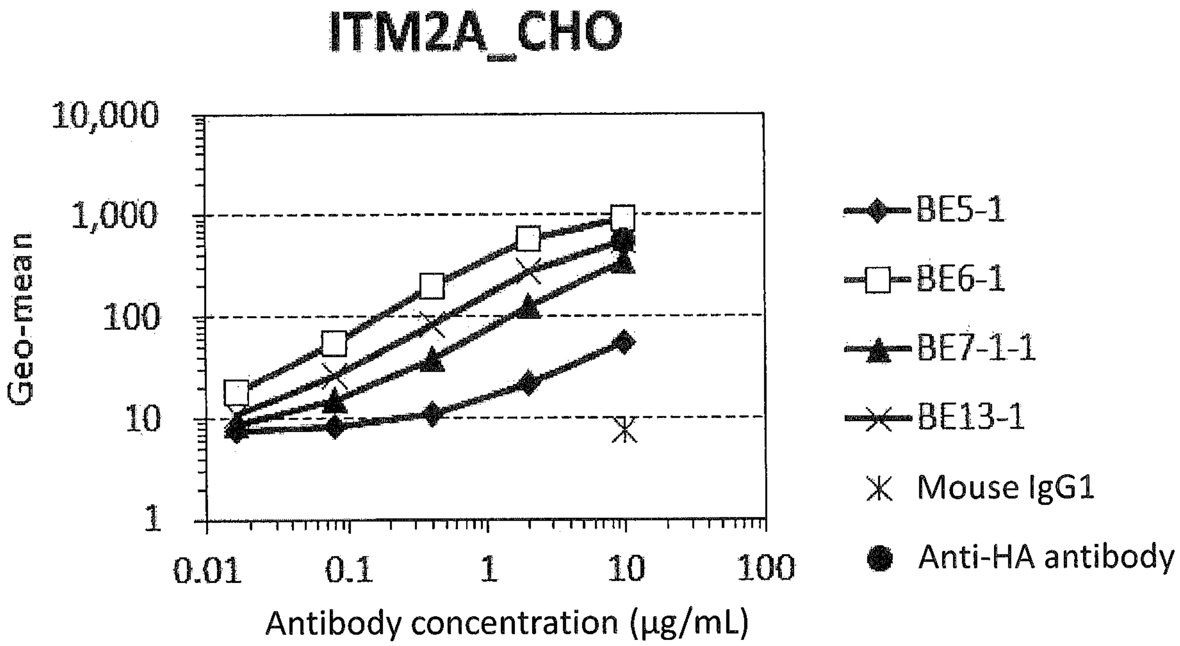
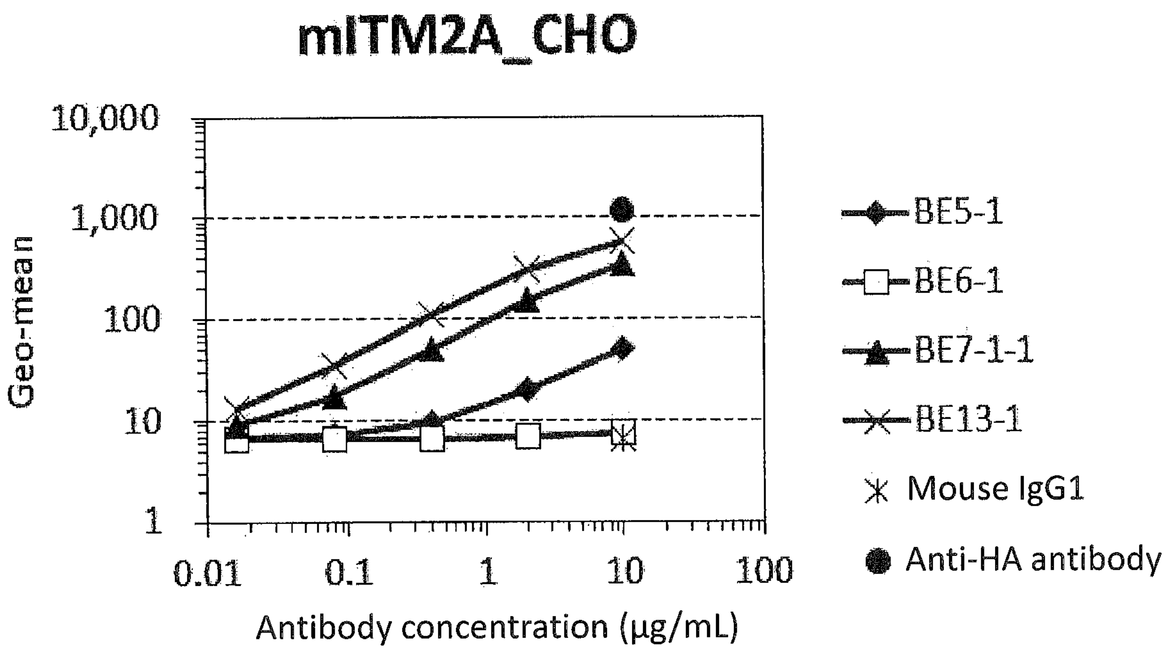

ITM2A_CHO
10,000
1,000
100
10
1
Geo-mean
0.01
0.1
1
10
100
Antibody concentration (μg/mL)
BE5-1
BE6-1
BE7-1-1
BE13-1
Mouse IgG1
Anti-HA antibody

FIG.4B


mITM2A_CHO
10,000
1,000
100
10
1
Geo-mean
0.01
0.1
1
10
100
Antibody concentration (μg/mL)
BE5-1
BE6-1
BE7-1-1
BE13-1
Mouse IgG1
Anti-HA antibody

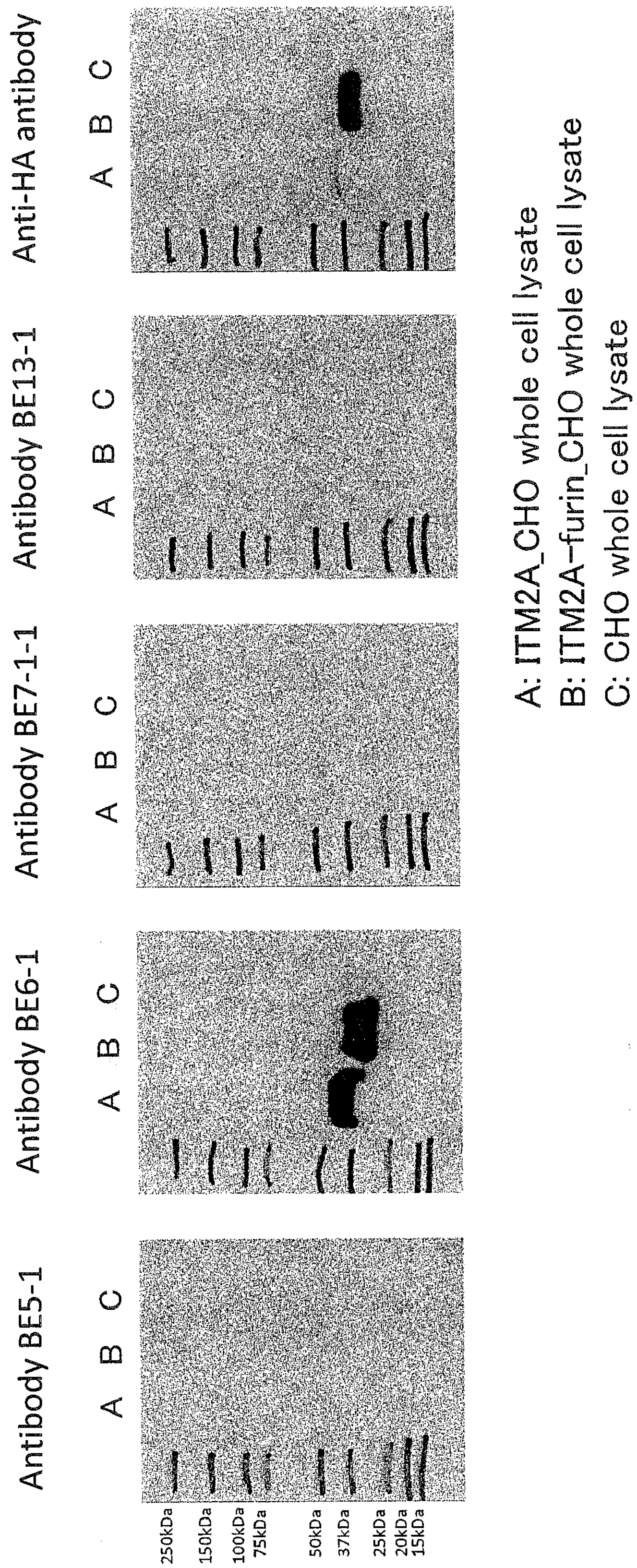


FIG.5A

FIG.5B

FIG.5C

FIG.5D

FIG.5E

FIG.6A

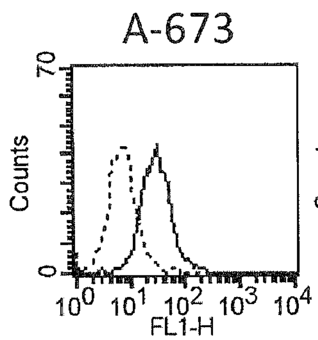


FIG.6B

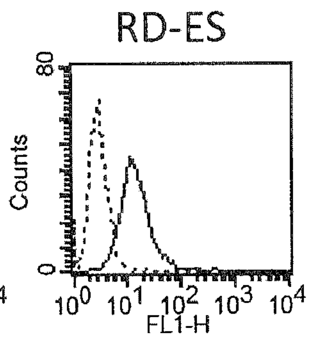


FIG.6C

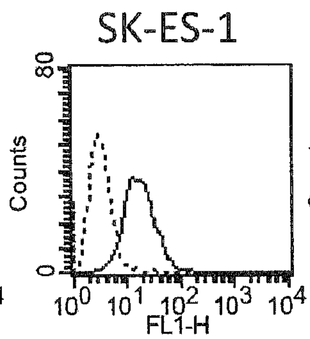


FIG.6D

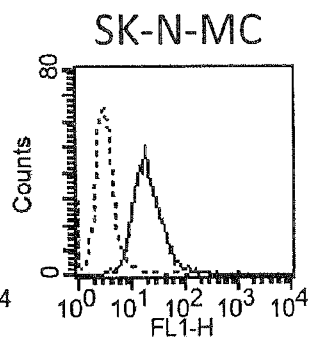


FIG.6E

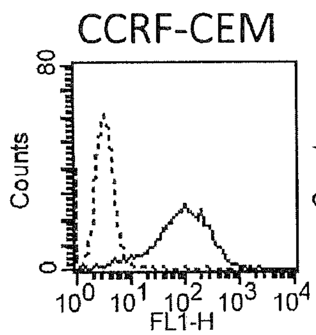


FIG.6F

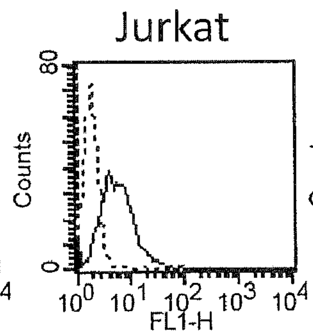


FIG.6G

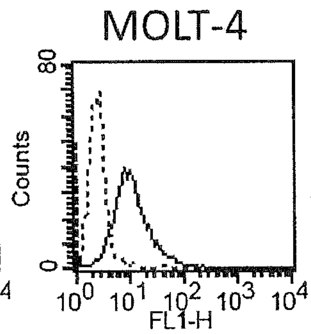


FIG.6H

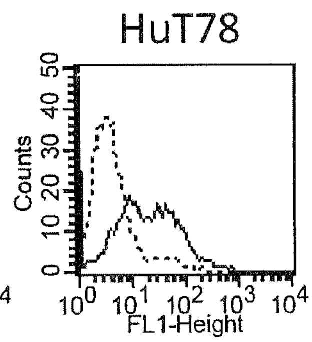


FIG.6I

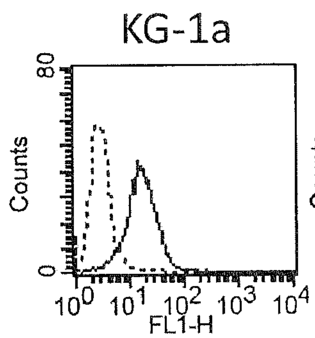
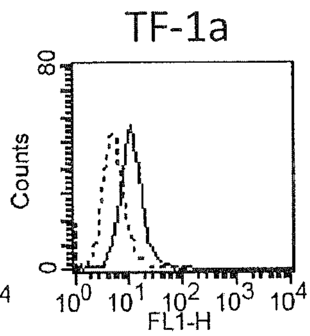


FIG.6J



——— BE6-1
········ Mouse IgG1

FIG.7A

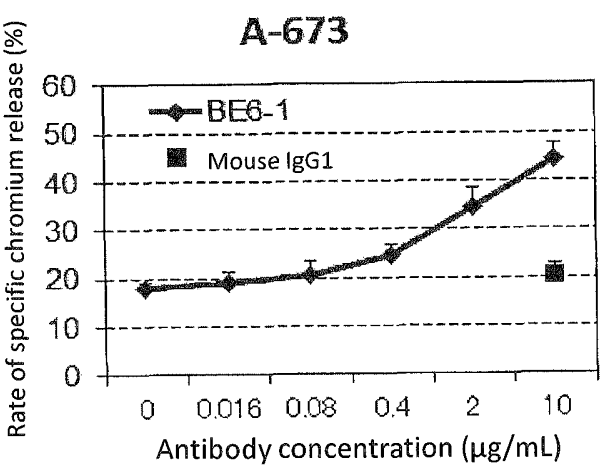


FIG.7B

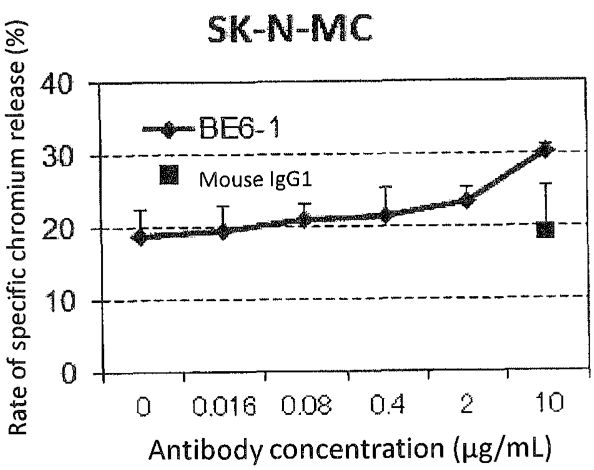


FIG.7C

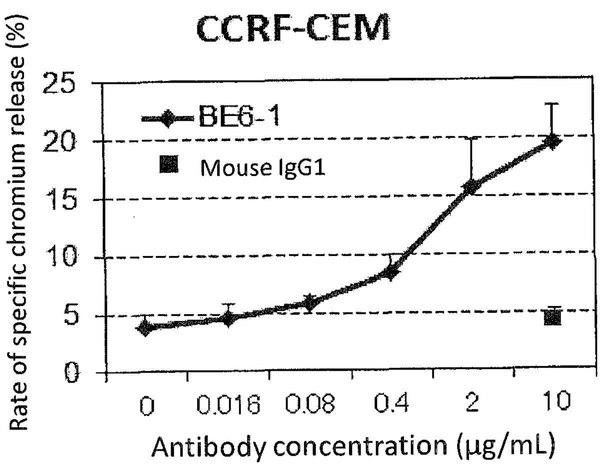


FIG.7D

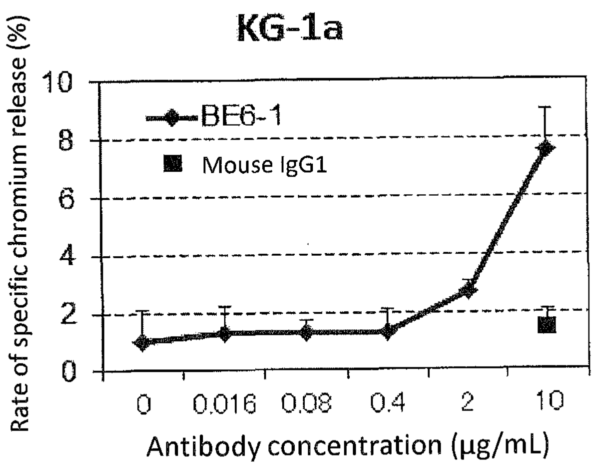


FIG.8A

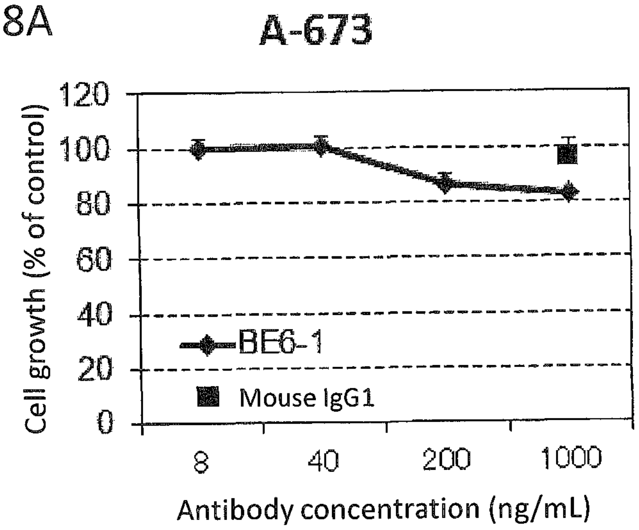


FIG.8B

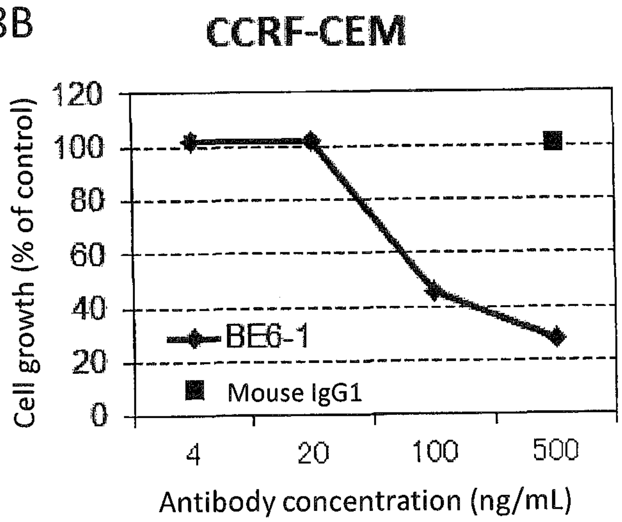


FIG.8C

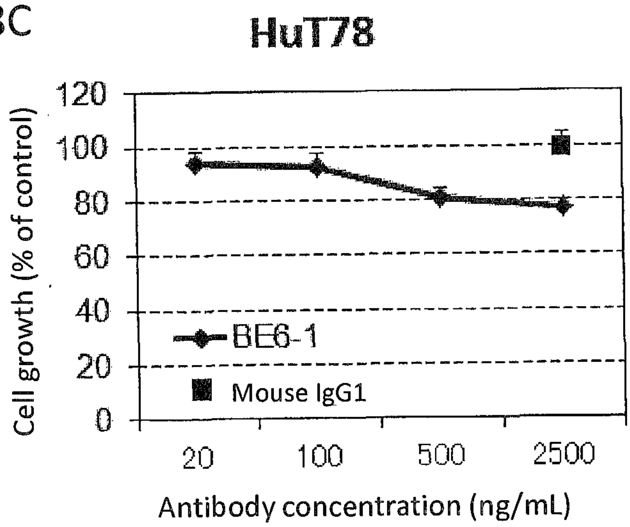


FIG.9A

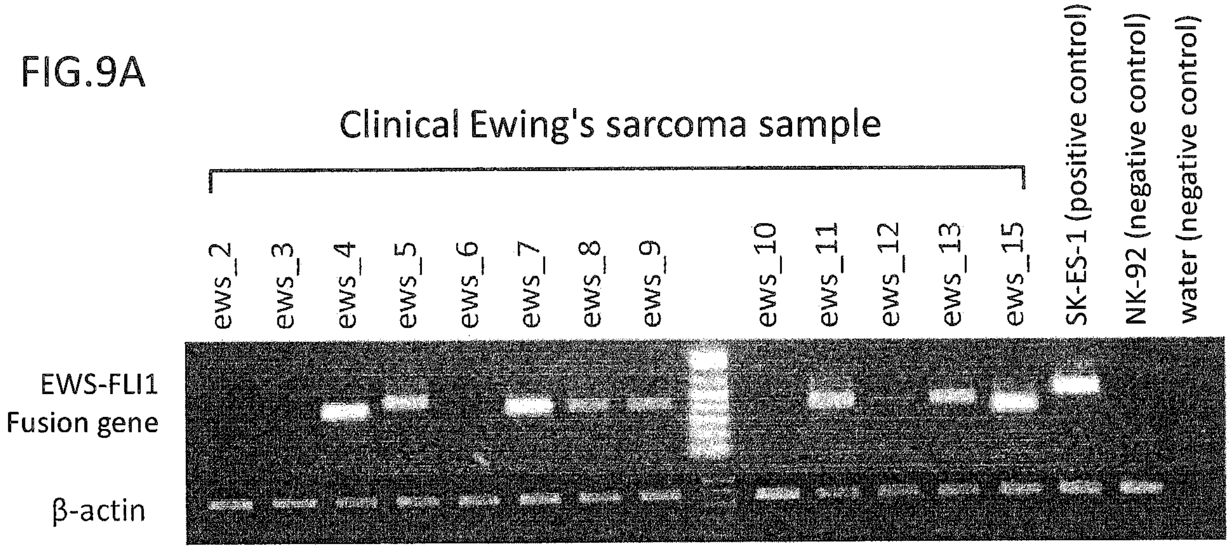
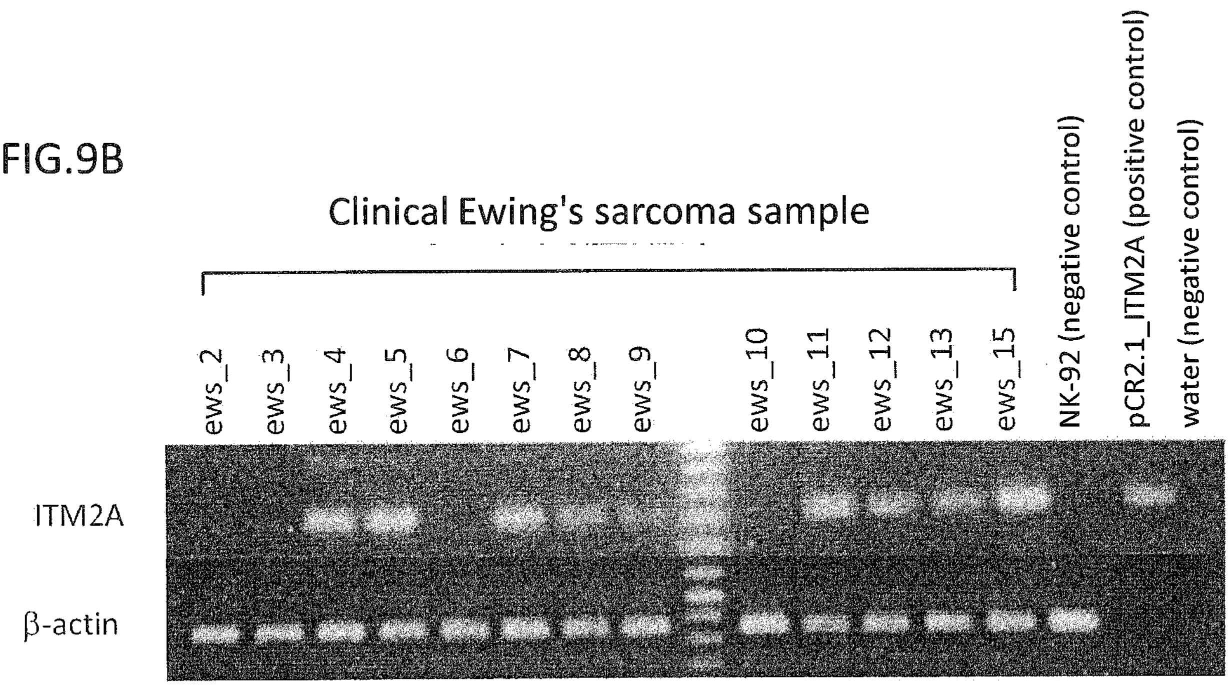

Clinical Ewing's sarcoma sample
ews_2
ews_3
ews_4
ews_5
ews_6
ews_7
ews_8
ews_9
ews_10
ews_11
ews_12
ews_13
ews_15
SK-ES-1 (positive control)
NK-92 (negative control)
water (negative control)
EWS-FLI1 Fusion gene
β-actin

FIG.9B


Clinical Ewing's sarcoma sample
ews_2
ews_3
ews_4
ews_5
ews_6
ews_7
ews_8
ews_9
ews_10
ews_11
ews_12
ews_13
ews_15
NK-92 (negative control)
pCR2.1_ITM2A (positive control)
water (negative control)
ITM2A
β-actin

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专利名称(译)	使用Anti-ITM2a抗体诊断和治疗癌症		
公开(公告)号	EP2700652B1	公开(公告)日	2018-12-19
申请号	EP2012774392	申请日	2012-04-18
[标]申请(专利权)人(译)	国立大学法人 东京大学		
申请(专利权)人(译)	东京大学 中外SEIYAKU株式会社		
当前申请(专利权)人(译)	东京大学 中外SEIYAKU株式会社		
[标]发明人	ABURATANI HIROYUKI ISHIKAWA SHUMPEI KAWAI SHIGETO		
发明人	ABURATANI, HIROYUKI ISHIKAWA, SHUMPEI KAWAI, SHIGETO		
IPC分类号	C07K16/28 C07K16/30 A61K39/395 A61P35/00 A61P35/02 C07K16/46 G01N33/53 G01N33/574 G01N33/577 C12N15/09 C12P21/08		
CPC分类号	C07K16/3061 C07K16/28 C07K2317/56 C07K2317/565 C07K2317/73 C07K2317/732 G01N33/57407 G01N33/57426 G01N2800/52		
代理机构(译)	J A KEMP		
优先权	2011092488 2011-04-18 JP		
其他公开文献	EP2700652A1 EP2700652A4		
外部链接	Espacenet		

摘要(译)

公开了结合ITM2A蛋白的单克隆抗体。该抗体可用于癌症的诊断，预防和治疗，例如尤文氏肉瘤，T细胞白血病，T细胞淋巴瘤，急性髓性白血病，B细胞瘤和多发性骨髓瘤。本发明还提供含有该抗体作为有效成分的药物组合物，细胞生长抑制剂和抗癌剂，治疗癌症的方法，预测癌症治疗功效的方法，以及确定其存在的方法使用抗体测试受试者中的癌症。

Ser,

Gly-Ser,

Gly-Gly-Ser,

Ser-Gly-Gly,

Gly-Gly-Gly-Ser (SEQ ID NO: 79),