(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 088 890 A1**

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 153(4) EPC

(43) Date of publication:
02.11.2016 Bulletin 2016/44

(21) Application number: **14874331.3**

(22) Date of filing: **25.06.2014**

(51) Int Cl.:
G01N 33/53 *(2006.01)*

(86) International application number:
PCT/JP2014/003409

(87) International publication number:
WO 2015/097928 (02.07.2015 Gazette 2015/26)

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR
Designated Extension States:
BA ME

(30) Priority: **24.12.2013 PCT/JP2013/007529**

(71) Applicants:
- **Chugai Seiyaku Kabushiki Kaisha**
  Kita-ku
  Tokyo 115-8543 (JP)
- **JSR Corporation**
  Minato-ku
  Tokyo 105-8640 (JP)
- **JSR Life Sciences Corporation**
  Tokyo 105-8640 (JP)

(72) Inventors:
- **OHTOMO, Toshihiko**
  Tokyo 103-8324 (JP)
- **AMANO, Jun**
  Gotemba-shi
  Shizuoka 412-8513 (JP)
- **ADACHI, Hideki**
  Gotemba-shi
  Shizuoka 412-8513 (JP)
- **SUZUKI, Tsukasa**
  Gotemba-shi
  Shizuoka 412-8513 (JP)
- **MIZUUCHI, Motoaki**
  Tokyo 105-8640 (JP)
- **YAMAGUCHI, Tetsuji**
  Tokyo 105-8640 (JP)
- **WAKUI, Seiki**
  Tokyo 105-8640 (JP)

(74) Representative: **Power, David**
J A Kemp
14 South Square
Gray's Inn
London WC1R 5JJ (GB)

(54) METHOD FOR MEASURING SOLUBLE GPC3 PROTEIN

(57) The present invention relates to a method for assaying soluble GPC3 protein in a test sample, comprising using two different antibodies binding to different epitopes present in the N-terminal region of GPC3 protein.

EP 3 088 890 A1

Description

Technical Field

[0001] The present invention relates to a method for assaying soluble GPC3 protein in a test sample, comprising using two different antibodies binding to different epitopes present in the N-terminal region of GPC3 protein.

Background Art

[0002] Deaths caused by hepatocellular carcinoma account for 600,000 deaths each year and are reportedly ranked the 5th most common cancer-related deaths worldwide (Non Patent Literature 1). Most cases of hepatocellular carcinoma die within 1 year after diagnosis as having the disease. Unfortunately, hepatocellular carcinoma patients are frequently diagnosed at a late stage where the patients rarely respond to therapy capable of curing. Medical procedures including chemotherapy, chemoembolization, cauterization, and proton beam therapy are still insufficiently effective for such patients. Many patients exhibit recurrence of the disease, which proceeds rapidly to an advanced stage with vascular invasion and multisite intrahepatic metastasis, resulting in its 5-year survival rate of only 7% (Non Patent Literature 2). Hepatocellular cancer patients with resectable local tumors have relatively good prognosis, but have a 5-year survival rate remaining at 15% to 39% (Non Patent Literature 3).

[0003] Glypican-3 (GPC3) is frequently expressed at a high level in liver cancer. It is therefore considered that GPC3 may be useful for the identification of GPC3 functions in liver cancer or as a target for the treatment of liver cancer or a target of the diagnosis of liver cancer.

[0004] GPC3 is known to be expressed on cell surface and then processed at the particular site by convertase, phospholipase D, or Notum (Non Patent Literature 4). A method for selecting an HCC patient by assaying GPC3 present in the plasma of patients is disclosed (Patent Literature 1) as a method based on such an event. A diagnostic product for liver cancer comprising an antibody binding to an epitope in a secreted form of GPC3 secreted into plasma, or a method for diagnosing liver cancer using the antibody has been developed (Patent Literatures 2 and 3). Also, a diagnostic product for liver cancer comprising an antibody binding to an epitope in an anchored form of GPC3 still present on cell surface after processing in a tissue specimen or the like isolated from a patient, or a method for diagnosing liver cancer using the antibody has been developed (Patent Literature 4). A method for selecting a prostate cancer patient by measuring a GPC3 level in blood is disclosed, in addition to liver cancer (Patent Literature 5).

[0005] All references cited herein are as given below. The contents described in these literatures are incorporated herein by reference in their entirety. However, this does not mean that any of these literatures are the prior art relative to the present specification.

Citation List

Patent Literature

[0006]

Patent Literature 1: WO2003/100429
Patent Literature 2: WO2004/038420
Patent Literature 3: WO2004/023145
Patent Literature 4: WO2009/116659
Patent Literature 5: WO2007/081790

Non Patent Literature

[0007]

Non Patent Literature 1: Llovet JM, Burroughs A, Bruix J; Lancet (2003), 362, 1907-17
Non Patent Literature 2: Bosch FX, Ribes J, Cleries R; Gastroenterology (2004), 127, S5-16
Non Patent Literature 3: Takenaka K, Kawahara N, Yamamoto K, Kajiyama K, Maeda T, Itasaka H, Shirabe K, Nishizaki T, Yanaga K, Sugimachi K; Arch Surg (1996), 131, 71-6
Non Patent Literature 4: Cheng AL, Chen Z, Tsao CJ, Qin S, Kim JS, et al. Efficacy and safety of sorefanib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomized, double-blind, placebo-controlled trial. Lancet Oncol. (2009) 10, 25-34

Summary of Invention

Technical Problem

[0008] No conventional technique is capable of detecting or accurately quantifying a low concentration of soluble GPC3 contained in a healthy subject body fluid. Therefore, early detection at an initial stage in the development of cancer, selection of an anticancer agent used, or post-treatment prognosis cannot be precisely carried out.

[0009] An object of the present invention is to provide a highly sensitive assay method for soluble GPC3 capable of quantitatively measuring the concentration of soluble GPC3 contained in a healthy subject body fluid.

Solution to Problem

[0010] The present inventors have conducted diligent studies to attain the object and consequently found that soluble GPC3 protein in a test sample can be assayed highly sensitively by using two different antibodies binding to different epitopes present in the N-terminal region of GPC3 protein.

[0011] Specifically, the present invention relates to the following [1] to [12]:

[1] A method for assaying soluble GPC3 protein in a test sample, comprising using two different antibodies binding to different epitopes contained in an amino acid sequence from position 128 to position 357 of GPC3 protein represented by SEQ ID NO: 70;

[2] The assay method according to [1], wherein one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 337, 339, 340, and 344 of the GPC3 protein;

[3] The assay method according to [1] or [2], wherein one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, and 309 of the GPC3 protein;

[4] The assay method according to [1], wherein the different epitopes are the following combination (A) or (B):

(A) one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 341, 343, 346, 347, 348, 349, and 350 of the GPC3 protein, and the other epitope is an epitope comprising at least one amino acid selected from amino acids at positions 297, 300, 304, 306, 311, 312, 313, 314, and 315 of the GPC3 protein; and

(B) one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 128, 129, 131, 132, 133, 134, 135, 171, 208, 209, 210, 211, 212, 214, 215, 218, 322, 325, 326, 328, 329, 330, 332, 333, 335, 336, and 338 of the GPC3 protein, and the other epitope is an epitope comprising at least one amino acid selected from amino acids at positions 220, 228, 231, 232, 235, 291, 294, and 295 of the GPC3 protein;

[5] The assay method according to [1], wherein the different epitopes are the following combination (A) or (B):

(A) one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 337, 339, 340, and 344 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 341, 343, 346, 347, 348, 349, and 350 thereof, and the other epitope is an epitope comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, and 309 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 297, 300, 304, 306, 311, 312, 313, 314, and 315 thereof; and

(B) one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 337, 339, 340, and 344 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 128, 129, 131, 132, 133, 134, 135, 171, 208, 209, 210, 211, 212, 214, 215, 218, 322, 325, 326, 328, 329, 330, 332, 333, 335, 336, and 338 thereof, and the other epitope is an epitope comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, and 309 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 220, 228, 231, 232, 235, 291, 294, and 295;

[6] The assay method according to [1], wherein the different epitopes are
an epitope that is bound by an antibody having a heavy chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 38 and a light chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 39, and an epitope that is bound by an antibody having a heavy chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 40 and a light chain variable region comprising a sequence having

80% or higher homology to the sequence represented by SEQ ID NO: 41, or an epitope that is bound by an antibody having a heavy chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 44 and a light chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 45, and an epitope that is bound by an antibody having a heavy chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 42 and a light chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 43;

[7] The assay method according to [1], wherein the two different antibodies are

a combination of an antibody having CDR regions identical to CDR regions contained in a heavy chain variable region shown in SEQ ID NO: 38 and CDR regions contained in a light chain variable region shown in SEQ ID NO: 39, and an antibody having CDR regions identical to CDR regions contained in a heavy chain variable region shown in SEQ ID NO: 40 and CDR regions contained in a light chain variable region shown in SEQ ID NO: 41, or

a combination of an antibody having CDR regions identical to CDR regions contained in a heavy chain variable region shown in SEQ ID NO: 44 and CDR regions contained in a light chain variable region shown in SEQ ID NO: 45, and an antibody having CDR regions identical to CDR regions contained in a heavy chain variable region shown in SEQ ID NO: 42 and CDR regions contained in a light chain variable region shown in SEQ ID NO: 43;

[8] The assay method according to [7], wherein the CDR regions are CDR1, CDR2, and CDR3 regions based on the Kabat numbering;

[9] The assay method according to [1], wherein the two different antibodies are

a combination of an antibody having a heavy chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 46, 47, and 48, respectively, and having a light chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 49, 50, and 51, respectively, and an antibody having a heavy chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 52, 53, and 54, respectively, and having a light chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 55, 56, and 57, respectively, or

a combination of an antibody having a heavy chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 58, 59, and 60, respectively, and having a light chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 61, 62, and 63, respectively, and an antibody having a heavy chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 64, 65, and 66, respectively, and having a light chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 67, 68, and 69, respectively;

[10] The assay method according to [1], wherein the two different antibodies are

a combination of an antibody having a heavy chain variable region shown in SEQ ID NO: 38 and a light chain variable region shown in SEQ ID NO: 39, and an antibody having a heavy chain variable region shown in SEQ ID NO: 40 and a light chain variable region shown in SEQ ID NO: 41, or

a combination of an antibody having a heavy chain variable region shown in SEQ ID NO: 44 and a light chain variable region shown in SEQ ID NO: 45, and an antibody having a heavy chain variable region shown in SEQ ID NO: 42 and a light chain variable region shown in SEQ ID NO: 43;

[11] The assay method according to any one of [1] to [10], wherein any one of the two different antibodies is bound with a magnetic particle; and

[12] The assay method according to any one of [1] to [11], wherein the test sample is a tissue sample, a whole blood sample, a plasma sample, or a serum sample isolated from a human.

Advantageous Effects of Invention

[0012] According to the present invention, soluble GPC3 protein in a test sample can be assayed conveniently and highly sensitively by using two different antibodies binding to different epitopes present in the N-terminal region of GPC3 protein. As a result, early detection at an initial stage in the development of cancer, selection of an anticancer agent used, and post-treatment prognosis can be precisely carried out.

Brief Description of Drawings

[0013]

[Figure 1] Figure 1 shows results of subjecting mouse antibodies obtained by screening to Western blotting for GPC3 and a C fragment and N fragment thereof under reductive conditions.

[Figure 2] Figure 2 shows results of subjecting GT30, GT114, GT607, and GT165 antibodies to Western blotting under reduction of pellets of CHO cells expressing either GPC3 or each fragment thereof (AW2, AW3, and AW5).
[Figure 3] Figure 3 shows results of a binding test between the GT30, GT114, GT607, or GT165 antibody and each fragment of GPC3.
[Figure 4] Figure 4 schematically shows a GT30, GT114, GT607, or GT165 antibody-binding region on GPC3.
[Figure 5] Figure 5 shows a GT30-binding region in the conformation of human GPC3.
[Figure 6] Figure 6 shows a GT165-binding region in the conformation of human GPC3.
[Figure 7] Figure 7 shows a GT607-binding region in the conformation of human GPC3.
[Figure 8] Figure 8 shows a GT114-binding region in the conformation of human GPC3.
[Figure 9] Figure 9 shows the GT30-, GT165-, GT607- and GT114-binding regions overlaid on each other in the conformation of human GPC3.
[Figure 10] Figure 10 is a graph showing the detection limit and the quantification limit of each assay system.

Description of Embodiments

Definition

[0014] In the present specification, the chemical terms and the technical terms used in relation to the present invention have meanings that are generally understood by those skilled in the art, unless otherwise specified.

Indefinite article

[0015] In the present invention, the indefinite article "a" or "an" refers to one or two or more (i.e., at least one) grammatical subject(s) of the indefinite article. For example, "a factor" means one factor or two or more factors.

Amino acid

[0016] In the present specification, each amino acid is indicated by a one-letter or three-letter code, or both, as indicated by, for example, Ala/A, Leu/L, Arg/R, Lys/K, Asn/N, Met/M, Asp/D, Phe/F, Cys/C, Pro/P, Gln/Q, Ser/S, Glu/E, Thr/T, Gly/G, Trp/W, His/H, Tyr/Y, Ile/I, or Val/V.

Alteration of amino acid

[0017] A method known in the art such as site-directed mutagenesis (Kunkel et al., Proc. Natl. Acad. Sci. USA (1985) 82, 488-492) or overlap extension PCR can be appropriately adopted for the alteration of an amino acid in the amino acid sequence of an antigen-binding molecule. A plurality of methods known in the art can also be adopted as alteration methods for amino acids to be substituted by amino acids other than natural amino acids (Annu. Rev. Biophys. Biomol. Struct. (2006) 35, 225-249; and Proc. Natl. Acad. Sci. U.S.A. (2003) 100 (11), 6353-6357). For example, a cell-free translation system (Clover Direct (Protein Express Co., Ltd.)) is preferably used which contains tRNA in which a non-natural amino acid is bound with amber suppressor tRNA complementary to a stop codon UAG (amber codon).

[0018] In the present specification, the term "and/or" used for indicating an amino acid alteration site includes every combination in which "and" and "or" are appropriately combined. Specifically, the phrase "amino acids at positions 43, 52, and/or 105 are substituted" includes the following variations of amino acid alteration:

(a) position 43, (b) position 52, (c) position 105, (d) positions 43 and 52, (e) positions 43 and 105, (f) positions 52 and 105, and (g) positions 43, 52, and 105.

Numbering of CDR

[0019] In the present invention, the amino acid positions assigned to antibody CDRs can be specified according to a method known in the art and can be specified according to, for example, Kabat (Sequences of Proteins of Immunological Interest, National Institute of Health, Bethesda, Md., 1987 and 1991).

Test sample

[0020] In the present invention, the term "test sample" refers to a sample of a tissue or fluid isolated from a subject. In a non-limiting embodiment, examples of such a sample include plasma, serum, spinal fluid, lymph, external sections of the skin, the respiratory tract, the intestinal tract, and the genitourinary tract, tear, saliva, sputum, milk, urine, whole

blood or any blood fraction, blood derivatives, blood cells, tumors, nervous tissues, organs or any type of tissue, any sample obtained by lavage (e.g., samples derived from the bronchi), and samples of components constituting cell cultures *in vitro*.

[0021] The concentration of soluble GPC3 can be measured in a biological sample (test sample) isolated from a patient. For example, the concentration of soluble GPC3 can be measured in a sample of whole blood or a sample of a blood fraction such as serum or plasma (in the present specification, also referred to as a whole blood sample, a serum sample, or a plasma sample, respectively). In a non-limiting embodiment, the concentration of soluble GPC3 in the whole blood sample, the serum sample, or the plasma sample of a patient can be measured using, for example, commercially available Human Glypican-3 ELISA kit (BioMosaic Inc.) or Enzyme-linked Immunosorbent Assay Kit For Glypican 3 (GPC3) (USCN Life Science Inc.) and an EDTA-treated whole blood sample, serum sample, or plasma sample.

[0022] The term "isolated" refers to causing "artificial" change from a natural state, i.e., shifting and/or removing a naturally occurring substance from its original environment. In the present invention, the term "isolated" means that, for example, a polynucleotide or a polypeptide present in an organism is unisolated, whereas the same polynucleotide or polypeptide thereas is isolated when separated from a material present with the polynucleotide or the polypeptide in a natural state. A polynucleotide or a polypeptide transferred to an organism by transformation, genetic manipulation, or any other recombination method is in an isolated state even if present in the organism (regardless of being alive or dead).

Soluble GPC3

[0023] In the present invention, the "soluble GPC3" refers to a soluble form of GPC3 unanchored to GPC3-expressing cells and includes fragments of a secreted form of GPC3 that can be easily dissociated from GPC3 anchored to GPC3-expressing cells under particular conditions *in vivo* or *in vitro*. In a non-limiting embodiment, examples of the "soluble GPC3" can include a polypeptide from the amino terminus to position 358 in GPC3 defined by SEQ ID NO: 70, a polypeptide from the amino terminus to position 374 in GPC3 defined by SEQ ID NO: 70, a GPC3 polypeptide liberated by the degradation of a GPI anchor present at the carboxy terminus, and their fragments (Patent Literature 2). Those skilled in the art can appropriately select an approach known in the art for determining the structure of soluble GPC3. In a non-limiting embodiment, a method therefor that may be appropriately used which involves, for example, directly detecting soluble GPC3 present in the serum or the plasma of a patient or a model animal by the method described in Patent Literature 2 and analyzing its structure, or which involves, for example, allowing an enzyme dissociating soluble GPC3, such as convertase, phospholipase D, or Notum, to act on GPC3 expressed in cells cultured *in vitro*, detecting the resulting soluble GPC3, and analyzing its structure (e.g., J. Cell. Biol. (2003) 163 (3), 625-635).

Method for measuring soluble GPC3 concentration

[0024] In the present invention, the soluble GPC3 concentration is measured by an immunological method using two different antibodies binding to different epitopes contained in an amino acid sequence from position 128 to position 357, preferably position 219 to position 357. Also, a method which involves detecting a fragment of soluble GPC3 further digested with an appropriate enzyme may be appropriately adopted.

[0025] Preferred examples of the method for assaying soluble GPC3 include enzyme immunoassay (ELISA or EIA), fluorescence immunoassay (FIA), radioimmunoassay (RIA), luminescence immunoassay (LIA), immunoenzymatic technique, fluorescent antibody technique, immunochromatography, immunoturbidimetry, latex turbidimetry, and latex agglutination assay. In the immunological method of the present invention, the soluble GPC3 can be assayed by procedures of manual operation or using an apparatus such as an analyzer.

[0026] The immunological method according to the present invention can be carried out according to a method known in the art, for example, sandwich technique. For example, a first antibody immobilized on a carrier, a biological sample, and a second antibody modified with a labeling material are reacted simultaneously or sequentially. This reaction forms a complex of the first antibody immobilized on a carrier, soluble GPC3, and the second antibody modified with a labeling material. The labeling material conjugated with the second antibody contained in this complex can be quantified to measure the amount (concentration) of the soluble GPC3 contained in the biological sample.

[0027] In the case of, for example, the enzyme immunoassay, a first antibody-immobilized microplate, serially diluted biological samples, a second antibody modified with an enzyme such as HRP, a washing buffer, and a solution containing a substrate reactive with the enzyme such as HRP are preferably used. In a non-limiting embodiment, the assay can involve reacting the enzyme modifying the second antibody under the optimum conditions thereof with the substrate, and measuring the amount of the resulting enzymatic reaction product by an optical method or the like. In the case of the fluorescence immunoassay, a first antibody-immobilized optical waveguide, serially diluted biological samples, a second antibody modified with a fluorescent material, and a washing buffer can be preferably used. In a non-limiting embodiment, the assay can involve irradiating the fluorescent material modifying the second antibody with excitation light to emit fluorescence, the intensity of which is then measured.

[0028] The radioimmunoassay involves measuring the amount of radiation from a radioactive substance. The luminescence immunoassay involves measuring luminescence intensity derived from a luminescent reaction system. For example, the immunoturbidimetry, the latex turbidimetry, or the latex agglutination assay involves measuring transmitted light or scattering light by an endpoint or rate method. The immunochromatography, for example, which is based on visual observation, involves visually measuring the color of the labeling material appearing on a test line. Alternatively, an instrument such as an analyzer may be appropriately used instead of this visual measurement.

[0029] In the immunological method of the present invention, the first antibody to be immobilized on a carrier can be adsorbed or bound to the carrier by a method such as physical adsorption, chemical binding, or a combination thereof. A method known in the art can be appropriately used as the method for immobilizing the antibody by physical adsorption. Examples thereof include a method which involves contacting the antibody with the carrier by mixing in a solution such as a buffer solution, and a method which involves contacting the antibody dissolved in a buffer or the like with the carrier. Alternatively, the antibody may be immobilized onto the carrier by chemical binding. Examples thereof include a method which involves mixing and contacting the antibody and the carrier with a divalent cross-linking reagent such as glutaraldehyde, carbodiimide, imide ester, or maleimide to react the reagent with amino groups, carboxyl groups, thiol groups, aldehyde groups, hydroxy groups, or the like in both of the antibody and the carrier. Such immobilization may require treatment for suppressing nonspecific reaction or the natural aggregation or the like of the antibody-immobilized carrier. In such a case, the aftertreatment of the immobilization can be carried out by a method known in the art. Examples thereof include a method which involves coating the surface or the inner wall of the antibody-immobilized carrier by contact with, for example, a protein (e.g., bovine serum albumin (BSA), casein, gelatin, egg albumin, or a salt thereof), a surfactant, or skimmed milk.

[0030] In the immunological method of the present invention, the second antibody to be modified with a labeling material can be adsorbed or bound to the labeling material by a method such as physical adsorption, chemical binding, or a combination thereof. A method known in the art can be appropriately used as the method for binding the labeling material to the antibody by physical adsorption. Examples of thereof include a method which involves contacting the antibody with the labeling material by mixing in a solution such as a buffer solution, and a method which involves contacting the antibody dissolved in a buffer or the like with the labeling material. When the labeling material is, for example, gold colloid or latex, the physical adsorption method is effective. The antibody can be mixed and contacted with the gold colloid in a buffer to obtain a gold colloid-labeled antibody. Alternatively, the antibody may be modified with the labeling material by chemical binding. Examples thereof include a method which involves contacting and mixing the antibody and the labeling material with a divalent cross-linking reagent such as glutaraldehyde, carbodiimide, imide ester, or maleimide to react the reagent with amino groups, carboxyl groups, thiol groups, aldehyde groups, hydroxy groups, or the like in both of the antibody and the labeling material. When the labeling material is, for example, a fluorescent material, an enzyme, or a chemiluminescent material, the chemical binding method is effective. Such modification may require treatment for suppressing nonspecific reaction or the natural aggregation or the like of the antibody modified with the labeling material. In such a case, the aftertreatment of the labeling can be carried out by a method known in the art. Examples thereof include a method which involves coating the labeling material-bound antibody by contacting with, for example, a protein (e.g., bovine serum albumin (BSA), casein, gelatin, egg albumin, or a salt thereof), a surfactant, or skimmed milk.

[0031] For example, peroxidase (POD), alkaline phosphatase (ALP), β-galactosidase, urease, catalase, glucose oxidase, lactate dehydrogenase, or amylase can be used as the labeling material for the enzyme immunoassay. For example, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, substituted rhodamine isothiocyanate, dichlorotriazine isothiocyanate, cyanine, or merocyanine can be used for the fluorescence immunoassay. For example, tritium, iodine 125, or iodine 131 can be used for the radioimmunoassay. For example, a luminol system, a luciferase system, an acridinium ester system, or a dioxetane compound system can be used for the luminescence immunoassay. Also, fine particles made of a material such as polystyrene, a styrene-styrene sulfonate copolymer, an acrylonitrile-butadiene-styrene copolymer, a vinyl chloride-acrylic acid ester copolymer, a vinyl acetate-acrylic acid copolymer, polyacrolein, a styrene-methacrylic acid copolymer, a styrene-glycidyl (meth)acrylate copolymer, a styrenebutadiene copolymer, a methacrylic acid polymer, an acrylic acid polymer, latex, gelatin, liposome, a microcapsule, silica, alumina, carbon black, a metal compound, a metal, a metal colloid, a ceramic, or a magnetic substance can be used for the immunochromatography, the immunoturbidimetry, the latex turbidimetry, or the latex agglutination assay.

[0032] A solid-phase carrier in the form of, for example, beads, a microplate, a test tube, a stick, a membrane, or a test pieces made of a material such as polystyrene, polycarbonate, polyvinyltoluene, polypropylene, polyethylene, polyvinyl chloride, nylon, polymethacrylate, polyacrylamide, latex, liposome, gelatin, agarose, cellulose, Sepharose, glass, a metal, a ceramic, or a magnetic substance can be appropriately used as the carrier in the immunological method of the present invention. Particularly, a magnetic substance such as magnetic particles is preferred. Polymer particles containing a magnetic substance or a superparamagnetic substance are preferred as the magnetic particles. Magnetic particles are more preferred in which a magnetic substance layer containing at least one of Fe_2O_3 and Fe_3O_4 is formed on the surface of a core particle and a polymer layer is further formed on the magnetic substance layer.

Two different antibodies

[0033] The "two different antibodies" used in the present invention bind to "different epitopes" contained in an amino acid region from position 128 to position 357 of GPC3. The "different epitopes" are not particularly limited as long as the different epitopes are in a relationship that does not mutually inhibit the binding of the "two different antibodies" to the soluble GPC3. Specific examples thereof can include a combination of an epitope on GPC3 that is bound by a GT30 antibody described in Examples and an epitope on GPC3 that is bound by a GT607 antibody described therein, and a combination of an epitope on GPC3 that is bound by a GT114 antibody described therein and an epitope on GPC3 that is bound by a GT165 antibody described therein. Examples of such a combination of the different epitopes include a combination of an epitope (for GT30 and GT165) comprising at least one amino acid selected from amino acids at positions 337, 339, 340, and 344 of the GPC3 protein and an epitope (for GT607 and GT114) comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, and 309 of the GPC3 protein. Alternative examples thereof include a combination of an epitope (for GT30) comprising at least one amino acid selected from amino acids at positions 343, 346, 347, 348, 349, and 350 of the GPC3 protein and an epitope (for GT607) comprising at least one amino acid selected from amino acids at positions 297, 300, 304, 306, 311, 312, 313, 314, and 315 of the GPC3 protein, and a combination of an epitope (for GT165) comprising at least one amino acid selected from amino acids at positions 128, 129, 131, 132, 133, 134, 135, 171, 208, 209, 210, 211, 212, 214, 215, 218, 322, 325, 326, 328, 329, 330, 332, 333, 335, 336, and 338 of the GPC3 protein and an epitope (for GT114) comprising at least one amino acid selected from amino acids at positions 220, 228, 231, 232, 235, 291, 294, and 295 of the GPC3 protein. Further examples thereof can include a combination of an epitope (for GT30) comprising at least one amino acid selected from amino acids at positions 337, 339, 340, and 344 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 341, 343, 346, 347, 348, 349, and 350 thereof, and an epitope (for GT607) comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, and 309 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 297, 300, 304, 306, 311, 312, 313, 314, and 315 thereof, and a combination of an epitope (for GT165) comprising at least one amino acid selected from amino acids at positions 337, 339, 340, and 344 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 128, 129, 131, 132, 133, 134, 135, 171, 208, 209, 210, 211, 212, 214, 215, 218, 322, 325, 326, 328, 329, 330, 332, 333, 335, 336, and 338 thereof, and an epitope (for GT114) comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, and 309 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 220, 228, 231, 232, 235, 291, 294, and 295 thereof.

[0034] The "two different antibodies" are not particularly limited as long as the different antibodies recognize different epitopes in the region described above and are in a relationship that does not mutually inhibit their binding to the soluble GPC3. Specific examples of the "two different antibodies" used in the present invention can include a combination of a GT30 antibody and a GT607 antibody, and a combination of a GT165 antibody and a GT 114 antibody.

GT30 antibody

[0035] The GT30 antibody recognizes an epitope comprising at least one amino acid selected from amino acids at positions 337, 339, 340, 341, 344, 343, 346, 347, 348, 349, and 350 of the GPC3 protein. The GT30 antibody has a heavy chain variable region shown in SEQ ID NO: 38 and a light chain variable region shown in SEQ ID NO: 39 and has heavy chain CDR1, heavy chain CDR2, and heavy chain CDR3 comprising the amino acid sequences represented by SEQ ID NO: 46, SEQ ID NO: 47, and SEQ ID NO: 48, respectively, and light chain CDR1, light chain CDR2, and light chain CDR3 comprising the amino acid sequences represented by SEQ ID NO: 49, SEQ ID NO: 50, and SEQ ID NO: 51, respectively (these sequences of CDR1, CDR2, and CDR3 are based on the Kabat numbering).

GT607 antibody

[0036] The GT607 antibody recognizes an epitope comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, 309, 297, 300, 304, 306, 311, 312, 313,314, and 315 of the GPC3 protein. The GT607 antibody has a heavy chain variable region shown in SEQ ID NO: 40 and a light chain variable region shown in SEQ ID NO: 41 and has heavy chain CDR1, heavy chain CDR2, and heavy chain CDR3 comprising the amino acid sequences represented by SEQ ID NO: 52, SEQ ID NO: 53, and SEQ ID NO: 54, respectively, and light chain CDR1, light chain CDR2, and light chain CDR3 comprising the amino acid sequences represented by SEQ ID NO: 55, SEQ ID NO: 56, and SEQ ID NO: 57, respectively (these sequences of CDR1, CDR2, and CDR3 are based on the Kabat numbering).

GT165 antibody

[0037] The GT165 antibody recognizes an epitope comprising at least one amino acid selected from amino acids at positions 337, 339, 340, 344, 128, 129, 131, 132, 133, 134, 135, 171, 208, 209, 210, 211, 212, 214, 215, 218, 322, 325, 326, 328, 329, 330, 332, 333, 335, 336, and 338 of the GPC3 protein. The GT165 antibody has a heavy chain variable region shown in SEQ ID NO: 44 and a light chain variable region shown in SEQ ID NO: 45 and has heavy chain CDR1, heavy chain CDR2, and heavy chain CDR3 comprising the amino acid sequences represented by SEQ ID NO: 58, SEQ ID NO: 59, and SEQ ID NO: 60, respectively, and light chain CDR1, light chain CDR2, and light chain CDR3 comprising the amino acid sequences represented by SEQ ID NO: 61, SEQ ID NO: 62, and SEQ ID NO: 63, respectively (these sequences of CDR1, CDR2, and CDR3 are based on the Kabat numbering).

GT114 antibody

[0038] The GT 114 antibody recognizes an epitope comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, and 309 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 220, 228, 231, 232, 235, 291, 294, and 295 thereof. The GT114 antibody has a heavy chain variable region shown in SEQ ID NO: 42 and a light chain variable region shown in SEQ ID NO: 43 and has heavy chain CDR1, heavy chain CDR2, and heavy chain CDR3 comprising the amino acid sequences represented by SEQ ID NO: 64, SEQ ID NO: 65, and SEQ ID NO: 66, respectively, and light chain CDR1, light chain CDR2, and light chain CDR3 comprising the amino acid sequences represented by SEQ ID NO: 67, SEQ ID NO: 68, and SEQ ID NO: 69, respectively (these sequences of CDR1, CDR2, and CDR3 are based on the Kabat numbering).

[0039] Hereinafter, the nucleotide sequences and the amino acid sequences of the heavy chain and light chain variable regions of each antibody will be described:

Nucleotide sequence of GT30 H chain (SEQ ID NO: 30)

ATGGAATGGATCTGGATCTTTCTCTTCATCCTGTCAGGAACTGCAGGTGTCCAATCC
CAGGTTCAGCTGCAGCAGTCTGGAGCTGAGCTGGCGAGGCCTGGGGCTTCAGTGAA
ACTGTCCTGCAGGGCTTCTGGCTACACCTTCACAAGCTATGGTATAAGCTGGATGAT
GCAGAGAACTGGACAGGGCCTTGAGTGGATTGGAGAGATTTATCCTAGAAGTGGTA
TTACTTACTACAATGAGAAGTTCAAGGGCAAGGCCACACTGACTGCAGACAAATCC
TCCAGCACAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCAGTCTAT
TTCTGTGCAAGAGATGTCTCTGATGGTTACCTTTTTCCTTACTGGGGCCAAGGGACTC
TGGTCACTGTCTCTGCAGCCAAA

Nucleotide sequence of GT30 L chain (SEQ ID NO: 31)

ATGAGTGTGCCCACTCAGGTCCTGGGGTTGCTGCTGCTGTGGCTTACAGGTGCCAGA
TGTGACATCCAGATGACTCAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAAACT

GTCACCATCACATGTCGAACAAGTGAGAATATTTACAGTTATTTAGCATGGTATCAG
CAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATAATGCAAAAACCTTACCAGA
AGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGCACACAGTTTTCTCTGAAGAT
CAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGTCAACATCATTATGGTAC
TCCTCCGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGGCT

Nucleotide sequence of GT607 H chain (SEQ ID NO: 32)

ATGAACTTCGGGCTCAGCTTGATTTTCCTTGCCCTCATTTTAAAAGGTGTCCAGTGTG
AGGTGCAGCTGGTGGAGTCTGGGGGAGACGTAGTGAGACCTGGAGGGTCCCTGAAA
CTCTCCTGTGCAGCCTCTGGATTCACTTTCAGTAGTTATGGCATGTCCTGGGTTCGCC
AGCTTCCAGACAAGAGGCTGGAGTGGGTCGCAAGTGTTGGTAATGGAGGTAGTTAC
AGGTACTATCCAGAGAATTTGAAGGGGCGGTTCACCATCTCCAGAGACAATACCAA
GAACACCCTATACCTGCAAATTAGTGGTCTGAAGTCTGAGGACACAGCCATTTATTA
CTGTGCAAGACGGGGGGCTTTCCCGTACTTCGATGTCTGGGGCGCAGGGACCACGG
TCACCGTCTCCTCAGCCAAA

Nucleotide sequence of GT607 L chain (SEQ ID NO: 33)

ATGGATTTTCAAGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATAGTAT
CCAGAGGACAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGG
AGAAGGTCACCCTGGCCTGCAGTGCCAGCTCAAGTGTAACTTACATGCACTGGTACC
AGCAGAAGTCAGGCACCTCCCCCAAAAGATGGATTTATGAAACATCCAAACTGGCT
TCTGGAGTCCCTCCTCGCTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAA
TCAGCACCATGGAGGCTGAAGATGCTGCCACTTATTACTGCCAACAGTGGAGTAGT
AACCCGCTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAACGGGCT

Nucleotide sequence of GT114 H chain (SEQ ID NO: 34)

ATGAGAGTGCTGATTCTTTTGTGGCTGTTCACAGCCTTTCCTGGTATCCTATCTGATG
TGCAGCTTCAGGAGTCGGGACCTGGCCTGGTGAAACCTTCTCAGTCTCTGTCCCTCA
CCTGCACTGTCACTGGCTACTCAATCACCAGTGATTCTGCCTGGAACTGGATCCGGC
AGTTTCCAGGAAACAAACTGGAGTGGATGGCCTACATAATGTACAGTGGTATCACT
AGCTACAATCCATCTCTCAAAAGTCGAATCTCTATCACTCGAGACACAGCCAAGAAC
CAGTTCTTTCTGCAGTTGAATTCTGTGACTACTGAGGACTCAGCCACATATTACTGTT
CACGAGGCTACTGGTACTTCGATGTCTGGGGCGCAGGGACTACGGTCACCGTCTCCT
CAGCCAAA

Nucleotide sequence of GT114 L chain (SEQ ID NO: 35)

ATGGATTTTCAGGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATAATGT
CCAGAGGACAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCTAGGGG
AGGAGATCACCCTAACCTGCAGTGCCAGCTCGAGTGTGAGTTACATGCACTGGTACC
AGCAGAAGTCAGGCACTTCTCCCAAACTCTTGATTTATAGCACATCCATCCTGGCTT

CTGGAGTCCCTTCTCGCTTCAGTGGCAGTGGGTCTGGGACCTTTTATTCTCTCACAAT
CAGCAGTGTGGAGGCTGAAGATGCTGCCGATTATTACTGCCTTCAGTGGATTACTTA
TCGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGGCT

Nucleotide sequence of GT165 H chain (SEQ ID NO: 36)

ATGTGTTGGAGCTGTATCATCCTCTTCCTGTTAGCAACAGCTGCACGTGTGCACTCCC
AGGTCCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGGGGCCTGGGGCCTCAGTGAAG
ATTTCCTGCAAGGCTTTTGGCTACACCTTCACAAACCATCATATAAACTGGGTGAAG
CAGAGGCCTGGACAGGGCCTGGACTGGATTGGATATATTAATCCTTATAATGATTAT
ACTAACTACAACCAGAAGTTCAAGGGCAAGGCCACATTGACTGTAGACAAATCCTC
CAGCACAGCCTATATGGAGCTTAGCAGCCTGACATCTGAGGACTCTGCAGTCTATTA
CTGTGCAAGATCAGACCCCGCCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCAC
TGTCTCTGCAGCCAAA

Nucleotide sequence of GT165 L chain (SEQ ID NO: 37)

ATGAGACCCTCCATTCAGTTCCTGGGGCTCTTGTTGTTCTGGCTTCATGGTGCTCAGT
GTGACATCCAGATGACACAGTCTCCATCCTCACTGTCTGCATCTCTGGGAGGCAAAG
TCACCATCACTTGCAAGGCAAGCCAAGACATTAACAAGAATATAGCTTGGTACCAA
CACAAGCCTGGAAAAGGTCCTAGGCTGCTCATATGGTACACATATACATTACAACC
AGGCATCCCATCAAGGTTCAGTGGAAGTGGATCTGGGAGAGATTATTCCTTCAGCAT
CAGCAACCTGGAGCCTGAAGATATTGCAACTTATTACTGTCTACAGTATGATAATCT
TCCATTCACGTTCGGCACGGGGACAAAATTGGAAATAAAACGGGCT

Amino acid sequence of GT30 H chain variable region (SEQ ID NO: 38)

QVQLQQSGAELARPGASVKLSCRASGYTFTSYGISWMMQRTGQGLEWIGEIYPRSGITY
YNEKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYFCARDVSDGYLFPYWGQGTLVTV
SAAK

Amino acid sequence of GT30 L chain variable region (SEQ ID NO: 39)

DIQMTQSPASLSASVGETVTITCRTSENIYSYLAWYQQKQGKSPQLLVYNAKTLPEGVPS
RFSGSGSGTQFSLKINSLQPEDFGSYYCQHHYGTPPTFGGGTKLEIKRA

Amino acid sequence of GT607 H chain variable region (SEQ ID NO: 40)

EVQLVESGGDVVRPGGSLKLSCAASGFTFSSYGMSWVRQLPDKRLEWVASVGNGGSY
RYYPENLKGRFTISRDNTKNTLYLQISGLKSEDTAIYYCARRGAFPYFDVWGAGTTVTV
SSAK

Amino acid sequence of GT607 L chain variable region (SEQ ID NO: 41)

QIVLTQSPAIMSASPGEKVTLACSASSSVTYMHWYQQKSGTSPKRWIYETSKLASGVPP
RFSGSGSGTSYSLTISTMEAEDAATYYCQQWSSNPLTFGAGTKLELKRA

Amino acid sequence of GT114 H chain variable region (SEQ ID NO: 42)

DVQLQESGPGLVKPSQSLSLTCTVTGYSITSDSAWNWIRQFPGNKLEWMAYIMYSGITS
YNPSLKSRISITRDTAKNQFFLQLNSVTTEDSATYYCSRGYWYFDVWGAGTTVTVSSAK

Amino acid sequence of GT114 L chain variable region (SEQ ID NO: 43)

QIVLTQSPAIMSASLGEEITLTCSASSSVSYMHWYQQKSGTSPKLLIYSTSILASGVPSRFS
GSGSGTFYSLTISSVEAEDAADYYCLQWITYRTFGGGTKLEIKRA

Amino acid sequence of GT165 H chain variable region (SEQ ID NO: 44)

QVQLQQSGAELVGPGASVKISCKAFGYTFTNHHINWVKQRPGQGLDWIGYINPYNDYT
NYNQKFKGKATLTVDKSSSTAYMELSSLTSEDSAVYYCARSDPAWFAYWGQGTLVTV
SAAK

Amino acid sequence of GT165 L chain variable region (SEQ ID NO: 45)

DIQMTQSPSSLSASLGGKVTITCKASQDINKNIAWYQHKPGKGPRLLIWYTYTLQPGIPS
RFSGSGSGRDYSFSISNLEPEDIATYYCLQYDNLPFTFGTGTKLEIKRA

[0040] Hereinafter, the CDR sequences of each antibody will be described.

GT30 H chain CDR sequences

CDR1 (SEQ ID NO: 46)	CDR2 (SEQ ID NO: 47)	CDR3 (SEQ ID NO: 48)
SYGIS	EIYPRSGITYYNEKFKG	DVSDGYLFPY

Amino acid sequence of GT30 L chain variable region

CDR1 (SEQ ID NO: 49)	CDR2 (SEQ ID NO: 50)	CDR3 (SEQ ID NO: 51)
RTSENIYSYLA	NAKTLPE	QHHYGTPPT

Amino acid sequence of GT607 H chain variable region

CDR1 (SEQ ID NO: 52)	CDR2 (SEQ ID NO: 53)	CDR3 (SEQ ID NO: 54)
SYGMS	SVGNGGSYRYYPENLKG	RGAFPYFDV

Amino acid sequence of GT607 L chain variable region

CDR1 (SEQ ID NO: 55)	CDR2 (SEQ ID NO: 56)	CDR3 (SEQ ID NO: 57)
SASSSVTYMH	ETSKLAS	QQWSSNPLT

Amino acid sequence of GT165 H chain variable region

CDR1 (SEQ ID NO: 58)	CDR2 (SEQ ID NO: 59)	CDR3 (SEQ ID NO: 60)
NHHIN	YINPYNDYTNYNQKFKG	SDPAWFAY

Amino acid sequence of GT165 L chain variable region (SEQ ID NO: 53)

CDR1 (SEQ ID NO: 61)	CDR2 (SEQ ID NO: 62)	CDR3 (SEQ ID NO: 63)

(continued)

KASQDINKNIA	YTYTLQP	LQYDNLPFTFGTGTKLEIK

Amino acid sequence of GT114 H chain variable region

CDR1 (SEQ ID NO: 64)	CDR2 (SEQ ID NO: 65)	CDR3 (SEQ ID NO: 66)
SDSAWN	YIMYSGITSYNPSLKS	GYWYFDV

Amino acid sequence of GT114L chain variable region (SEQ ID NO: 51)

CDR1 (SEQ ID NO: 67)	CDR2 (SEQ ID NO: 68)	CDR3 (SEQ ID NO: 69)
SASSSVSYMH	STSILAS	LQWITYRT

[0041] For the assay of soluble GPC3 protein according to the present invention, antibodies having heavy chain and light chain variable regions having amino acid sequences with high homology or identity to the amino acid sequences of the heavy chain and light chain variable regions, respectively, of the antibodies described above can be used instead of the antibodies described above. The homology to the amino acid sequences of the heavy chain and light chain variable regions of the antibodies described above is at least 70% or higher, preferably 80% or higher, more preferably, for example, 90%, further preferably 95%, particularly preferably 98% or higher. The homology or the identity (similarity) can be calculated using any well-known algorithm, for example, Needleman-Wunsch, Smith-Waterman, BLAST, or FASTA and is calculated using, for example, the BLAST program (Atschul et al., J. Molec. Biol., 1990; 215: 403-410) under default conditions.

Antibody recognizing same epitopes

[0042] A combination of antibodies respectively binding to the same epitopes as those for the antibodies described above can also be used as the "two different antibodies" of the present invention. The antibody binding to the same epitope as that for each antibody can be obtained by using a method known in the art such as competitive ELISA or by assaying competition (cross-reactivity) with each antibody described above for the soluble GPC3 protein or an epitope fragment recognized by this antibody.

Recombinant antibody

[0043] A combination of antibodies each having heavy chain and light chain variable regions identical to those of the GT30 antibody, the GT607 antibody, the GT165 antibody, or the GT114 antibody, or a combination of antibodies each having heavy chain CDR (CDR1, CDR2, and CDR3) and light chain CDR (CDR1, CDR2, and CDR3) regions identical to those of any of these antibodies can also be used as the "two different antibodies" of the present invention. A combination of chimeric antibodies, humanized antibodies, or human antibodies of these antibodies can also be used as the "two different antibodies" of the present invention.

[0044] Each antibody having heavy chain and light chain variable regions identical to those of the GT30 antibody, the GT607 antibody, the GT165 antibody, or the GT 114 antibody, or each antibody having heavy chain CDRs and light chain CDRs identical to those of any of these antibodies can be prepared by a recombination technique. Specifically, DNAs encoding the heavy chain and light chain variable regions of the anti-GPC3 N-terminal peptide antibody or the anti-GPC3 C-terminal peptide antibody of interest are incorporated into expression vectors having DNAs encoding desired antibody constant regions (C regions), and host cells are transformed with the resulting expression vectors and allowed to express antibodies.

[0045] For the antibody gene expression, the antibody heavy chain (H chain)- and light chain (L chain)-encoding DNAs can be separately incorporated into different expression vectors, with which a host cell can be co-transfected. Alternatively, the H chain- and L chain-encoding DNAs may be incorporated into a single expression vector, with which a host cell can be transformed (see WO 94/11523).

[0046] In addition to the host cells, transgenic animals can be used for the recombinant antibody production. For example, the antibody gene is inserted to a midpoint in a gene encoding a protein (goat β casein, etc.) specifically produced in milk to prepare a fusion gene. A DNA fragment containing the fusion gene having the antibody gene insert is injected into a goat embryo, which is in turn introduced into a female goat. The desired antibody is obtained from milk

produced by transgenic goats (or progeny thereof) brought forth by the goat that has received the embryo. In addition, hormone may be appropriately used for the transgenic goats in order to increase the amount of milk containing the desired antibody produced from the transgenic goats (Ebert, K. M. et al., Bio/Technology (1994) 12, 699-702).

[0047] In the present invention, genetically recombinant antibodies that have been engineered artificially, for example, chimeric antibodies (humanized antibodies, etc.) can be used, in addition to the antibodies described above. These engineered antibodies can be produced by use of a known method. In the case of using the antibodies of the present invention as antibodies for treatment, genetically recombinant antibodies are preferably used.

[0048] The chimeric antibodies are obtained by linking the antibody V region-encoding DNAs obtained as described above to human antibody C region-encoding DNAs and incorporating the resulting products into expression vectors, which are then transferred to hosts to produce the chimeric antibodies. Chimeric antibodies useful for the present invention can be obtained by this known method.

[0049] The humanized antibodies, also called reshaped human antibodies, comprise complementarity determining regions (CDRs) of a non-human animal (e.g., mouse) antibody grafted in human antibodies, and a general genetic recombination method therefor is also known (see European Patent Application Publication No. EP 125023 and WO 96/02576).

[0050] The assay method of the present invention is an invention useful for a method for examining the possibility of liver cancer in a test subject by detecting solubilized GPC3 protein in a test sample isolated from the test subject, as with an invention described in, for example, Japanese Patent No. 4283227. Also, the assay method of the present invention is an invention also useful for a drug for predicting or determining recurrence after treatment of liver cancer, containing an anti-GPC3 antibody, as with an invention described in Japanese Patent No. 4658926. The assay method of the present invention can be used in a diagnosis method and a method for predicting or determining recurrence, comprising the assay method of the present invention. As shown later in Examples, the assay method of the present invention is capable of assaying soluble GPC3 protein contained in healthy subject blood on the order of several pg/mL and thus, is a more useful technique for early detection and prognosis of liver cancer as compared with the inventions related to the two patents described above.

[0051] More specifically, since the concentration range of soluble GPC3 in healthy subjects is approximately 15 to 174 pg/mL, soluble GPC3 detected at a level exceeding this range indicates the possibility that liver cancer has developed or recurred, and suggests that the risk can be reduced by early detection.

[0052] The present invention also provides an assay kit for use in the method for assaying soluble GPC3 protein according to the present invention, comprising the two different antibodies binding to different epitopes. These antibodies may be provided in a state immobilized on the carrier mentioned above or may be provided independently of the carrier. The kit may additionally comprise standard solutions of serially diluted soluble GPC3. Assay principles, etc., for use in the immunological assay kit of the present invention are the same as in the immunological method mentioned above. In the assay kit of the present invention, any of various aqueous solvents may be used as a solvent. Examples of the aqueous solvents include purified water, saline, and various buffers such as tris buffers, phosphate buffers, and phosphate-buffered saline. The pH of this buffer can be appropriately selected from among suitable pHs. The pH value used is not particularly limited and is generally selected within the range of pH 3 to 12.

[0053] The assay kit of the present invention may appropriately contain, in addition to the components mentioned above, one or two or more components selected from proteins (e.g., bovine serum albumin (BSA), human serum albumin (HSA), casein, and salts thereof), various salts, various sugars, skimmed milk, various animal sera (e.g., normal rabbit serum), various antiseptics (e.g., sodium azide and antibiotics), activators, reaction accelerants, sensitivity-increasing substances (e.g., polyethylene glycol), nonspecific reaction-inhibiting substances, various surfactants (e.g., nonionic surfactants, amphoteric surfactants, and anionic surfactants), and the like. The concentrations of these components contained in the assay reagent are not particularly limited and are preferably 0.001 to 10% (W/V). Particularly preferred concentrations are appropriately selected within the range of 0.01 to 5% (W/V).

[0054] The assay kit of the present invention may be further combined with other reagents, in addition to the components mentioned above. Examples of these other reagents include buffers, diluting solutions for biological samples, reagent diluting solutions, reagents containing labeling materials, reagents containing substances that generate signals such as color, reagents containing substances involved in the generation of signals such as color, reagents containing substances for calibration, and reagents containing substances for accuracy control.

[0055] The assay kit of the present invention is not particularly limited by its form and may be provided as an integral-type assay kit comprising all of the components constituting the assay kit of the present invention in order to carry out assay conveniently in a short time. Examples of the integral-type assay kit include ELISA kits, fluorescence immunoassay kits, and immunochromatography kits. The ELISA kit form comprises, for example, a first antibody-immobilized microplate, standard solutions of serially diluted soluble GPC3, a second antibody modified with an enzyme such as HRP, a washing buffer, and a substrate solution for the enzymatic reaction. The fluorescence immunoassay kit comprises, for example, a first antibody-immobilized optical waveguide, standard solutions of serially diluted soluble GPC3, a second antibody modified with a fluorescent material, and a washing buffer. The immunochromatography kit comprises a membrane

housed in a reaction cassette where the first antibody is immobilized at one end (downstream) of the membrane. In an exemplary embodiment, a developing solution is placed at the other end (upstream) of the membrane; a pad supplemented with a substrate for the labeling agent is disposed in proximity (downstream) to the developing solution; and a pad supplemented with the second antibody labeled as described above is disposed in the central part of the membrane.

Examples

[0056] Hereinafter, the present invention will be described in detail with reference to Examples. However, the present invention is not intended to be limited by these Examples.

[Example 1]

[0057] A Balb/c or MRL/lpr mice were immunized a total of 6 to 9 times with GPC3 core (sGPC3-His) protein prepared by the C-terminal His tagging of the extracellular domain (positions 25 to 563) of GPC3 core variant derived from human glypican-3 (GPC3; SEQ ID NO: 70) by the exchange of serine at two heparan sulfate-binding sites (positions 495 and 509) to alanine. 3 days after the final immunization, the splenocytes of this mouse were fused with mouse myeloma cells SP2/0 by a routine method using PEG1500 or by using HVJ-E (Ishihara Sangyo Kaisha, Ltd.).

[0058] Next, hybridoma cells were screened by ELISA using sGPC3-His directly immobilized on a solid phase, ELISA using sGPC3-His bound with an anti-His antibody immobilized on a solid phase, or Cell ELISA using CHO cells stably expressing GPC3 (see WO 2006/006693).

[Example 2]

[0059] The sGPC3-His protein has been found to yield bands of approximately 86 kDa, approximately 43 kDa, and approximately 33 kDa, which correspond to a full length, a protease-cleaved N-terminal fragment, and a protease-cleaved C-terminal fragment, respectively, in SDS-PAGE under reductive conditions (WO 2006/006693). Mouse antibodies thus obtained by the screening were used in Western blot for sGPC3-His to select antibodies that did not recognize the C-terminal fragment. As a result, GT30, GT114, and GT607 as N-terminal fragment-recognizing antibodies, and GT165 whose recognition site was unidentified were obtained (Figure 1).

[0060] Subsequently, C-terminally His-tagged recombinant products of positions 1 to 196 (AW2), positions 1 to 218 (AW3), and positions 1 to 357 (AW5) of GPC3 were each expressed in CHO cells, and pellets of the CHO cells expressing each recombinant product were used in Western blot. As a result, as shown in Figure 2 and Table 1, GT30, GT114, and GT607 were confirmed to have reactivity with AW5 or sGPC3-His, but no reactivity with AW2 and AW3, indicating that these antibodies bind to positions 219 to 357 of GPC3. On the other hand, GT165 was confirmed to have reactivity with none of these products, suggesting the possibility that this antibody strongly recognizes conformation.

[Table 1]

Clone	Immunized mouse	Isotype	Western blot analysis			
			sGPC3-His	AW2 (1-196)	AW3 (1-218)	AW5 (1-357)
GT114	BALB/c	IgG1	++	-	-	+
GT607	BALB/c	IgG1	++	-	-	+
GT30	MRL	IgG1	++	-	-	++
GT165	MRL	IgG1	+/-	-	-	-

[Example 3]

[0061] In order to identify more detailed epitopes for the antibodies, the amino acid sequence of GPC3 was divided into peptides of 30 residues containing 10-residue overlaps, and the corresponding peptides were synthesized (SEQ ID NOs: 1 to 29 in Table 2). Each peptide thus synthesized was dissolved at a concentration of 1 mg/mL in dimethyl sulfoxide and then diluted to 1 μg/mL with PBS. The diluted peptide solution was added at 70 μL/well to a 96-well plate and immobilized thereon at room temperature for 1 hour. After removal of unimmobilized peptides, 20% Blocking-one (Nacalai Tesque, Inc.) was added thereto as a blocking buffer solution at 200 μL/well, and the plate was blocked overnight. After removal of the blocking buffer solution, each well was washed with TBS-T three times, and each antibody diluted to 2 μg/mL with a blocking buffer was then added thereto at 70 μL/well. One hour later, the antibody was removed, and

each well was washed three times by the addition of TBS-T at 200 μL/well. An HRP-bound anti-mouse antibody was added at 70 μL/well to each well and reacted therewith for 1 hour. After removal of the antibody, each well was washed with 200 μL/well of TBS-T three times, and a chromogenic reagent ABTS Peroxidase Substrate System (1 component, KPL, Kirkegaard & Perry Laboratories, Inc.) was then added thereto at 70 μL/well and reacted at room temperature for 15 to 30 to develop color. The reaction was terminated by the addition of 1% SDS at 70 μL/well, and the absorbance at 405 nm was measured using a plate reader. As a result, as shown in Figure 3, GT30 was found to bind to the peptide of SEQ ID NO: 17, whereas the specific binding of GT114, GT165, and GT607 was not found for these peptides.

[0062] These results suggest that GT30 binds to a site from position 321 to position 350 of GPC3 and also suggest the possibility that GT165 as well as GT 114 and GT607 recognize conformation.

[Table 2]

Amino acid position	Sequence	SEQ ID NO
1-30	MAGTVRTACLVVAMLLSLDFPGQAQPPPPP	1
21-50	PGQAQPPPPPPDATCHQVRSFFQRLQPGLK	2
41-70	FFQRLQPGLKWVPETPVPGSDLQVCLPKGP	3
61-90	DLQVCLPKGPTCCSRKMEEKYQLTARLNME	4
81-110	YQLTARLNMEQLLQSASMELKFLHQNAAV	5
101-130	KFLIIQNAAVFQEAFEIVVRHAKNYTNAMF	6
121-150	HAKNYTNAMFKNNYPSLTPQAFEFVGEFFT	7
141-170	AFEFVGEFFTDVSLYILGSDINVDDMVNEL	8
161-190	INVDDMVNELFDSLFPVIYTQLMNPGLPDS	9
181-210	QLMNPGLPDSALDINECLRGARRDLKVFGN	10
201-230	ARRDLKVFGNFPKLIMTOVSKSLQVTRIFL	11
221-250	KSLQVTRIFLQALNLGIEVINTTDHLKFSK	12
241-270	NTTDHLKFSKDCGRMLTRMWYCSYCQGLMM	13
261-290	YCSYCQGLMMVKPCGGYCNVVMQGCMAGVV	14
281-310	VMQGCMAGVVEIDKYWREYILSLEELVNGM	15
301-330	LSLEELVNGMYRIYDMENVLLGLFSTIHDS	16
321-350	LGLFSTIHDSIQYVQKNAGKLTTTIGKLCA	17
341-370	LTTTIGKLCAHSQQRQYR^SAYYPEDLFIDK	18
361-390	YYPEDLFIDKKVLKVAHVEHEETLSSRRRE	19
381-410	EETLSSRRRELIQKLKSFISFYSALPGYIC	20
401-430	FYSALPGYICSHSPVAENDTLCWNGQELVE	21
421-450	LCWNGQELVERYSQKAARNGMKNQFNLHEL	22
441-470	MKNQFNLHELKMKGPEPVVSQIIDKLKHIN	23
461-490	QIIDKLKHINQLLRTMSMPKGRVLDKNLDE	24
481-510	GRVLDKNLDEEGFESGDCGDDEDECIGGSG	25
501-530	DEDECIGGSGDGMIKVKNQLRFLAELAYDL	26
521-550	RFLAELAYDLDVDDAPGNSQQATPKDNEIS	27
541-570	QATPKDNEISTFHNLGNVHSPLKLLTSMAI	28
551-580	TFHNLGNVHSPLKLLTSMAISVVCFFFLVH	29

[Example 4]

[0063] Next, in order to analyze the binding competition of each antibody recognizing the GPC3 N-terminal fragment, each antibody was used in a binding competition test.

[0064] Specifically, a 1 μg/mL sGPC3 solution (in PBS) was added at 50 μL/well to a 96-well transparent microplate (MaxiSoap manufactured by Thermo Fisher Scientific Inc.), and the microplate was left standing overnight at room temperature. Subsequently, the microplate was washed with a washing solution (TBS containing 0.01% Triton X-100) once. Then, a blocking solution (Blocking-One manufactured by Nacalai Tesque, Inc.) was added thereto at 200 μL/well, and the microplate was left standing at room temperature for 1 hour. The microplate was washed with a washing solution three times. Then, a 50 μg/mL unlabeled anti-GPC3 antibody solution (in PBS containing 20% Blocking-One) was added thereto at 25 μL/well, and the mixture was stirred at room temperature for 1 minute. A 0.5 μg/mL biotin-labeled anti-GPC3 antibody solution (in PBS containing 20% Blocking-One) was further added thereto at 25 μL/well, and the mixture was stirred at room temperature for 1 hour. Subsequently, the microplate was washed with a washing solution three times. Then, a solution of StreptAvidin-HRP (manufactured by Prozyme) diluted 10,000-fold with a diluting solution (TBS containing 10% Block Ace and 0.1 % Tween 20) was added thereto at 50 μL/well, and the mixture was stirred at room temperature for 30 minutes. The microplate was washed with a washing solution five times. Then, a chromogenic substrate solution (1-Step Turbo TMB manufactured by Thermo Fisher Scientific Inc.) was added thereto at 50 μL/well, and the absorbance at 450 nm (O.D. 450) was measured using a microplate reader.

[Table 3]

			Labeled antibody			
			GT30	GT114	GT607	GT1 65
O.D. 450		-	1.840	2.299	2.181	1.678
	Unlabeled antibody (added in 100-fold amount)	GT30	0.070	2.315	2.125	0.098
		GT114	2.140	0.251	0.307	1.818
		GT607	2.064	1.132	0.135	0.129
		GT165	0.846	2.280	1.248	0.111
Inhibitory effect (%)		GT30	96%	0%>	3%	94%
	Unlabeled antibody (added in 100-fold amount)	GT114	0%>	89%	86%	0%>
		GT607	0%>	51%	94%	92%
		GT165	54%	1%	43%	93%

[0065] As shown in Table 3, GT30 and GT165 mutually inhibited their binding to GPC3. Therefore, the epitopes for GT165 and GT30 seem to be located in proximity. Although GT114 and GT607 were free from inhibition by GT30, their binding to GPC3 was inhibited in the coexistence of GT114 and GT607. Therefore, the epitopes for GT114 and GT607 seem to be located in proximity. On the other hand, GT114 was also free from inhibition by GT165, whereas GT607 underwent the inhibition of binding by GT165. Therefore, the binding site of GT607 was found to not overlap with the binding site of GT30, but to be located near the binding region of GT165 (Figure 4).

[Example 5]

[0066] The results described above suggested that GT114, GT165, and GT607 recognize conformation, and, particularly, GT165 binds to near the site from position 321 to position 350, which is an epitope region for GT30. Therefore, a possible binding region was identified using a conformational model of human GPC3.

[0067] First, on the hypothesis that the structure of fruit fly Dally-like protein (PDB ID: 3ODN; Kim M.S. et al., Proc Natl Acad Sci USA 2011; 108: 13112-13117) having high homology to human GPC3 and having reported results of X-ray crystallography would be the conformation of human GPC3 (containing a proline residue at position 29 to a lysine residue at position 486 except for tyrosine at position 357 to valine at position 372), the interaction of each antibody was predicted using PyMOL Molecular Graphics System, Version 1.5.0.4 (manufactured by Schrodinger LLC). The conformation of the glypican molecule has been reported to be well conserved across species (Svensson G. et al., J Biol Chem 2012; 287: 14040-14051).

[0068] As a result, the epitope region for GT30 from position 321 to position 350 was presumed to have an α-helix structure. In order to analyze a binding mode for this region, three mouse monoclonal antibody Fabs (PDB IDs: 2CMR, 2XRA, and 3V6Z) found to recognize an α-helix structure and having reported results of X-ray crystallography were used as helix structure recognition models. The X-ray crystal structure of a mouse IgG1 antibody (PDB ID: 1IGY) was

further overlaid with each of these three Fabs. As a result, a total of 12 possible binding modes including all combinations of two types of helix-binding sites for the region from position 321 to position 350, the binding modes of 3 types of Fabs (2CMR, 2XRA, and 3V6Z), and two mouse IgG1 Fabs were predicted for mouse IgG1 antibodies recognizing two helix moieties in the region from position 321 to position 350 of human GPC3.

[0069] For each of these 12 IgG1 binding models, a region on human GPC3 that came in contact with Fab within 4 angstroms was identified as a binding region for GT165, which is an antibody competing with GT30 for antigen binding. Likewise, a region capable of competing with the binding region for GT165 was identified as a binding region for GT607. Subsequently, on the basis of the binding region for GT607, a region capable of competing therewith was identified as a binding region for GT114. From these results, the results of Examples 2 and 3, and the results about competitive relationships shown in Table 3 and Figure 4, regions shown in Figures 5 to 9 were identified as binding regions for GT30, GT165, GT607, and GT114. Of these recognition regions identified here, human GPC3 amino acid residues that are important for the antigen binding of each antibody are described in Table 4, in light of the results of Examples 2 and 3. All human GPC3 amino acid residues that may serve as recognition regions of each antibody predicted from the structural models are shown in Table 5.

[Table 4]

GT30	GT165		GT607	GT114
N337	A128	G322	K221	S220
G339	M129	S325	R297	K221
K340	K131	T326	E298	I228
L341	N132	H328	I300	Q231
T343	N133	D329	L301	A232
T344	Y134	S330	S302	L235
G346	P135	Q332	E304	E291
K347	F171	Y333	E305	K294
L348	F208	Q335	L306	Y295
C349	G209	K336	N308	E298
A350	N210	N337	G309	L301
	F211	A338	Y311	S302
	P212	G339	R312	E305
	L214	K340	I313	N308
	I215	T344	Y314	G309
	Q218		D315	
	N318		N318	

[Table 5]

Human GPC3 amino acid position	Human GPC3 amino acid residue
129	Met
133	Asn
134	Tyr
182	Leu
186	Gly
194	Ile
205	Leu
208	Phe
209	Gly
210	Asn
211	Phe

(continued)

Human GPC3 amino acid position	Human GPC3 amino acid residue
214	Leu
215	Ile
218	Gln
318	Asn
351	His

[Example 6]

(Construction of ELISA assay system using magnetic particle)

[0070] On the basis of the results of Example 4, two types shown in Table 6 were selected as combinations of antibodies capable of constructing soluble GPC3 assay systems.

[Table 6]

Assay system No.	On solid-phase side	On label side
1	GT114	GT165
2	GT30	GT607

[0071] For each assay system shown in Table 6, the antibody on a slid-phase side was immobilized on a magnetic particle (Magnosphere MS300/Carboxyl manufactured by JSR Life Sciences Corp.), while the antibody on a label side was labeled with alkaline phosphatase in order to achieve high sensitivity.

[Example 7]

(Assay of soluble GPC3 by chemiluminescent enzyme immunoassay using magnetic particle)

[0072] The soluble GPC3 was assayed by chemiluminescent enzyme immunoassay using magnetic particles according to the following method:

[0073] 0.06% (w/v) GT30 or GT114 antibody-bound magnetic particles were added at 25 μL/wee to a 96-well white microplate (manufactured by Corning Inc.). Subsequently, a solution sample containing soluble GPC3 was added thereto at 25 μL/well. Each solution of the GT607 or GT165 antibody labeled with alkaline phosphatase was further added thereto at 25 μL/well. Then, the 96-well white microplate was stirred at 25°C for 20 minutes. The antibody-bound magnetic particles were washed with a washing solution (TBS containing 0.01% Triton X-100) five times while magnetism was collected with a microplate washer (HydroFlex manufactured by Tecan Trading AG). A luminescent substrate solution (Lumipulse substrate solution manufactured by FUJIREBIO Inc.) was further added thereto at 50 μL/well. Five minutes later, the luminescence intensity was measured using a chemiluminescence detector (GloMax 96 manufactured by Promega Corp.).

[Example 8]

(Detection limit and quantification limit)

[0074] For the detection limit, soluble GPC3 solution samples prepared at 0 to 10 pg/mL were each assayed 10 times. A mean and standard deviation (SD) of the luminescence intensity values measured at each concentration were calculated. Then, the lowest concentration at which the range of the mean measured luminescence intensity value ± 3SD did not overlap with the range of the mean measured luminescence intensity at the concentration 0 pg/mL ± 3SD was defined as the detection limit.

[0075] For the quantification limit, soluble GPC3 solution samples prepared at 0 to 10 pg/mL were each assayed 10 times. The concentration at which CV of the measurement values calculated from a calibration curve was 10% or less was defined as the quantification limit.

[0076] As a result, as shown in Figure 10, all of the constructed assay systems had a detection limit and a quantification

limit of 10 pg/mL or lower and were confirmed to be capable of assaying soluble GPC3 highly sensitively.

[Example 9]

(Dilution linearity test of liver cancer specimen)

[0077] A calibration curve was prepared from soluble GPC3 solutions each prepared at 0, 10, 25, 50, 100, 250, 500, or 1000 pg/mL. Soluble GPC3 in serially diluted liver cancer specimens was assayed by the same procedures as in Example 7.

[0078] As a result, as shown in Table 7, values were calculated by multiplying each measurement value by each dilution ratio and evaluated for dilution linearity. Consequently, the dilution linearity was confirmed.

[Table 7]

	Dilution ratio	GT114-GT165	GT30-GT607
Mean measurement value (pg/mL)	1	773	763
	2	395	370
	4	192	181
	8	121	92
	16	52	53
Measurement value × dilution ratio (pg/mL)	1	773	763
	2	790	740
	4	768	724
	8	968	736
	16	832	848
Linearity evaluation (%)	1	100	100
	2	102	97
	4	99	95
	8	125	96
	16	108	111

[Example 10]

(Assay using healthy subjects' specimens)

[0079] A calibration curve was prepared from soluble GPC3 solutions each prepared at 0, 10, 25, 50, 100, 250, 500, or 1000 pg/mL. Soluble GPC3 in healthy subject sera was assayed by the same procedures as in Example 7. 21 healthy subjects' sera were used in the assay, and a mean of the measurement values was determined.

[Table 8]

		GT114-GT165	GT30-GT607
Measurement value (pg/mL)	Normal human 1	32	103
	Normal human 2	53	99
	Normal human 3	32	107
	Normal human 4	15	109
	Normal human 5	35	171
	Normal human 6	21	129
	Normal human 7	55	119
	Normal human 8	20	138
	Normal human 9	58	174
	Normal human 10	25	86
	Normal human 11	21	66
	Normal human 12	41	125

(continued)

	GT114-GT165	GT30-GT607
Normal human 13	64	165
Normal human 14	55	122
Normal human 15	16	47
Normal human 16	17	128
Normal human 17	73	142
Normal human 18	52	127
Normal human 19	16	88
Normal human 20	51	116
Normal human 21	31	104
Mean	37	117

[0080] As a result, as shown in Table 8, all of the measurement values of the soluble GPC3 in the healthy subjects' sera were above the detection limit and the quantification limit of each assay system, and the concentration range of the soluble GPC3 was 15 to 174 pg/mL. From these results, all of the constructed assay systems were confirmed to be capable of detecting soluble GPC3 contained at as trace as several tens to several hundreds of pg/mL in healthy subject serum.

[Example 11]

Sequence analysis of obtained antibody

[0081] Total RNA was extracted from each of the hybridomas producing the antibodies GT30, GT114, GT165, GT607, and GT30 obtained as described above, and cDNA was synthesized therefrom using reverse transcriptase. Then, the mouse antibody genes were amplified by PCR, and nucleotide sequences each encoding the amino terminus to the variable region were determined (SEQ ID NOs: 30 to 37). The amino acid sequences of the variable regions are shown in SEQ ID NOs: 38 to 45. The sequences of their CDR regions are shown in SEQ ID NOs: 46 to 69.

[Example 12]

[0082] In order to confirm the efficacy and safety of GC33 in advanced and/or recurrent hepatocellular carcinoma (HCC) patients, a multicenter, randomized, double-blind, placebo-controlled phase-II clinical trial involving administering 1600 mg of GC33 every other week was conducted targeting previously treated human adult patients with unresectable advanced or metastatic hepatocellular carcinoma (NP27884 study). GC33 is a genetically recombinant humanized IgG1 monoclonal antibody binding to human GPC3 with high affinity (WO2006/006693).

[0083] The concentration of soluble GPC3 in serum before the first dose for the cases given GC33 or a placebo in the GPC3-targeting treatment was confirmed to be able to be assayed with a combination of two different antibodies binding to soluble GPC3 (combination of the GT30 antibody and the GT607 antibody or a combination of GT 114 and GT165).

[0084] An antibody-bound particle solution containing GT30 or GT114 bound with magnetic particles (MS300 manufactured by JSR Life Sciences Corp.) was added at 25 μL/well to a 96-well microplate. Subsequently, a standard sample solution for a calibration curve or an appropriately diluted serum sample was added thereto at 25 μL/well, and alkaline phosphatase-labeled GT607 or GT165 was further added thereto at 25 μL/well. After shaking at 25°C for 20 minutes, each well was washed with a washing solution five times with magnetism collected using Dyna-Mag-96 Side Skirted (manufactured by VERITAS Corp.). A luminescent substrate solution preheated to 37°C was added thereto at 50 μL/well. The plate was shaken at room temperature for 1 minute and then left standing for 4 minutes to develop light. The chemiluminescence intensity was measured using a luminometer (manufactured by VERITAS Corp.). The GPC3 standard used in the standard sample solution for a calibration curve was recombinant GPC3 with serine residues at positions 495 and 509 substituted by alanine residues so as to prevent the binding of a heparan sulfate sugar chain (Hippo et al., Cancer Res. (2004) 64, 2418-2423).

[0085] The calibration curve (standard curve) prepared on the basis of standard samples containing the recombinant GPC3 was used to calculate the GPC3 antigen in the serum of each patient from the obtained chemiluminescence intensity of each well.

Industrial Applicability

[0086] The present invention is useful for early detection at an initial stage in development, selection of an anticancer agent used, and post-treatment prognosis for cancer, particularly, liver cancer.

Claims

1. A method for assaying soluble GPC3 protein in a test sample, comprising using two different antibodies binding to different epitopes contained in an amino acid sequence from position 128 to position 357 of GPC3 protein represented by SEQ ID NO: 70.

2. The method according to claim 1, wherein one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 337, 339, 340, and 344 of the GPC3 protein.

3. The method according to claim 1 or 2, wherein one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, and 309 of the GPC3 protein.

4. The method according to claim 1, wherein the different epitopes are the following combination (A) or (B):

   (A) one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 341, 343, 346, 347, 348, 349, and 350 of the GPC3 protein, and the other epitope is an epitope comprising at least one amino acid selected from amino acids at positions 297, 300, 304, 306, 311, 312, 313, 314, and 315 of the GPC3 protein; and
   (B) one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 128, 129, 131, 132, 133, 134, 135, 171, 208, 209, 210, 211, 212, 214, 215, 218, 322, 325, 326, 328, 329, 330, 332, 333, 335, 336, and 338 of the GPC3 protein, and the other epitope is an epitope comprising at least one amino acid selected from amino acids at positions 220, 228, 231, 232, 235, 291, 294, and 295 of the GPC3 protein.

5. The method according to claim 1, wherein the different epitopes are the following combination (A) or (B):

   (A) one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 337, 339, 340, and 344 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 341, 343, 346, 347, 348, 349, and 350 thereof, and the other epitope is an epitope comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, and 309 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 297, 300, 304, 306, 311, 312, 313, 314, and 315 thereof; and
   (B) one of the different epitopes is an epitope comprising at least one amino acid selected from amino acids at positions 337, 339, 340, and 344 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 128, 129, 131, 132, 133, 134, 135, 171, 208, 209, 210, 211, 212, 214, 215, 218, 322, 325, 326, 328, 329, 330, 332, 333, 335, 336, and 338 thereof, and the other epitope is an epitope comprising at least one amino acid selected from amino acids at positions 221, 298, 301, 302, 305, 308, and 309 of the GPC3 protein and comprising at least one amino acid selected from amino acids at positions 220, 228, 231, 232, 235, 291, 294, and 295.

6. The method according to claim 1, wherein the different epitopes are an epitope that is bound by an antibody having a heavy chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 38 and a light chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 39, and an epitope that is bound by an antibody having a heavy chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 40 and a light chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 41, or an epitope that is bound by an antibody having a heavy chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 44 and a light chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 45, and an epitope that is bound by an antibody having a heavy chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 42 and a light chain variable region comprising a sequence having 80% or higher homology to the sequence represented by SEQ ID NO: 43.

7. The method according to claim 1, wherein the two different antibodies are
a combination of an antibody having CDR regions identical to CDR regions contained in a heavy chain variable region shown in SEQ ID NO: 38 and CDR regions contained in a light chain variable region shown in SEQ ID NO: 39, and an antibody having CDR regions identical to CDR regions contained in a heavy chain variable region shown in SEQ ID NO: 40 and CDR regions contained in a light chain variable region shown in SEQ ID NO: 41, or
a combination of an antibody having CDR regions identical to CDR regions contained in a heavy chain variable region shown in SEQ ID NO: 44 and CDR regions contained in a light chain variable region shown in SEQ ID NO: 45, and an antibody having CDR regions identical to CDR regions contained in a heavy chain variable region shown in SEQ ID NO: 42 and CDR regions contained in a light chain variable region shown in SEQ ID NO: 43.

8. The method according to claim 7, wherein the CDR regions are CDR1, CDR2, and CDR3 regions based on the Kabat numbering.

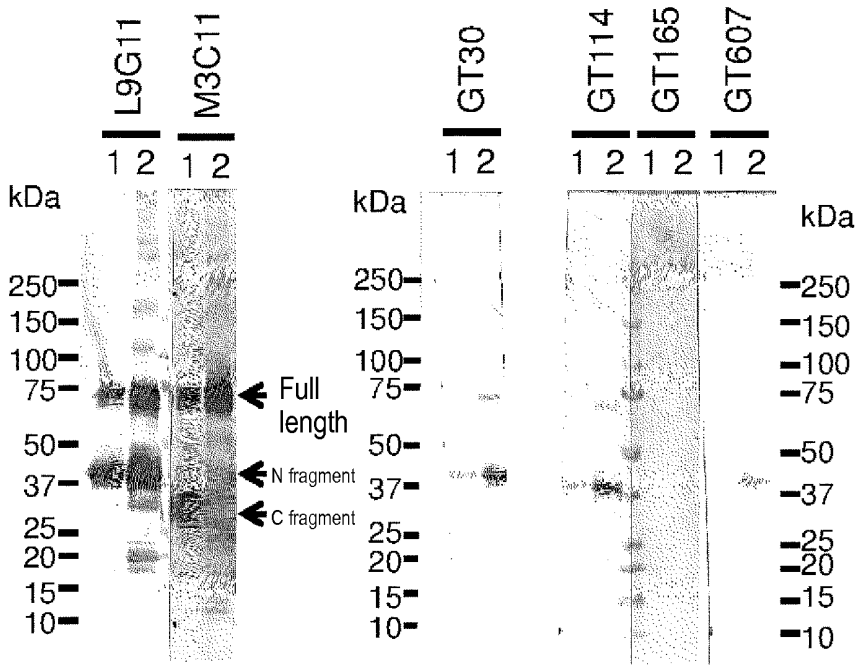
9. The method according to claim 1, wherein the two different antibodies are
a combination of an antibody having a heavy chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 46, 47, and 48, respectively, and having a light chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 49, 50, and 51, respectively, and an antibody having a heavy chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 52, 53, and 54, respectively, and having a light chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 55, 56, and 57, respectively, or
a combination of an antibody having a heavy chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 58, 59, and 60, respectively, and having a light chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 61, 62, and 63, respectively, and an antibody having a heavy chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 64, 65, and 66, respectively, and having a light chain variable region whose CDR1, CDR2, and CDR3 comprise the amino acid sequences represented by SEQ ID NOs: 67, 68, and 69, respectively.

10. The method according to claim 1, wherein the two different antibodies are
a combination of an antibody having a heavy chain variable region shown in SEQ ID NO: 38 and a light chain variable region shown in SEQ ID NO: 39, and an antibody having a heavy chain variable region shown in SEQ ID NO: 40 and a light chain variable region shown in SEQ ID NO: 41, or
a combination of an antibody having a heavy chain variable region shown in SEQ ID NO: 44 and
a light chain variable region shown in SEQ ID NO: 45, and an antibody having a heavy chain variable region shown in SEQ ID NO: 42 and a light chain variable region shown in SEQ ID NO: 43.

11. The method according to any one of claims 1 to 10, wherein any one of the two different antibodies is bound with a magnetic particle.

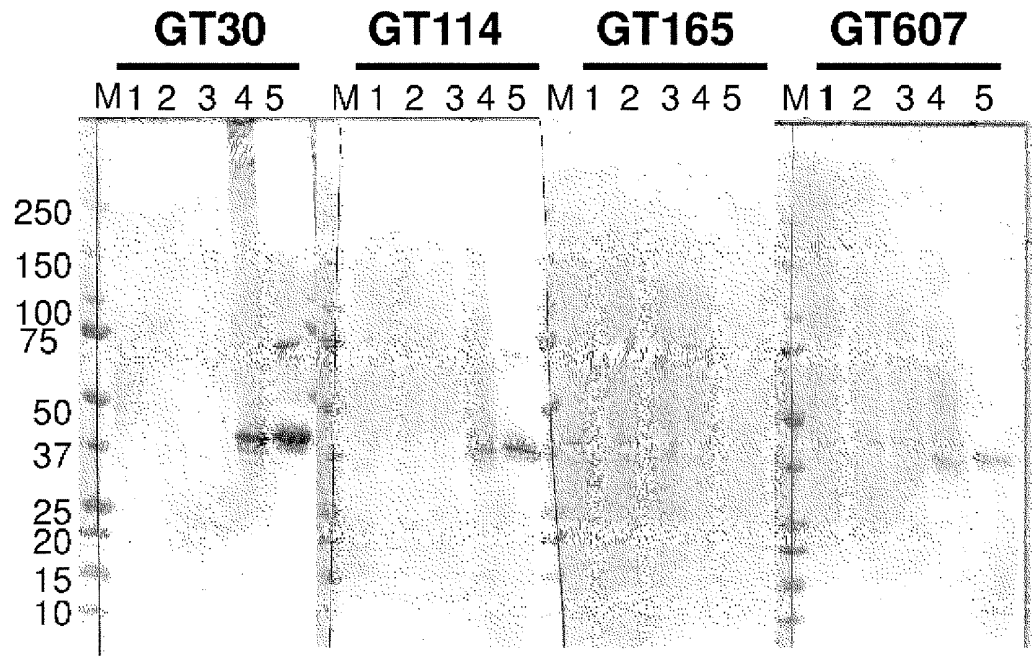
12. The method according to any one of claims 1 to 11, wherein the test sample is a tissue sample, a whole blood sample, a plasma sample, or a serum sample isolated from a human.

Fig 1



Lane 1 ; GPC3 core 50 ng, 2; GPC3 core 500 ng

Fig 2



M; marker, 1; CHO, 2; AW2, 3; AW3, 4; AW5, 5; GPC3

Fig 3

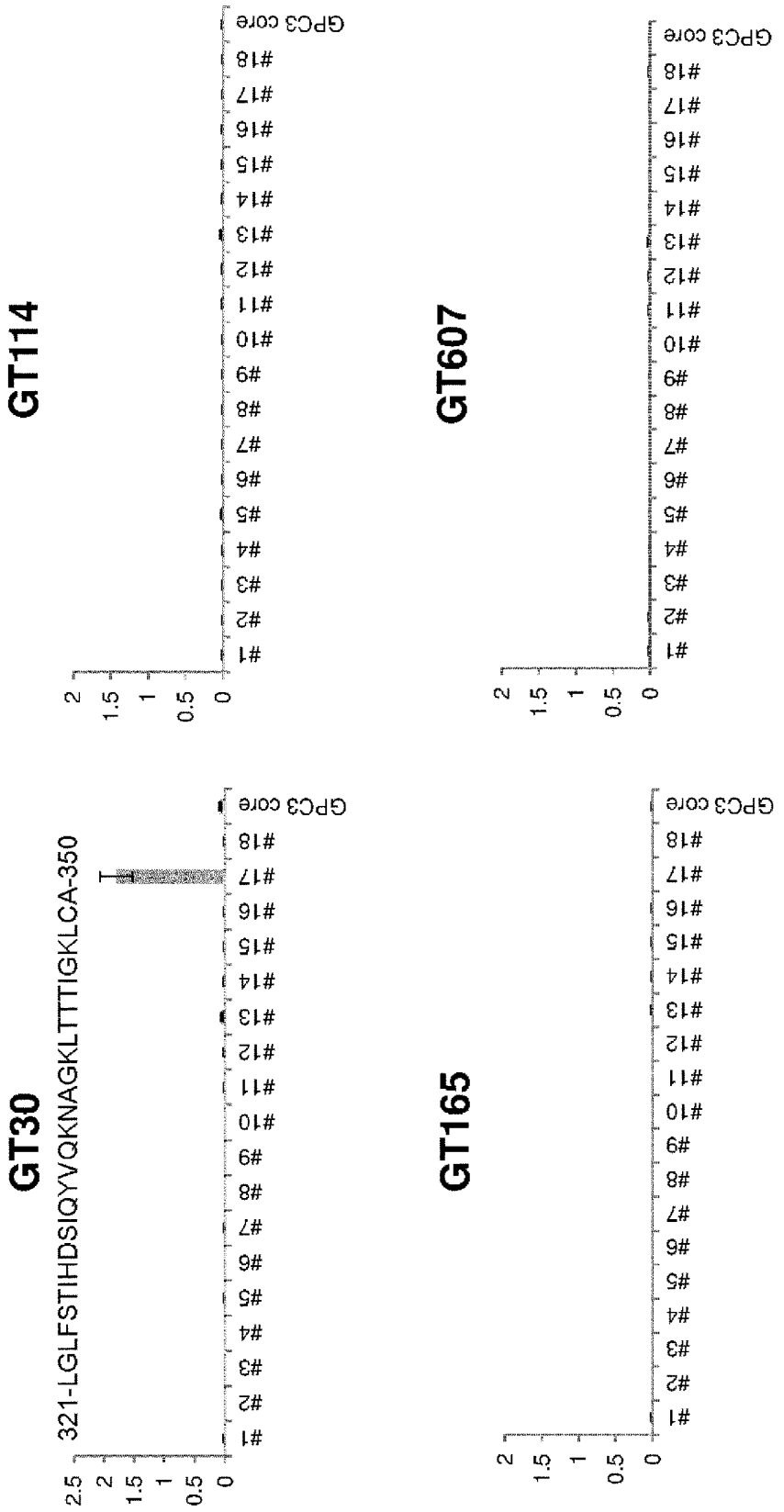

GT114
GT607
GT30
321-LGLFSTIHDSIQYVQKNAGKLTTTIGKLCA-350
GT165
GPC3 core
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
#13
#14
#15
#16
#17
#18
0
0.5
1
1.5
2
2.5

Fig 4

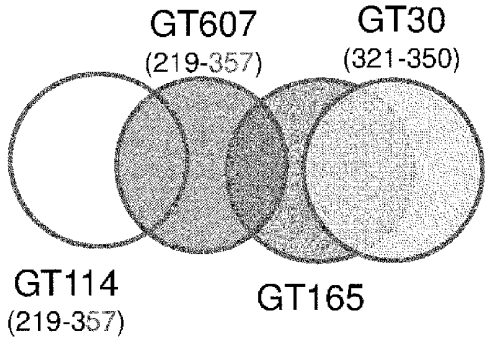


Fig 5

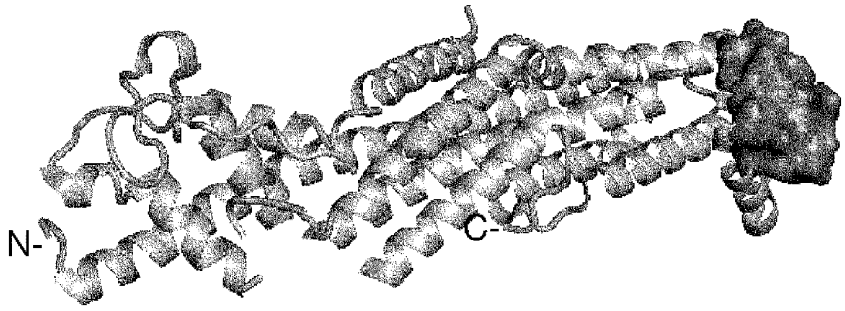


Fig 6

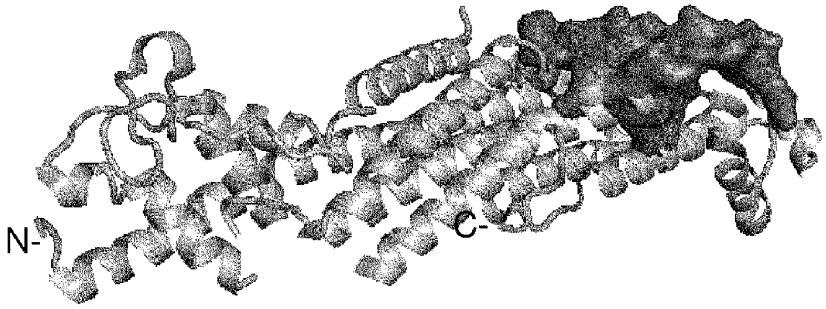


Fig 7

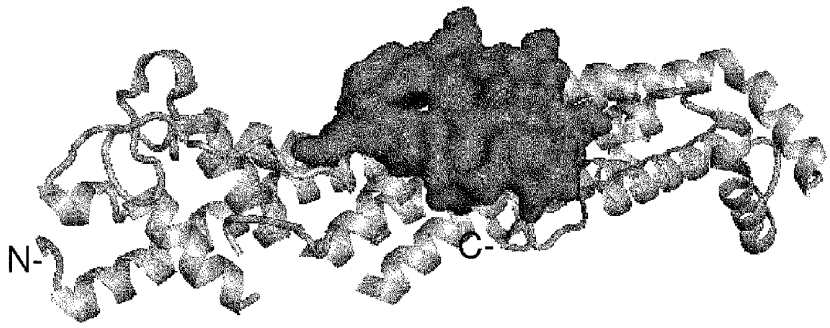


Fig 8

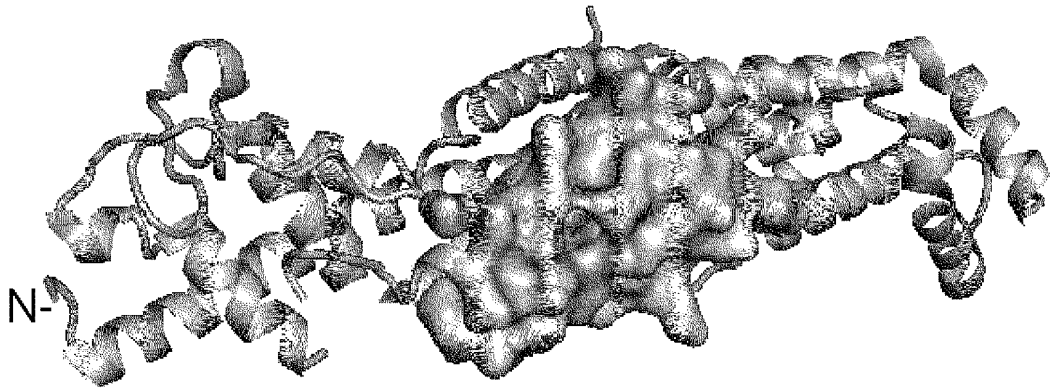

N-

Fig 9

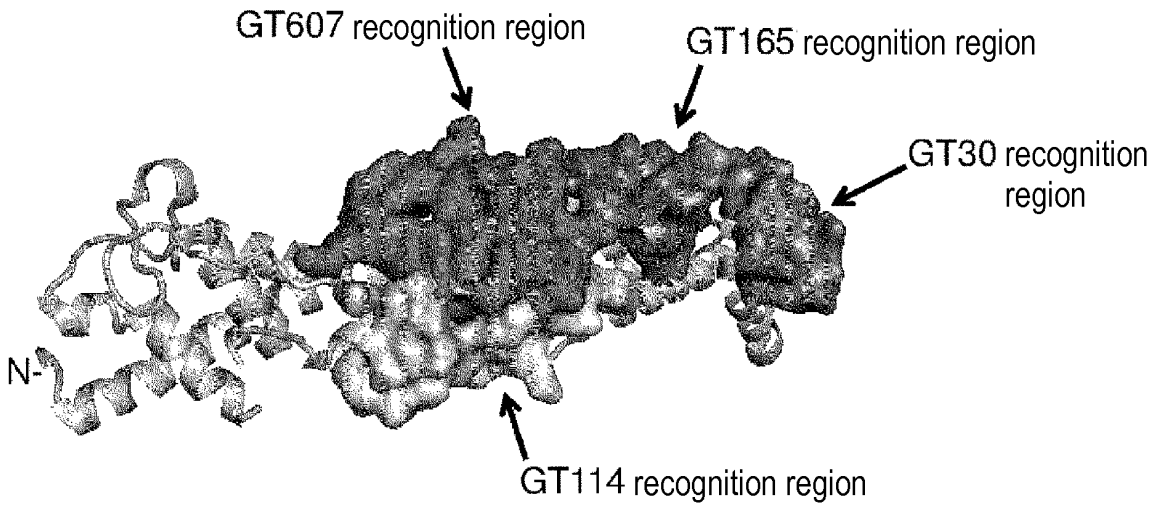
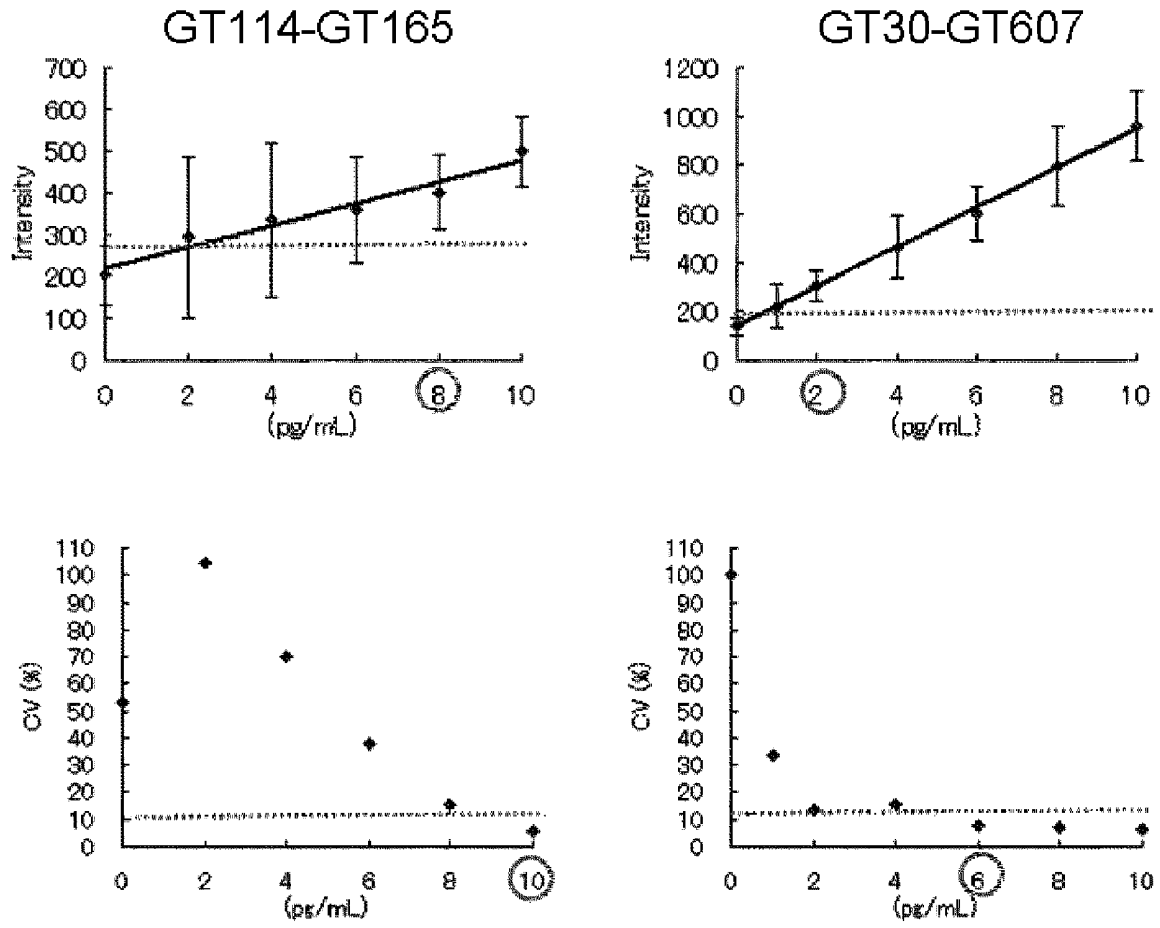

GT607 recognition region
GT165 recognition region
GT30 recognition region
N-
GT114 recognition region

Fig 10


GT114-GT165
Intensity
700
600
500
400
300
200
100
0
0
2
4
6
8
10
(pg/mL)
CV (%)
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
(pg/mL)
GT30-GT607
Intensity
1200
1000
800
600
400
200
0
0
2
4
6
8
10
(pg/mL)
CV (%)
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
(pg/mL)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2014/003409

A. CLASSIFICATION OF SUBJECT MATTER
G01N33/53(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2014
Kokai Jitsuyo Shinan Koho 1971-2014 Toroku Jitsuyo Shinan Koho 1994-2014

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus/JMEDPlus/JST7580(JDreamIII), PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	JP 2009-232848 A (Chugai Pharmaceutical Co., Ltd.), 15 October 2009 (15.10.2009), paragraphs [0030] to [0031], [0165] to [0169] & US 2007/0190599 A1 & EP 1674111 A1 & WO 2006/006693 A1	1-4,11-12 5-10
X A	WO 2006/038588 A1 (Perseus Proteomics Inc.), 13 April 2006 (13.04.2006), paragraph [0093]; claims & US 2008/0003623 A1 & EP 1813944 A1 & CN 101052878 A	1-4,11-12 5-10
X A	WO 2004/038420 A1 (Perseus Proteomics Inc.), 06 May 2004 (06.05.2004), example 2; claims & US 2006/0014223 A1 & EP 1548442 A1	1-4,11-12 5-10

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 September, 2014 (17.09.14)	30 September, 2014 (30.09.14)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2014/003409

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2013/070468 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA), 16 May 2013 (16.05.2013), entire text; all drawings (Family: none)	1-12

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO 2003100429 A **[0006]**
- WO 2004038420 A **[0006]**
- WO 2004023145 A **[0006]**
- WO 2009116659 A **[0006]**
- WO 2007081790 A **[0006]**
- WO 9411523 A **[0045]**
- EP 125023 A **[0049]**
- WO 9602576 A **[0049]**
- JP 4283227 B **[0050]**
- JP 4658926 B **[0050]**
- WO 2006006693 A **[0058] [0059] [0082]**

Non-patent literature cited in the description

- **LLOVET JM ; BURROUGHS A ; BRUIX J.** *Lancet,* 2003, vol. 362, 1907-17 **[0007]**
- **BOSCH FX ; RIBES J ; CLERIES R.** *Gastroenterology,* 2004, vol. 127, 5-16 **[0007]**
- **TAKENAKA K ; KAWAHARA N ; YAMAMOTO K ; KAJIYAMA K ; MAEDA T ; ITASAKA H ; SHIRABE K ; NISHIZAKI T ; YANAGA K ; SUGIMACHI K.** *Arch Surg,* 1996, vol. 131, 71-6 **[0007]**
- **CHENG AL ; CHEN Z ; TSAO CJ ; QIN S ; KIM JS et al.** Efficacy and safety of sorefanib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomized, double-blind, placebo-controlled trial. *Lancet Oncol.,* 2009, vol. 10, 25-34 **[0007]**
- **KUNKEL et al.** *Proc. Natl. Acad. Sci. USA,* 1985, vol. 82, 488-492 **[0017]**
- *Annu. Rev. Biophys. Biomol. Struct.,* 2006, vol. 35, 225-249 **[0017]**
- *Proc. Natl. Acad. Sci. U.S.A.,* 2003, vol. 100 (11), 6353-6357 **[0017]**
- **KABAT.** Sequences of Proteins of Immunological Interest. National Institute of Health, 1987 **[0019]**
- *J. Cell. Biol.,* 2003, vol. 163 (3), 625-635 **[0023]**
- **ATSCHUL et al.** *J. Molec. Biol.,* 1990, vol. 215, 403-410 **[0041]**
- **EBERT, K. M. et al.** *Bio/Technology,* 1994, vol. 12, 699-702 **[0046]**
- **KIM M.S. et al.** *Proc Natl Acad Sci USA,* 2011, vol. 108, 13112-13117 **[0067]**
- **SVENSSON G. et al.** *J Biol Chem,* 2012, vol. 287, 14040-14051 **[0067]**
- **HIPPO et al.** *Cancer Res.,* 2004, vol. 64, 2418-2423 **[0084]**

专利名称(译)	测量可溶性GPC3蛋白的方法		
公开(公告)号	EP3088890A1	公开(公告)日	2016-11-02
申请号	EP2014874331	申请日	2014-06-25
[标]申请(专利权)人(译)	杰瑟股份有限公司 JSR生命科学株式会社		
申请(专利权)人(译)	中外SEIYAKU株式会社 JSR株式会社 JSR生命科学株式会社		
当前申请(专利权)人(译)	中外SEIYAKU株式会社 JSR株式会社 JSR生命科学株式会社		
[标]发明人	OHTOMO TOSHIHIKO AMANO JUN ADACHI HIDEKI SUZUKI TSUKASA MIZUUCHI MOTOAKI YAMAGUCHI TETSUJI WAKUI SEIKI		
发明人	OHTOMO, TOSHIHIKO AMANO, JUN ADACHI, HIDEKI SUZUKI, TSUKASA MIZUUCHI, MOTOAKI YAMAGUCHI, TETSUJI WAKUI, SEIKI		
IPC分类号	G01N33/53		
CPC分类号	G01N33/57438 C07K16/303 G01N33/53 G01N2333/4722		
代理机构(译)	POWER，DAVID		
优先权	PCT/JP2013/007529 2013-12-24 WO		
其他公开文献	EP3088890A4		
外部链接	Espacenet		

摘要(译)

本发明涉及一种用于测定测试样品中可溶性GPC3蛋白的方法，包括使用结合存在于GPC3蛋白N末端区域中的不同表位的两种不同抗体。

ATGGAATGGATCTGGATCTTTCTCTTCATCCTGTCAGGAACTGCAGGTGTCCAATCC

CAGGTTCAGCTGCAGCAGTCTGGAGCTGAGCTGGCGAGGCCTGGGGCTTCAGTGAA

ACTGTCCTGCAGGGCTTCTGGCTACACCTTCACAAGCTATGGTATAAGCTGGATGAT

GCAGAGAACTGGACAGGGCCTTGAGTGGATTGGAGAGATTTATCCTAGAAGTGGTA

TTACTTACTACAATGAGAAGTTCAAGGGCAAGGCCACACTGACTGCAGACAAATCC

TCCAGCACAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCAGTCTAT

TTCTGTGCAAGAGATGTCTCTGATGGTTACCTTTTTCCTTACTGGGGCCAAGGGACTC

TGGTCACTGTCTCTGCAGCCAAA