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(54) **BIOMARKER FOR DIAGNOSIS OF LIVER DISEASE**

(57) Disclosed are: a marker for the diagnosis of a liver disease, which can determine the disease in a simple manner; an antibody directed against the marker; a diagnostic agent; a diagnosis method; and a method for marker detection in blood or serum. Proteome analysis revealed that quantities of the full-length kininogen and three partial peptides thereof (sequence A: position-440 to position-456, sequence B: position-439 to position-456, and sequence C: position-43 to position-456) in sera of patients with non-alcoholic fatty liver disease are signif-

icantly different from those in sera of healthy individuals; and a diagnostic agent and a detecting method for the non-alcoholic fatty liver disease that can be conveniently used for medical examination are established. The use of a combination of a kininogen-based marker and a C4-based marker (the full length sequence or partial peptides thereof) enables identification of chronic hepatitis and an asymptomatic virus carrier, as well as non-alcoholic fatty liver disease.

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Description

TECHNICAL FIELD

5 **[0001]** The present invention relates to a biomarker for diagnosis of liver diseases (hereinafter referred to as marker (s)), more specifically, to a marker, an antibody, a diagnostic agent, and a detecting method for discrimination between a non-alcoholic fatty liver disease (hereinafter referred to as "NAFLD"), chronic hepatitis, and an asymptomatic carrier (hereinafter referred to as "ASC").

10 BACKGROUND ART

[0002] In general, it is said that NAFLD includes simple steatosis (hereinafter, "SS") and non-alcoholic steatohepatitis (hereinafter referred to as "NASH"), which is developed from SS. NASH has a possibility to progress to poor prognosis diseases such as cirrhosis, and hepatocellular carcinoma. However, at present, there are no useful diagnostic markers for diagnosing NAFLD. NAFLD is usually diagnosed by ultrasonography, but pathological findings of liver biopsy are necessary for its correct diagnosis. The liver biopsy imposes a heavy burden on patients and lacks convenience. Accordingly, it is unsuitable for medical examination of lifestyle-related diseases. If NAFLD is detected at an earlier stage, NAFLD can be immediately prevented from progressing and can be treated, which is, needless to say, very advantageous.

15 **[0003]** Regarding a kininogen, Japanese Unexamined Patent Application Publication No. 04-110660 (Patent Literature 1) discloses a liver disease diagnostic agent composed of a kininogen/calpain complex. Patent Literature 1 states that the complex diagnostic agent is useful for diagnosing liver diseases such as chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, hepatitis A, and fulminant hepatitis, but not NAFLD. Furthermore, there is no description about the use of full-length kininogen or a specific biological material derived from the kininogen as a NAFLD diagnostic marker.

20 **[0004]** C. Cordova, et al. (Non-Patent Literature 1) report on that the level of kininogen decreases in patients with, chronic hepatitis or liver cirrhosis, compared to that in healthy individuals, but do not mention or suggest any relationship between hepatic steatosis (including NAFLD) and the kininogen.

25 **[0005]** Thus, a marker is desired for discrimination between, for example, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma that have been developed from NAFLD. A known marker of such a type is complement C4, which is a protein generated in the liver and present in the serum and gets involved in immunoreaction and prevention of infection. Japanese Unexamined Patent Application Publication No. 2006-300689 (Patent Literature 2), a report on the use of C4 as a liver disease marker, states that the marker can be detected in patients with chronic hepatitis or liver cirrhosis, but not in healthy individuals or that the marker can be detected in healthy individuals and patients with chronic hepatitis, but not in patients with liver cirrhosis. Patent Literature 2, however, does not mention the discrimination of healthy individuals, patients with NAFLD, and patients with chronic hepatitis, including ASCs of hepatitis virus, from one another. Furthermore, Japanese Unexamined Patent Application Publication No. 2006-308533 (Patent Literature 3) states that the presence or absence or the amounts of complement C4 and its partial peptides are different between healthy individuals and liver cancer patients and are therefore useful as markers for discriminating patients with liver cancer from healthy individuals. Patent Literature 3, however, does not mention the discrimination of healthy individuals, patients with NAFLD, patients with chronic hepatitis, and ASCs from one another at all.

35 **[0006]** Furthermore, Dumestre-Perard, et al. (Non-Patent Literature 2) report on a method for determining the results of treatment of chronic hepatitis caused by hepatitis C virus through monitoring the correlation between C4 and a rheumatoid factor during the process of treating with, for example, interferon or ribavirin, but do not mention the discrimination of healthy individuals, patients with NAFLD, patients with chronic hepatitis, and ASCs from one another at all.

45 Patent Document 1: Japanese Unexamined Patent Application Publication No. 04-110660

Patent Document 2: Japanese Unexamined Patent Application Publication No. 2006-300689

Patent Document 3: Japanese Unexamined Patent Application Publication No. 2006-308533

Non Patent Document 1: C Cordova, et al., "Hageman factor, high molecular weight kininogen, and prekallikrein in chronic liver disease", J Clin Pathol, 39, 1003-1005, (1986)

50 Non Patent Document 2: Dumestre-Perard, et al., Clin Exp Immunol, 127, 131-136, (2002)

DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

55 **[0007]** It is an object of the present invention to provide a marker, an antibody, a diagnostic agent, and a detecting method that may be conveniently used for identification of NAFLD.

[0008] It is an object of the present invention to provide a marker, an antibody, a diagnostic agent, and a detecting

method that may be conveniently used for identification of chronic hepatitis and an ASC.

[0009] It is an object of the present invention to provide a diagnostic agent and a detecting method that are suitable for the purpose of combined use of the above-mentioned markers.

5 MEANS FOR SOLVING THE PROBLEMS

[0010] The present invention, which achieves the above-mentioned objects, includes the following aspects:

[0011] Aspect 1: A biomarker for identification of NAFLD, the biomarker comprising a full-length high-molecular-weight kininogen and/or a partial peptide derived from the high-molecular-weight kininogen, wherein the partial peptide derived from the high-molecular-weight kininogen is any one of the following sequences A, B, and C:

sequence A: Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln,

sequence B: His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln, and

sequence C: Lys His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln;

Aspect 2: The biomarker according to Aspect 1, wherein the full-length high-molecular-weight kininogen and/or the partial peptide derived from the high-molecular-weight kininogen include a modified form thereof;

[0012] Aspect 3: An antibody for identification of NAFLD, the antibody recognizing at least one of the biomarkers according to Aspect 1 or 2 as an antigen;

[0013] Aspect 4: The antibody according to Aspect 3, wherein the antibody is a polyclonal antibody that is obtained by immunizing a rabbit with at least one selected from the group consisting of the full-length kininogen and the partial peptide of the sequence A, the partial peptide of the sequence B, and the partial peptide of the sequence C;

[0014] Aspect 5: The antibody according to Aspect 3, wherein the antibody is a monoclonal antibody that is obtained by immunizing a mouse with at least one selected from the group consisting of the full-length kininogen and the partial peptide of the sequence A, the partial peptide of the sequence B, and the partial peptide of the sequence C;

[0015] Aspect 6: A diagnostic agent for identification of NAFLD, the agent comprising at least one selected from the group consisting of the biomarkers according to Aspect 1 or 2 and the antibodies according to any of Aspects 3 to 5;

[0016] Aspect 7: A biomarker for identification of hepatitis and an ASC, the biomarker being complement C4 and/or a partial peptide derived from C4, wherein the partial peptide derived from C4 is at least one selected from the group consisting of C4a, C4b, C4c, and the following sequences D and E:

sequence D: Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile, and

sequence E: Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile Arg;

Aspect 8: An antibody for identification of chronic hepatitis and an ASC, the antibody recognizing at least one of the biomarkers according to Aspect 7 as an antigen;

[0017] Aspect 9: The antibody according to Aspect 8, wherein the antibody is a polyclonal antibody;

[0018] Aspect 10: The antibody according to Aspect 8, wherein the antibody is a monoclonal antibody;

[0019] Aspect 11: A diagnostic agent for identification of chronic hepatitis and an ASC, the agent comprising at least one selected from the group consisting of the biomarker according to Aspect 7 and the antibodies according to any of Aspects 8 to 10;

[0020] Aspect 12: A diagnostic agent for identification of NAFLD, chronic hepatitis, and an ASC, the agent comprising a combination of at least one of the biomarkers, the antibodies, and the diagnostic agent according to any of Aspects 1 to 6 and at least one of the biomarker, the antibodies, and the diagnostic agent according to any of Aspects 7 to 11;

[0021] Aspect 13: A detecting method for identification of NAFLD, the method using at least one of the biomarkers, the antibodies, and the diagnostic agent according to any of Aspects 1 to 6;

[0022] Aspect 14: A detecting method for identification of chronic hepatitis and an ASC, the method using at least one of the biomarkers, the antibodies, and the diagnostic agent according to any of Aspects 7 to 11;

[0023] Aspect 15: A detecting method for identification of NAFLD, chronic hepatitis, and an ASC, the method including a combination of the detecting method according to Aspect 13 and the detecting method according to Aspect 14;

[0024] Aspect 16: The detecting method according to any of Aspects 13 to 15, the method using an antibody that recognizes the high-molecular-weight kininogen in a sample but does not recognize any of the partial peptide of the sequence A, the partial peptide of the sequence B, and the partial peptide of the sequence C;

[0025] Aspect 17: The detecting method according to Aspect 16, wherein the detection is performed by ELISA; and

[0026] Aspect 18: The diagnostic agent according to Aspect 6 or 12, wherein the antibody recognizes the high-molecular-weight kininogen in a sample but does not recognize any of the partial peptide of the sequence A, the partial peptide of the sequence B, and the partial peptide of the sequence C.

[0027] Hereinafter, in the present invention, the term "kininogen-based marker" means a full-length kininogen, a partial

peptide thereof consisting of the sequence A, B, or C (hereinafter referred to as partial peptide A, B or C), or a partial peptide belonging to the kininogen D5 region described below, unless specifically stated otherwise. In addition, in the present invention, the term "C4-based marker" means complement C4 including C4A or C4B, C4a, C4b, or C4c, or a partial peptide consisting of the sequence D or E (hereinafter referred to as partial peptide D or E), unless specifically stated otherwise.

[0028] In addition to the above aspects, the present invention may preferably include the following embodiments:

a) A kininogen-based marker comprising the full-length high-molecular-weight kininogen according to Aspect 1 or 2, which decreases or disappears in a biological specimen collected from a patient with NAFLD compared to that from a healthy individual;

b) A kininogen-based marker being at least one selected from the group consisting of the partial peptides A, B, and C according to Aspect 1 or 2, used for determining that a subject is suffering from NAFLD when the marker increases in a biological specimen collected from a patient with NAFLD compared to that from a healthy individual;

c) A detecting method using a kininogen-based marker, in which a combination of the full-length high-molecular-weight kininogen with at least one selected from the group consisting of the partial peptides A, B, and C is used, and a subject is determined as suffering from NAFLD when the full-length marker decreases and at least one selected from the group consisting of the partial peptides A, B, and C increases compared to those in a healthy individual;

d) A C4-based marker contained in the full-length complement C4 according to Aspect 7, which decreases or disappears in a biological specimen from a patient with chronic hepatitis or an ASC compared to that in a healthy individual or a patient with any other liver disease;

e) A C4-based marker derived from complement C4, being at least one selected from the group consisting of C4a, C4b, and C4c and the partial peptides D and E according to Aspect 7, used for determining that a subject is suffering from chronic hepatitis or is an ASC when the marker increases in a biological specimen from a patient with chronic hepatitis or an ASC compared to that from a healthy individual;

f) A detecting method using a C4-based marker, in which a combination of the full-length complement C4 with at least one selected from the group consisting of the partial peptides C4a, C4b, C4c, D and E derived from the C4 is used, and a subject is determined to be suffering from chronic hepatitis or is an ASC when the C4-based marker contained in the full-length decreases and at least one selected from the group consisting of partial peptides C4a, C4b, C4c, D, and E increases compared to those in a healthy individual;

g) A detecting method using both the kininogen-based marker and the C4-based marker for identification of NAFLD, chronic hepatitis, and an ASC according to Aspect 14, comprising detecting a decrease or disappearance of the full-length high-molecular-weight kininogen and/or an increase or expression of at least one of the partial peptides A, B, and C; and detecting a decrease of the C4-based marker contained in the full-length C4 and an increase or expression of at least one of the partial peptides C4a, C4b, C4c, D, and E derived from C4;

h) A detecting method using both the kininogen-based marker and the C4-based marker for identification of a healthy individual, a patient with NAFLD, a patient with chronic hepatitis, and an ASC according to Aspect 14, comprising detecting an increase or expression of the full-length high-molecular-weight kininogen and/or a decrease or disappearance of at least one of the partial peptides A, B, and C; and detecting an increase or expression of the C4 and/or a decrease or disappearance of at least one of the partial peptides C4a, C4b, C4c, D, and E; and

i) A detecting method using both the kininogen-based marker and the C4-based marker for identification of a healthy individual, a patient with NAFLD, a patient with chronic hepatitis, and an ASC according to Aspect 14, comprising detecting an increase or expression of the full-length high-molecular-weight kininogen and/or detecting a decrease or disappearance of at least one of the partial peptides A, B, and C; and also detecting a decrease or disappearance of the C4 and/or an increase or expression of at least one of the partial peptides D and E.

ADVANTAGES OF THE INVENTION

[0029] The present invention is advantageous in that not only diagnosis of NAFLD but also diagnosis of progress thereafter can be expected.

[0030] (1) Since the level of expression of the kininogen-based marker is largely different between patients with NAFLD and healthy individuals, NAFLD may be conveniently and correctly diagnosed without liver biopsy imposing heavy burden on patients.

[0031] (2) Since the partial peptides A, B, and C of the kininogen include a large number of polar amino acids in their sequences and are thereby excellent in antigenicity, useful antibodies may be easily produced, and a kit thereof may be produced. In particular, the partial peptide C is excellent in recognition by an antibody and is therefore preferred.

[0032] (3) This enables insurance medical care for many people in pre-disease conditions, including prevention of lifestyle-related diseases such as metabolic syndromes, as a result.

[0033] (4) A multi-marker system composed of a combination of the kininogen-based marker and the C4-based marker

enables diagnosis of the progress of a patient with NAFLD to chronic hepatitis or detection of an ASC, and, thereby, it is easy to determine a treatment principle according to the progress of a liver disease at an early stage.

BRIEF DESCRIPTION OF THE DRAWINGS

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[0034]

Fig. 1 is a schematic diagram illustrating domain structures of amino acid sequences of kininogens.

Fig. 2 is a schematic diagram illustrating domain structures of complement C4 (C4A and C4B) and its decomposition products.

Fig. 3 includes graphs showing spectral comparison of patients with NAFLD and healthy individuals using ClinProt; Graph (A) shows typical spectral patterns of a patient with NAFLD and a healthy individual measured by a linear mode of Autoflex; Graph (B) shows an enlarged view of the region near 1800 to 2300 m/z in Graph (A), and three peaks observed in the patient with NAFLD are peaks at 1942 m/z, 2079 m/z, and 2207 m/z from the left; the vertical axis represents peak intensity, and the horizontal axis represents molecular weight in Graphs (A) and (B); and Graph (C) plots peak intensities of the patients with NAFLD and the healthy individuals at 1942 m/z (a), 2079 m/z (b), and 2207 m/z (c).

Fig. 4 includes graphs showing MS/MS spectra of marker candidate peptides at 1942 m/z Graph (A), 2079 m/z Graph (B), and 2207 m/z Graph (C).

Fig. 5 is a photograph showing the results of Western blotting using RABBIT ANTI HUMAN HMW-KINNOGEN (anti-kininogen polyclonal antibody) manufactured by AbD Serotec.

Fig. 6 is a photograph showing the results of Western blotting using Rabbit-anti-human-kininogen (anti-kininogen polyclonal antibody) manufactured by Sigma-Aldrich Japan.

Fig. 7 is a photograph showing the results of Western blotting using HMW Kininogen Light Chain antibody [1.B.709] (anti-kininogen monoclonal antibody) manufactured by GeneTex.

Fig. 8 is a photograph showing the results of Western blotting using Anti-human-kininogen/Kininostatin Antibody (anti-kininogen polyclonal antibody) manufactured by R&D Systems.

Fig. 9 is a graph showing the results of measurement of 1942 m/z (a), 2079 m/z (b), and 2207 m/z (c) peaks by immunoprecipitation using anti-kininogen polyclonal antibodies (an AbD Serotec product and a Sigma-Aldrich contract product), the vertical axis represents peak intensity, and the horizontal axis represents molecular weight.

Fig. 10 is a graph showing the results of measurement of 1942 m/z (a), 2079 m/z (b), and 2207 m/z (c) peaks by immunoprecipitation using anti-kininogen monoclonal antibodies (products of GeneTex and R&D Systems), the vertical axis represents peak intensity, and the horizontal axis represents molecular weight.

Fig. 11 includes graphs showing spectral comparison of patients with NAFLD and healthy individuals using a protein chip; Graph (A) shows typical spectral patterns of a patient with NAFLD and a healthy individual measured by SELDI; Graph (B) shows an enlarged view of a region near 1800 to 2300 m/z in Graph (A), and three peaks observed in the patient with NAFLD are peaks at 1942 m/z, 2079 m/z, and 2207 m/z from the left; and Graph (C) plots peak intensities of the patients with NAFLD and the healthy individuals at 1942 m/z (a), 2079 m/z (b), and 2207 m/z (c).

Fig. 12 includes graphs showing spectral comparison of ASCs and healthy individuals using a protein chip; Graph (A) shows typical spectral patterns of an ASC and a healthy individual measured by SELDI; Graph (B) shows an enlarged view of the region near 1600 to 2100 m/z in Graph (A), and two peaks observed in the ASC are peaks at 1738 m/z and 1896 m/z from the left; the vertical axis represents peak intensity, and the horizontal axis represents molecular weight in Graphs (A) and (B); and Graph (C) plots peak intensities of the ASCs and the healthy individuals at 1738 m/z (a) and 1896 m/z (b).

Fig. 13 includes graphs showing MS/MS spectra of marker candidate peptides identified in ASCs using a database of Sw-issProt at 1738 m/z (A) and 1896 m/z (B).

Fig. 14 includes the results of analysis of peaks at 1738 m/z (a) and 1896 m/z (b) using a ClinProt system.

Fig. 15 is a graph showing the results of detection of the C4a protein level in sera by ELISA (BD OptEIA ELISA Kit).

Fig. 16 includes graphs showing spectral comparison of ASCs and healthy individuals using ClinProt; Graph (A) shows typical spectral patterns of an ASC and a healthy individual measured by a linear mode of Autoflex; Graph (B) shows an enlarged view of the region near 1600 to 2000 m/z in Graph (A), and two peaks observed in the ASC are peaks at 1738 m/z and 1896 m/z from the left; the vertical axis represents peak intensity; and Graph (C) plots peak intensities of the ASCs and the healthy individuals at 1738 m/z (a) and 1896 m/z (b).

Fig. 17 is a schematic diagram (multimarker system) of a flow for identification of liver diseases using kininogen-based and C4-based markers of the present invention.

Fig. 18 is a graph showing discrimination between patients with NAFLD and healthy individuals by kininogen-based ELISA.

BEST MODE FOR CARRYING OUT THE INVENTION

5 [0035] Full-length kininogen or its partial peptide includes post-translational modified forms of a high-molecular-weight Kininogen, such as glycosylated forms. That is, the full-length high-molecular-weight kininogen is a protein consisting of a sequence of 644 amino acids shown in Sequence Listing 1. The kininogen gene contains 11 exons (E1 to E11) and, as shown in Fig. 1, are roughly classified into a high-molecular-weight kininogen and a low-molecular-weight kininogen due to a difference in splicing in transcription (Robert W. Colman, et al., "Contact System: A Vascular Biology Modulator With Anticoagulant, Profibrinolytic, Antiadhesive, and Proinflammatory Attributes", Blood, 90(10), 3819-3833, (1997)). The high-molecular-weight kininogen is a kininogen (644 amino acids: about 71 kDa, its glycosylated form: about 120 kDa) of Accession No. P01042 (SwissProt: Expercy) and consists of six functional domains (D1 to D6), whereas the low-molecular-weight kininogen consists of five domains (D1 to D5). In the both kininogens, D1 to D3 transcribed and translated from the same exons (E1 to E9) have the same amino acid sequence, while their sequences from D4 to the C-terminal side are different from each other because of a difference in mRNA splicing. In many cases, these kininogens include their post-translational modified forms, such as glycosylated forms. The partial peptides of the present invention have sequences A, B, and C as shown in Sequence Listings 2 to 4, and they are peptides which belong to the D5 region of the high-molecular-weight kininogen.

[Antibody for detecting markers]

20 [0036] In the identification of NAFLD according to the present invention, the above-mentioned full-length kininogen as well as partial peptides A, B, and C in sera may be directly used as markers. However, it is convenient and desirable to produce an antibody recognizing such markers by a common method and use it for diagnosis. The antibody may be produced by a known technology. Examples of the antibody include, but are not limited to, a polyclonal antibody, a monoclonal antibody, an F(ab) fragment, an Fv fragment, a single-chain antibody, a chimera antibody, a humanized antibody, and a Fab expression library and also include an antigen-binding protein. The monoclonal antibody is preferred in order to specifically recognize the kininogen.

25 [0037] The polyclonal antibody of the present invention is produced by administering the full-length kininogen or the partial peptide A, B, or C together with an immune-adjuvant to a host animal, such as rabbit, mouse, rat, guinea pig, or goat, for immunization. According to need, the immunogen may be conjugated to a high-molecular-weight carrier and be used for immunization. The immunization may be performed by, for example, repeating intracutaneous administration to many sites or direct administration to lymph nodes. Through the immunization, the IgM class is mainly produced in the primary immune response, and the IgG class is mainly produced in the secondary immune response. In order to extract the produced antibodies, a serum separated from the collected blood is subjected to ammonium sulfate precipitation according to need and then purification. The purification may be performed by dialysis, gel filtration, Protein A/G column chromatography, or antigen column chromatography.

30 [0038] The monoclonal antibody used in the method of the present invention can be prepared by a common method that includes immunizing a mouse with the full-length kininogen or peptide C of the present invention, fusing antibody-producing cells with myeloma cells, selecting a hybridoma that produces an anti-kininogen monoclonal antibody from the resulting hybridomas, and collecting the monoclonal antibody produced by culturing the hybridoma.

35 [0039] A typical method for producing a monoclonal antibody will be described below. A monoclonal antibody can be prepared by producing hybridomas through cell fusion of antibody-producing cells obtained from an animal immunized with an antigen and myeloma cells, and selecting a clone that produces an antibody that specifically recognizes the antigen from the resulting hybridomas.

40 [0040] The antigen used for immunization of an animal is the full-length kininogen or the partial peptide A, B, or C. The antigen is administered to a host animal such as mouse, rat, guinea pig, horse, monkey, rabbit, goat, sheep, or pig. Other immunized animals such as chicken can be also used. The immunization may be performed by any known method, but is mainly performed by, for example, intravenous injection, intracutaneous injection, or intraperitoneal injection. The immunization interval is not particularly limited and ranges from several days to several weeks, and immunization is preferably performed at intervals of 4 to 21 days.

45 [0041] Antibody-producing cells are collected after a predetermined period from the final immunization. Examples of the antibody-producing cells include spleen cells, lymph node cells, and peripheral blood cells. Spleen cells are usually used. The antigen is used, for example, in an amount of 100 μ g per mouse in each immunization.

50 [0042] The antibody titer in blood of an immunized animal or the antibody titer in culture medium supernatant of antibody-producing cells is measured for confirmation of the immune response level of the immunized animal or selection of a target hybridoma from the cells after cell fusion treatment. Examples of the method for detecting the antibody include known assays such as enzyme immunoassay (EIA), radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (hereinafter referred to as "ELISA").

55 [0043] The myeloma cells to be fused with the antibody-producing cells are derived from various animals such as

mouse, rat, and human, and a cell line that is usually available to those skilled in the art is used. Cell lines having the following characteristics are used: the lines have drug resistance, cannot survive in a selection medium (for example, HAT medium) in their unfused state, and can survive in their fused state. In general, an 8-azaguanine resistant line is often used, and this cell line is deficient in hypoxanthine-guanine-phosphoribosyltransferase and thus cannot grow in a hypoxanthine-aminopterin-thymidine (HAT) medium.

5 [0044] Examples of the myeloma cell include P3x63Ag8.653, P3x63Ag8U.1, NS-1, MPC-11, SP2/0, F0, S194, and R210.

10 [0045] The antibody-producing cell may be obtained from, for example, a spleen cell or a lymph node cell. That is, the desired antibody-producing cell is prepared by extracting or collecting, for example, the spleen or the lymph node from an animal such as mentioned above, fracturing the tissue, suspending the resulting homogenate in a medium or buffer such as PBS, DMEM, or RPMI-1640, and subjecting the suspension to filtration through a stainless mesh or the like and then centrifugation.

15 [0046] Then, the myeloma cell and the antibody-producing cell are fused. The cell fusion is performed by bringing the myeloma cell into contact with the antibody-producing cell in a medium for culturing animal cells, such as an MEM, DMEM, or RPMI-1640 medium, for example, at a mixing ratio of 1:1 to 1:10 in the presence of a fusion-accelerating agent at 30 to 37°C for 1 to 15 minutes. In order to accelerate the cell fusion, a fusion accelerating agent or a fusing virus, such as a polyethylene glycol or polyvinyl alcohol having an average molecular weight of 1000 to 6000 or Sendai virus, may be used. In addition, the antibody-producing cell and the myeloma cell may be fused using a commercially available cell fusion apparatus utilizing electric stimulation (for example, electroporation).

20 [0047] The intended hybridoma is selected from the cells after cell fusion treatment. The selection is performed by, for example, selective proliferation of cells using a selection medium. That is, the cell suspension is diluted with an appropriate medium and then is spread on a microtiter plate. A selection medium (such as HAT medium) is added to each well, and then, the cells are cultured by appropriately replacing the selection medium. As a result, survived cells are obtained as hybridomas.

25 [0048] The screening of the hybridoma is performed by, for example, a limiting dilution method or a fluorescence-activated cell sorting method, in order to obtain a monoclonal antibody-producing hybridoma finally. Examples of the method for harvesting the monoclonal antibody from the resulting hybridoma include usual cell culturing and ascitic fluid formation. In the cell culturing, the hybridoma is cultured, for example, in a medium for culturing animal cells, such as RPMI-1640 or MEM containing 10 to 20% fetal bovine serum, or a serum-free medium, under usual culture conditions (for example, at 37°C, 5% CO₂) for 2 to 14 days, and the antibody is collected from the culture supernatant. In the ascitic fluid formation, the hybridoma is administered in the abdominal cavity of the same type animal as a mammal from which the myeloma cells are derived, and a large amount of the hybridoma is proliferated. Then, the ascitic fluid or the serum is collected one to four weeks later.

30 [0049] In the extraction of the antibody, if purification of the antibody is necessary, any known purification process such as ammonium sulfate precipitation, ion exchange chromatography, affinity chromatography, or a combination thereof is selected.

35 [0050] The anti-kininogen antibody of the present invention may also be selected from commercially available or publicly reported polyclonal or monoclonal antibodies recognizing the kininogen of the present invention or its biological decomposition product, for example, the partial peptide A, B, or C, in addition to the antibody produced by the above-mentioned method of the present invention. Alternatively, an antibody may be newly produced by the above-mentioned method.

40 [0051] The polyclonal antibody is preferably produced by immunizing a rabbit with at least one selected from the group consisting of the full-length kininogen, the partial peptide A, the partial peptide B, and the partial peptide C. For example, preferred are rabbit-anti-hunrtan-HMW-kininogen (RABBIT ANTI HUMAN HMW-KININOGEN 5575-4957, manufactured by AbD Serotec) and rabbit-anti-human-kininogen that is specific to K438-Q456 peptides (Sigma-Aldrich Japan special order product). The monoclonal antibody is preferably produced by immunizing a mouse with at least one selected from the group consisting of the full-length kininogen, the partial peptide A, the partial peptide B, and the partial peptide C. Specifically, preferred are (mouse-monoclonal) HMW Kininogen Light Chain antibody [1.B.709] (manufactured by Gene-Tex Inc.) and (mouse-monoclonal) Anti-human-kininogen/Kininostatin antibody (manufactured by R&D Systems) specific to K438-S531 peptides.

45 [0052] A diagnostic agent may be prepared using the above-described marker of the present invention by a common process. Furthermore, a kit with the above-described various antibodies may be used as for convenient diagnosis.

50 [0053] In the present invention, a comparison was made between healthy individuals and NAFLD patients with respect to peak intensities in spectra of proteomic analysis on sera, and candidates of markers for NAFLD diagnosis are determined on the basis of statistic test values or absolute values of peak intensities or a significant difference therebetween. The NAFLD diagnostic marker in the present invention is a protein or a partial peptide identified from the thus selected marker candidates. Various well-known methods of qualitative and quantitative determination of protein for those skilled in the art may be used without particular limitation. For example, proteomic analysis involving gel electrophoresis such

as two-dimensional electrophoresis, shotgun analysis by LC-MS, or a method using an antibody against a specific biological material may be used. Specifically, immunochemical detection processes using an antibody, for example, ELISA, radioimmunoassay (RIA), a method using an antibody chip (protein chip in which antibodies are densely immobilized on a surface of a solid-phase such as glass), Western blotting, or immunostaining of a tissue section, may be used. Furthermore, in mass spectrometric (MS) detection, for example, after fractionation on a chip or with a column, which will be described in detail below, the protein on the chip or in the eluate from the column is subjected to molecular weight measurement by MS. Liquid chromatography or dot blotting may also be employed.

[0054] Regarding the full-length kininogen and its partial peptides A, B, and C, which serve as the NAFLD diagnostic markers according to the present invention, the detection, selection, and identification thereof will be described below.

[0055] Sera of healthy individuals or patients with NAFLD are prepared by leaving the collected blood to stand or centrifuging the blood. In order to detect the protein or the partial peptide in the sera, a ClinProt system (Bruker Daltonics) or a Protein Chip system (Bio-Rad) is used. In these systems, exchangers having surfaces modified with various functional groups, for example, a cation exchanger and a copper-ion exchanger (IMAC-Cu) may be used alone or in combination. These exchangers can capture proteins and partial peptides having affinities to the functional groups.

[0056] Preferably, the ClinProt system is used together with ClinProt Profiling Kit 100MB-WCX (Bruker Daltonics, hereinafter referred to as "WCX beads"), which is a bead type cation exchanger exclusive for the system. The captured proteins and partial peptides are used as test specimens and subjected to mass spectrometry to obtain peaks and peak intensities on a spectrum.

[0057] The WCX beads are used in accordance with the manual of the kit. First, the WCX beads are mixed with the prepared serum. The beads and the WCX binding solution provided in the kit are mixed in a washing vessel, for example, in a PCR tube, and the serum is added thereto, followed by leaving to stand (incubation) for a predetermined time. After the leaving to stand, the supernatant is removed using a pipette or any other tool. The WCX beads are washed, usually, two or more times to give a ClinProt eluate.

[0058] The resulting ClinProt eluate is mixed with, for example, a mixed solution (hereinafter referred to as "CCA solution") of α -cyano-4-hydroxycinnamic acid (Bruker Daltonics, hereinafter referred to as "CCA") and an organic solvent. A predetermined amount of the CCA solution is placed dropwise on a thin film, followed by leaving to stand for a predetermined time for crystallizing the proteins and the partial peptides in the eluate and CCA to give a test specimen for mass spectrometry. From the viewpoint that the target protein in the test specimen is not required to be subjected to complicated processes such as purification and can be directly identified by MS/MS ion search, a peak intensity of 3000 m/z or less is desirable.

[0059] Also, in cases of other protein chip systems, the same procedures are basically performed in accordance with their manuals. Examples of the exchanger include a cation exchange chip, a reversed-phase chip, and a metal modify chip, which may be used alone or in combination. The concentration of the serum to be treated with a protein chip is preferably 1% (vol/vol) or more from the viewpoint of obtaining a satisfactory MS/MS spectrum. The peak sensitivity may be improved by controlling the concentration of the serum to be treated.

[0060] The ionization principle employed in the mass spectrometry is preferably matrix-assisted laser desorption/ionization (hereinafter referred to as "MALDI") from the viewpoint of convenience in sample preparation. The mass separation principle employed is preferably a time-of flight type (hereinafter referred to as "TOF"). Therefore, a preferred mass spectrometer is a MALDI-TOF-MS, and more specifically, an Autoflex TOF-TOF (Bruker Daltonics, hereinafter referred to as "Autoflex") or Ultraflex TOF-TOF apparatus (Bruker Daltonics). The detecting procedure may be either a linear mode or a reflector mode, and the linear mode is preferred from the viewpoint of detection sensitivity. In addition, a protein chip system consisting of a protein chip and a surface-enhanced time-of-flight mass spectrometer (Bio-Rad, SELDI-TOF-MS, hereinafter referred to as "SELDI") may be used.

[0061] For example, in the use of Autoflex, first, the measurement is performed in the linear mode to obtain a mass spectrum. The linear mode measurement, as shown in Table 1 of Example 1 described below, produces a large number of peaks having different intensities between a healthy individual serum and an NAFLD patient serum. In the present invention, marker candidate proteins and partial peptides are selected from these peaks based on a statistical test, the absolute values of the peak intensities, or combination thereof. In comparison of a plurality of mass spectra, peaks having m/z values that are in agreement with each other within an error of 0.1% may be recognized as being derived from the same molecule. The following three partial peptides having sequences identified in the present invention are candidates of the NAFLD diagnostic markers of the present invention selected in accordance with the above-mentioned criteria: partial peptide A (approximately 1942 m/z), partial peptide B (approximately 2079 m/z), and partial peptide C (approximately 2207m/z).

[0062] In the present invention, the kininogen that is a candidate of these markers is identified by, for example, MS/MS analysis. The test specimen for the identification may be prepared by a thin film technique. For example, a saturated acetone solution of CCA is applied to the anchor surface of an anchor chip in advance to form a thin film therefrom. Then, about 1 μ L of a WCX bead eluate of an NAFLD patient serum is placed dropwise on the thin film, followed by leaving to stand for about 5 minutes to crystallize the proteins and partial peptides in the eluate and CCA. The crystal

is then washed with 3 μ L of 0.1% trifluoroacetic acid (hereinafter referred to as "TFA") around three times for desalination.

[0063] In acquisition of an MS/MS spectrum, for example, high-accuracy measurement is performed in the reflector mode of Autoflex to obtain molecular weights of a target peak (parent ion) and their fragments (ions of partial peptides). The correction (calibration) of the molecular weight may be performed by peptide calibration standard 2 (Bruker Daltonics, hereinafter referred to as "PCS-2"). Based on the observed MS/MS spectrum, a peak list of the parent ions and their fragment ions may be made using BioTools (Bruker Daltonics), and these peaks may be identified by MS/MS ion search of Mascot search (Matrix Science).

[0064] As shown in Fig. 3, in addition to the parent ion peaks (1942, 2079, and 2207 m/z), a large number of ion peaks of partial peptides are generally detected. A peak list is prepared based on these spectrum, and the partial peptides come from the peaks are identified by MS/MS ion search. As a result, it was revealed that all the peak (A) at 1942 m/z, the peak (B) at 2079 m/z, and the peak (C) at 2207 m/z of the three candidates of the NAFLD diagnostic markers of the present invention correspond to the following partial peptides belonging to the domain D5, which is a part of the high-molecular-weight kininogen.

[0065]

Sequence A: Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln (440th to 456th).

Sequence B: His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln (439th to 456th), and

Sequence C: Lys His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln (438th to 456th).

[0066] The diagnosis of NAFLD using the marker of the present invention may be performed by the following process. For example, in the case of mass spectrometry, a threshold value is set using a cut-off value calculated by adding the standard deviation to the average of the peak intensities of healthy individuals and is used for diagnosing whether a subject is NAFLD or not. In the cases where each partial peptide A, B, or C of the kininogen is used alone as a marker for diagnosing NAFLD, diagnosis with high sensitivity (correct-positive rate) and specificity (correct-negative rate) may be performed through determination of the sensitivity and the specificity by a common method. For example, as shown in Table 1, 40 subjects among the 44 NAFLD subjects were diagnosed as NAFLD (sensitivity: 90.99%), and 21 subjects among the 24 healthy individual subjects were diagnosed as healthy individuals (specificity: 87.5%), using the peak at 1942 m/z.

[0067] In addition, in the present invention, a combination of two or more of the partial peptides A, B, and, of the kininogen as a multimarker enables more correct diagnosis of NAFLD and grasp of its condition. Furthermore, multimarker diagnosis by a combination with an NAFLD diagnostic marker other than the partial peptides A, B, and C of the present invention or combined diagnosis by a combination with another blood or serum test results is also possible.

[0068] In particular, the kininogen partial peptides A, B, and C of the present invention are assumed to be generated by decomposition of the full-length kininogen with the progress of NAFLD, as shown in the analytical results of the decomposition pattern of the kininogen in "[3] Decomposition of full-length kininogen in NAFLD patient serum" in Example 1 described below. This suggests that the use in a combination with the full-length kininogen enables correct diagnosis of NAFLD or grasp of a progress in its condition by an increase in at least one of the partial peptides A, B, and C and a decrease in the full-length kininogen. Since NAFLD may be regarded as an initial stage that progresses to liver cirrhosis and hepatocellular carcinoma, the multimarker may be used as an early diagnosis marker of hepatocellular carcinoma.

[0069] As shown by C. Cordova, et al. (Non-Patent Literature 1), since the full-length kininogen tends to decrease with progress of liver cirrhosis or hepatocellular carcinoma, the extent of progress from NAFLD to liver cirrhosis or hepatocellular carcinoma may be diagnosed by combination with the partial peptide A, B, or C of the present invention or another marker or another test result.

[0070] In the present invention, at least one protein and/or partial peptide of the full-length kininogen and the partial peptides may be used as the marker. Patients with NAFLD and healthy individuals may be discriminated with high accuracy from each other using, preferably two or more types, more preferably three or more types or four types of the protein and/or the partial peptides. In such a case, the combination may be of the full length and the partial peptide or of the partial peptides only. Thus, NAFLD may be more correctly detected using the full length and/or the partial peptide as the marker, and the degree of its progress may be accurately determined.

[0071] In addition, in the present invention, the marker may be quantitatively measured or its presence or absence may be determined by qualitative measurement. The use of the full-length kininogen or the partial peptide as a marker enables correct diagnosis with a protein/partial peptide profile prepared based on the correct level obtained by quantitative measurement of the marker. Use of an increased number of markers enables correct diagnosis by qualitative measurement of the makers to obtain a protein/partial peptide profile regarding the presence or absence of each maker.

[0072] As described above, recently, methods by multimarker systems have been proposed for detecting various types of diseases with high accuracy. The present invention using these antibodies may be also widely applied to such assay or measurement of blood or serum for detection of the presence or absence or the amount of an NAFLD diagnostic marker in human blood or serum. In this point, it is advantageous to use at least one of the kininogen-based markers in

combination with a marker of another system. For example, a combination, as a multi marker, with the below-described C4-based marker including a series of specific biological materials relating to C4 found by the present inventor as markers may discriminate between a healthy individual, a patient with NAFLD, a patient with chronic hepatitis, and an ASC.

[0073] The term "C4" means the fourth component of the complement (C4 fraction of complement) and is a glycoprotein with a molecular weight of 198000 and a structure in which three polypeptide chains are coupled with disulfide bonds by processing after translation. Among the complement components in blood, C4 is abundant next to C3. Known isoforms of C4 include, for example, C4A with Accession No. P0C0L4 (SwissProt: Experc) and C4B with Accession No. P0C0L5 (SwissProt: Experc). The C4 gene is located together with C2 and factor B genes on an HLA region of chromosome 6 and is synthesized as a single chain proC4 in a liver cell, monocyte, or macrophage, and is secreted as C4 after binding with sugar chains and fragmentation to three chains. It is said that C4A is involved in removal of pathogens while C4B is mainly involved in disinfection and cytolysis (for example, hemolysis). In the description of the present invention, C4 encompasses these two isoforms, unless specifically stated otherwise. The sequences of C4 (C4A and C4B) and C4A and C4B, which are decomposition products of the C4 as shown in Fig. 2 (decomposition process of C4), are shown in Sequence Listings 5 and 6. C4, as well as C1 and C2, is called an initial response complement component in a classical pathway and plays an important role in transmission of the activity of C1 to C3. After C1 is activated, C4 is decomposed to C4a and C4b. C4 is also similarly decomposed by various serine proteases other than C1. The C4b produced as a result of the activation of C4 forms, for example, (1) C4bC2 by binding with C2, (2) a conjugate with a C4b-binding protein (C4bp), or (3) a conjugate with a C4b receptor (the same as CR1). The C4a is released to a liquid phase and shows an anaphylatoxin activity. By the mechanism (1), the activity is transmitted, and the C4b is decomposed, through several reactions, to C4c, which is released to a liquid phase, and C4d, which remains on a cell membrane. As a result, the C4b activity is lost.

[0074] In the present invention, C4 or the above-mentioned partial peptide as the C4-based marker is used in combination with a kininogen-based marker, so that a healthy individual, a patient with NAFLD, a patient with chronic hepatitis, and an ASC are determined through comparison of the levels thereof in a patient to those in a healthy individual. Various known methods may be used for such determination. That is, a protein or peptide may be quantitatively determined for the measurement of C4, C4a, C4b, or C4c by the above-mentioned various detecting methods.

[0075] The partial peptides D and E derived from C4 according to the present invention are identified by preparing a test specimen for the identification and then subjecting the specimen to MS/MS analysis (below-described Example 2), as in the partial peptides of the kininogen.

[0076] Accordingly, a large number of peaks having different intensities were found in sera of a healthy individual, a patient with NAFLD, a patient with chronic hepatitis, and an ASC. In the present invention, proteins and partial peptides are selected as marker candidates from these peaks based on a statistical test or the absolute values of peak intensities or combination of the both. In the case of comparison of a plurality of mass spectra, peaks having m/z values that are in agreement with each other within an error of 0.1% may be recognized as being derived from the same molecule. The following partial peptides are candidates of the marker for discriminating between liver diseases of the present invention selected in accordance with such criteria.

Sequence D: Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile, and Sequence E: Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile Arg. According to the present invention, as shown in Fig. 16, healthy individuals may be first discriminated from patients with NAFLD using the kininogen-based marker, and then, patients with chronic hepatitis may be conveniently discriminated from ASCs in the patients with NAFLD by combining the C4-based markers. In particular, progress of chronic hepatitis may be conveniently determined, resulting in planning of an appropriate treatment in an early stage.

EXAMPLES

[Example 1] (Kininogen)

[1] Detection serum peptide by ClinProt system

(1) Material and method

[0077] As serum samples, sera of 44 patients with NAFLD and 24 healthy individuals were used. Five microliters of each serum were added to WCX beads for adsorbing the peptides of serum proteins on the WCX beads. Unadsorbed peptides were removed by washing, and then the peptides adsorbed on the WCX beads were eluted by an elution solution.

[0078] Then, crystals of the peptides and a matrix were prepared. In the preparation, 1 mg of CCA was added to 1 mL of acetone, and 300 μ L of the mixture and 600 μ L of ethanol were well mixed. Then, 2 μ L of the ClinProt eluate was mixed with 18 μ L of the prepared CCA solution. One microliter of the resulting mixture was placed dropwise on a thin film, followed by air drying to crystallize the peptides and CCA.

[0079] The peaks were detected by the linear mode measurement of Autoflex to obtain a mass spectrum. The mass spectrum of a healthy individual was compared with that of a patient with NAFLD using a ClinPro tool (Bruker Daltonics), and the peaks increased in the patient with NAFLD were selected as candidates of NAFLD markers. Diagnostic marker candidates were screened in a mass range of 3000 m/z or less. In comparison of a plurality of mass spectra, peaks having m/z values that were in agreement with each other within an error of 0.2% were recognized as being derived from the same molecule. The significant difference between the NAFLD patient group and the healthy individual group was investigated by Student's t-test, and peaks of $P < 0.05$ were determined to be significant.

(2) Results

[0080] Peaks ($P < 0.05$) that significantly increased in the NAFLD patient group were further determined from the spectra of the NAFLD patient group and the healthy individual group, and peaks having an intensity of 50 or more were selected. Table 1 shows a list of the peaks. Fig. 3(A) shows typical spectral patterns of a patient with NAFLD and a healthy individual. In these peaks, the peaks at 1942 m/z, 2079 m/z, and 2207 m/z (Fig. 3(B)) had particularly high intensities compared to other peaks (Table 1).

[0081]

[Table 1]
Peaks increased in expression in patients with NAFLD (ClinProt)

m/z	Patient with NAFLD (n = 44)		Healthy individual (n = 24)	P value
	Peak intensity		Peak intensity	
1942.88	435.01±213.79		45.44±71.52	2.61×10^{-14}
2079.79	334.72±187.24		69.65±115.17	9.57×10^{-9}
2207.68	158.52±96.78		63.05±143.36	0.0154
2858.61	151.37±82.25		77.01±49.46	6.92×10^{-5}
2928.29	83.05±42.66		59.67±21.07	0.00854
2986.07	56.58±50.66		24.07±18.35	0.000823

* data shows average ± standard deviation

[0082] The plot of the intensities of these three peaks of each sample shows remarkably high values in patients with NAFLD as shown by (a), (b), and (c) in Fig. 3(C). Then, the cut-off value for diagnosis was set to [(average peak intensity of healthy individuals) + $2 \times$ (standard deviation)]. A specimen with a value not lower than the cut-off value was determined to be NAFLD, and a specimen with a value lower than the value was determined to be a healthy individual, and 44 patients with NAFLD and 24 healthy individuals were subjected to diagnosis. First, in diagnosis using the peak at 1942 m/z, 40 subjects among the 44 NAFLD subjects were diagnosed as NAFLD (sensitivity: 90.9%), and 20 subjects among the 24 healthy individual subjects were diagnosed as healthy individuals (specificity: 87.5%). In diagnosis using the peak at 2079 m/z, the NAFLD diagnosis showed a sensitivity of 72.7% and a specificity of 87.5%. Furthermore, in the peak at 2207 m/z, the NAFLD diagnosis showed a sensitivity of 70.5% and a specificity of 91.7%. Based on these results, the peaks at 1942 m/z, 2079 m/z, and 2207 m/z were determined as candidates of NAFLD markers, and peptides come from these peaks were identified.

[2] Identification of kininogen as NAFLD marker

[0083] In order to identify the peptides come from the peaks at 1942 m/z, 2079 m/z, and 2207 m/z, MS/MS ion search was performed. The detail will be described below.

(1) Material and method

[0084] An MS/MS spectrum was acquired as follows: First, crystals of the peptides and a matrix were prepared by a thin film technique. A saturated acetone solution of CCA was applied to the anchor surface of an anchor chip in advance to form a thin film of CCA. Then, 1 μ L of ClinProt eluate of an NAFLD patient serum was placed dropwise on the thin film, followed by leaving to stand for about 5 minutes to crystallize the peptides in the eluate and CCA. Then, the crystal was washed with 3 μ L of a 0.1 % TFA three times.

[0085] The molecular weight of the target peak was measured with high accuracy in the reflector mode of Autoflex. The MS/MS spectrum was acquired by lift mode measurement for obtaining the molecular weights of the target peak

(parent ions) and their fragments (ions of partial peptides). The molecular weight was corrected (calibrated) by peptide calibration standard 2 (Bruker Daltonics). Based on the observed MS/MS spectrum, a peak list of the parent ions and their fragment ions was made using BioTools (Bruker Daltonics), and the peaks were identified by MS/MS ion search of Mascot search (Matrix Science). In the identification, the database of SwissProt was used.

(2) Results

[0086] Fig. 4 shows MS/MS spectra at 1942 m/z (A), 2079 m/z (B), and 2207 m/z (C). Screening of all the peptides that agree with peak information of these spectra revealed that all of the three peaks were parts of a high-molecular-weight kininogen. Table 2 shows Accession Numbers and peptide sequences of the high-molecular-weight kininogen.

[0087]

[Table 2]
Identification of NAFLD marker by MS/MS ion search

m/z	Results of identification	Accession No. (SwissProt)	Sequence
1942	Kininogen-1precursor	P01042	Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln
2079	Kininogen-1 precursor	F01042	His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln
2207	Kininogen-1 precursor	P01042	Lys His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln

[0088] The results demonstrate that any of the peptides belongs to domain 5 of the high-molecular-weight kininogen and that 1942 m/z, 2079 m/z, and 2207 m/z correspond to 440th to 456th, 439th to 456th, and 438th to 456th amino acid sequences, respectively.

[3] Decomposition of full-length kininogen in NAFLD patient serum

[0089] The identified peptides are parts of domain 5 of the high-molecular-weight kininogen. The molecular weight of the full-length kininogen protein is about 120 kDa. The identified three peaks, which are parts of the full length, suggest a possibility of facilitated decomposition of the kininogen by the onset of NAFLD. The relationship between NAFLD and the kininogen has not been known yet. Consequently, the decomposition of the full-length kininogen was investigated in patients with NAFLD by Western blotting using an anti-kininogen antibody.

(1) Material and method (polyclonal antibody)

[0090] A sample for electrophoresis was prepared as follows. After 5 μ L of a serum of a patient with NAFLD or a healthy individual was mixed with 45 μ L of PBS, 400 μ L of acetone was added thereto. The mixture was left at -80°C overnight for acetone precipitation of serum proteins. The precipitated proteins were collected by centrifugation and were dissolved in an electrophoresis sample buffer, followed by treatment at 100°C for 5 minutes. The completely denatured and reduced proteins were subjected to SDS-PAGE on 8% acrylamide gel for separating the proteins. The separated proteins were transferred to a PVDF membrane. After being blocked with 5% skim milk/0.05% Tween 20/PBS, the proteins were reacted for 1 hour with RABBIT ANTI HUMAN HMW-KININOGEN Catalog Number 5575-4957 (manufactured by AbD Serotec) for the full-length kininogen and rabbit-anti-human-kininogen (Sigma-Aldrich Japan contract product), which is specific to K438-Q456 peptides, as an antibody specifically recognizing the identified partial peptide C. After treatment with an HRP-labeled secondary antibody, specific bands were detected with ECL Western Blotting Detection System (GE Healthcare).

(2) Results

[0091] Fig. 5 shows the results of Western blotting using an anti-kininogen polyclonal antibody (the above-mentioned RABBIT ANTI HUMAN HMW-KININOGEN manufactured by AbD Serotec). In 18 subjects among the 19 healthy individual subjects, the full-length high-molecular-weight kininogen of about 120 kDa was detected, and a band of the high-molecular-weight kininogen heavy chain of about 65 kDa and a band of the light chain of about 45 kDa were also detected.

On the other hand, in patients with NAFLD, the band of the full-length high-molecular-weight kininogen was not detected in 36 subjects among the 37 subjects. Similarly, Fig. 6 shows the results of the rabbit-anti-human-kininogen (Sigma-Aldrich Japan contract product), which specifically recognizes the identified peptide. In the healthy individuals, the full-length high-molecular-weight kininogen of about 120 kDa was detected in all of four subjects. In the patients with NAFLD, the band of the full-length high-molecular-weight kininogen was not detected in all of eight subjects. These results reveal that the full-length high-molecular-weight kininogen decreases in the patients with NAFLD.

(3) Material and method (monoclonal antibody)

[0092] A sample for electrophoresis was prepared as follows. After 5 μ L of a serum of a patient with NAFLD or a healthy individual was mixed with 45 μ L of PBS, 400 μ L of acetone was added thereto. The mixture was left at -80°C overnight for acetone precipitation of serum proteins. The precipitated proteins were collected by centrifugation and were dissolved in an electrophoresis sample buffer, followed by treatment at 100°C for 5 minutes. The completely denatured and reduced proteins were subjected to SDS-PAGE on 8% acrylamide gel for separating the proteins. The separated proteins were transferred to a PVDF membrane. After being blocked with 5% skim milk/0.05% Tween 20/PBS, the proteins were reacted with an anti-kininogen monoclonal antibody. The anti-kininogen, antibody used was, (mouse-monoclonal) HMW Kininogen Light Chain antibody [1.B.709]: Catalog Number: GTX 14514 (manufactured by GeneTex Inc.). The anti-kininogen monoclonal antibody specifically recognizing a portion containing the identified peptide C used was (mouse-monoclonal) Anti-human-kininogen/Kininostatin Antibody: Catalog Number: MAB1569 (manufactured by R&D Systems), which is specific to K438-S531 peptides. The reaction with the antibody was performed for 1 hour. After treatment with an HRP-labeled secondary antibody, specific bands were detected with ECL Western Blotting Detection System (GE Healthcare).

(4) Results

[0093] Fig. 7 shows the results of Western blotting using the anti-kininogen monoclonal antibody (the above-mentioned HMW Kininogen Light Chain antibody [1.B.709] manufactured by GeneTex Inc.). The band of the light chain of about 45 kDa was detected, but a significant difference was not found between the healthy individuals and patients with NAFLD. Fig. 8 shows the results of anti-kininogen monoclonal antibody (the above-mentioned Anti-human-kininogen/Kininostatin Antibody manufactured by R&D Systems), which specifically recognizes the portion containing the identified partial peptide C. In the healthy individuals, the full-length high-molecular-weight kininogen of about 120 kDa was detected in 15 subjects among the 16 subjects. In the patients with NAFLD, the band of the full-length high-molecular-weight kininogen was not detected in 36 subjects among the 40 subjects. These results reveal that the full-length high-molecular-weight kininogen decreases in patients with NAFLD.

[5] Detection of kininogen peptide with anti-kininogen antibody

[0094] In order to use these markers for diagnosis, it is necessary to detect specific peaks using antibodies against the markers. Accordingly, the inventors tried to detect the kininogen by immunoprecipitation using an anti-kininogen antibody.

(1) Material and method

[0095] The specific detection of kininogen partial peptides was performed by immunoprecipitation using anti-kininogen antibodies. Ten microliters of an NAFLD patient serum was mixed with 40 μ L of PBS, followed by addition of 10 μ L of RABBIT ANTI HUMAN HMW-KININOGEN (manufactured by AbD Serotec) or rabbit-anti-human-kininogen (Sigma-Aldrich Japan contract product) as the polyclonal antibody or HMW Kininogen Light Chain antibody [1.B.709] (manufactured by GeneTex Inc.) or Anti-human-kininogen/Kininostatin Antibody: Catalog Number: MAB 1569 (manufactured by R&D Systems) as the monoclonal antibody. The mixture was left to stand on ice bath (4°C) for 1 hour for a reaction of the antigen in the serum and the antibody. After 30 μ L of 50% protein A sepharose beads were added thereto, the mixture was left to stand on an ice bath for 1 hour while being inverted for mixing every 10 minutes. Then, the mixture was separated by centrifugation into a precipitate and a supernatant. The precipitated beads were washed five times with 400 μ L of PBS to remove unadsorbed proteins and peptides, and the proteins and peptides adsorbed onto the beads were eluted by addition of 30 μ L of 50% acetonitrile and centrifugation. Twenty microliters of the eluate was diluted with 80 μ L of 50 mM sodium acetate (pH 4.5), followed by application to CM10 by shaking treatment for 30 minutes. The spots were washed and air-dried, followed by addition of 0.5 μ L of 20% saturated CCA/50% acetonitrile/0.5% TFA twice. The identified kininogen partial peptides were detected by SELDI measurement.

(2) Results

[0096] Fig. 9 shows the results of immunoprecipitation with polyclonal antibodies. The peak intensities of NAFLD patient sera treated with the rabbit-anti-human-kininogen manufactured by Sigma-Aldrich Japan were higher than those treated with RABBIT ANTI HUMAN HMW-KININOGEN manufactured by AbD Serotec.

[0097] Similarly, Fig. 10 shows the results of immunoprecipitation with monoclonal antibodies. The peak intensities of sera treated with the Anti-human-kininogen/Kininostatin Antibody manufactured by R&D Systems are higher than those treated with the HMW Kininogen Light Chain antibody [1.B.709] manufactured by GeneTex. That is, antibodies specifically recognizing the identified portion more remarkably react with the identified kininogen peptide portion.

[6] Detection of kininogen-derived peak using protein chip system (SELDI)

[0098] A protein chip system (Bio-Rad) consists of a protein chip and SELDI and is useful for screening serum diagnostic markers as in ClinProt. The chip surface is labeled with various functional groups and captures proteins and peptides in a serum applied thereto. Peaks are detected by measurement with the SELDI. The inventors then tried to investigate whether partial peptide peaks (1942 m/z, 2079 m/z, and 2207 m/z) derived from the kininogen are also detected by the protein chip system.

(1) Material and method

[0099] The serum was applied to the protein chip as follows. Five microliters of the serum were added to 45 μ L of a urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholyte). The mixture was left to stand on an ice bath for 10 minutes to denature the serum proteins and then diluted with 450 μ L of 50 mM sodium acetate having a pH of 4.5, followed by centrifugation at 10000 rpm for 5 minutes with being cooled (4°C). The supernatant was transferred to a tube and stored on an ice bath. One hundred microliters of the diluted supernatant were added to a cation exchange chip CM10 (Bio-Rad) equilibrated in advance. After osmotic treatment at room temperature for 30 minutes, the CM10 was washed with 100 μ L of 50 mM sodium acetate at a pH of 4.5 three times and then with ultrapure water twice. After air drying, 0.5 μ L of a 50% saturated CCA were added to the spot twice to prepare a crystal mixture of the peptides and CCA. The peaks were detected with SELDI.

[0100] The peak intensities of the NAFLD patient group and the healthy individual group were investigated with marker Wizard software (Bio-Rad). In comparison of a plurality of mass spectra, peaks having m/z values that were in agreement with each other within an error of 0.3% were recognized as being derived from the same molecule. The significant difference between these groups was determined by a Mann-Whitney U test, and a peak was determined to be significant at $P < 0.05$.

(2) Results

[0101] Peaks ($P < 0.05$) that significantly increases in the NAFLD patient group were further selected from the spectra of the NAFLD patient group and the healthy individual group, and peaks having an intensity of 5 or more were selected. Table 3 shows a list of the peaks. Fig. 11 (A) shows typical spectral patterns of a patient with NAFLD and a healthy individual. Like Example 1, the results reveal that the peaks at 1942 m/z, 2079 m/z, and 2207 m/z (Fig. 11 (B)) derived from the high-molecular-weight kininogen are significantly higher in patients with NAFLD compared to those in healthy individuals (Table 3). The peak intensities plotted for each sample are also remarkably high in patients with NAFLD (Fig. 11(C)).

[0102]

[Table 3]

Peaks with increased expression in patient with NAFLD (Protein chip)

m/z	Patient with NAFLD (n = 44)		Healthy individual (n = 24)	P value
	Peak intensity	Peak intensity		
1942	25.57 \pm 9.13	6.74 \pm 5.40	2.0 \times 10 ⁻¹⁰	
1965	10.57 \pm 3.79	4.98 \pm 2.28	2.4 \times 10 ⁻⁸	
2019	10.70 \pm 10.23	2.34 \pm 2.38	3.6 \times 10 ⁻⁵	
2079	19.75 \pm 10.34	3.42 \pm 7.48	1.9 \times 10 ⁻⁸	
2207	14.63 \pm 9.02	4.55 \pm 9.81	1.0 \times 10 ⁻⁶	
2656	8.49 \pm 4.04	5.72 \pm 6.89	0.00112	

(continued)

Peaks with increased expression in patient with NAFLD (Protein chip)

m/z	Patient with NAFLD (n = 44)		Healthy individual (n = 24)
	Peak intensity	Peak intensity	P value
2858	7.04±4.35	2.27±2.99	1.2×10 ⁻⁶
2949	24.58±9.80	13.78±8.02	5.0×10 ⁻⁵

[Example 2] (Complement C4)

[1] Detection of serum peptide with ClinProt system

(1) Material and method

[0103] As serum specimens, sera of 19 ASCs and 24 healthy individuals were used. Five microliters of each serum was added to WCX beads for adsorbing the peptides of serum proteins to the WCX beads. Unadsorbed peptides were washed out, and then the peptides adsorbed to the WCX beads were eluted by adding an elution solution.

[0104] Then, crystals of the peptides and a matrix were prepared. In the preparation, 1 mg of CCA was added to 1 mL of acetone, and 300 μL of the mixture and 600 μL of ethanol were well mixed to each other. Then, 2 μL of the ClinProt eluate was mixed with 18 μL of the prepared CCA solution. One microliter of the resulting mixture was placed dropwise on a thin film, followed by air drying to crystallize the peptides and CCA.

[0105] The peaks were detected by the linear mode measurement of Autoflex to obtain a mass spectrum. The mass spectra of a healthy individual and ASC were compared with each other using a ClinPro tool (Bruker Daltonics), and the peaks increasing in the ASC were determined as candidates of ASC markers. Candidates of diagnostic markers were screened in a mass range of 3000 m/z or less. In comparison of a plurality of mass spectra, peaks having m/z values that were in agreement with each other within an error of 0.2% were recognized as being derived from the same molecule. The significant difference between the ASC group and the healthy individual group was investigated by Student's t-test, and peaks were determined to be significant at P < 0.05.

(2) Result

[0106] Significantly increasing peaks (P < 0.05) were detected from the spectra of the ASC group and the healthy individual group, and peaks having an intensity of 50 or more were selected. Table 4 shows a list of the peaks, and Fig. 12(A) shows typical spectral patterns of ASC and a healthy individual. In these peaks, the peaks at 1738 m/z and 1896 m/z (Fig. 12(B)) have higher peak intensities compared to other peaks (Table 4).

[0107]

[Table 4]

Peaks with increasing expression in asymptomatic carrier (ClinProt)

m/z	ASC (n = 44)		Healthy individual (n = 24)
	Peak intensity	Peak intensity	P value
1497.98	15.76±12.8	4.66±2.92	6.1×10 ⁻⁵
1738.06	100.88±63.16	57.79±44.48	0.00971
1777.32	26.12±15.93	16.27±13.49	0.0286
1864.04	50.84±35.4	21.35±21.11	7.2×10 ⁻⁴
1895.98	94.68±58.7	31.63±21.1	4.0×10 ⁻⁶
1942.69	180.35±137.12	39.8±41.5	6.3×10 ⁻⁶
2079.63	162.02±62.26	56.33±67.19	4.0×10 ⁻⁶
2207.47	124.1±70.24	46.93±89.11	0.0041

* data shows average ± standard deviation

[0108] These two peaks obviously exhibit remarkably high values in ASC (Fig. 12(C)) in the plot of the peak intensities for each sample. Then, the cut-off value for diagnosis was set to [(average peak intensity of healthy individuals) + 2×(standard deviation)]. A specimen with a value not lower than the cut-off value was determined to be an ASC, and a specimen with a value lower than the value was determined to be a healthy individual, and 19 ASC subjects and 24

healthy individuals were subjected to diagnosis.

[0109] First, in diagnosis using the peak at 1738 m/z, 15 subjects among the 19 ASC subjects were diagnosed as ASC (sensitivity: 78.9%), and 21 subjects among the 24 healthy individuals were diagnosed as healthy individuals (specificity: 87.5%). The diagnostic results at the peak at 1896 m/z were similar to the above. Based on these results, the peaks at 1738 m/z and 1896 m/z were determined as candidates of ASC markers, and peptides come from these peaks were identified.

[2] Identification of sequences D and E as liver disease marker

[0110] In order to identify peptides come from the peaks at 1738 m/z and 1896 m/z, MS/MS ion search was performed. The detail will be described below.

(1) Material and method

[0111] An MS/MS spectrum was acquired as follows. First, crystals of the peptides and a matrix were prepared by a thin film technique. A saturated acetone solution of CCA was applied to the anchor surface of an anchor chip in advance to form a thin film of CCA. Then, 1 μL of ClinProt eluate of an ASC serum was placed dropwise on the thin film, followed by leaving to stand for about 5 minutes to crystallize the peptides in the eluate and CCA. Then, the crystal was washed three times with 3 μL of 0.1% TFA.

[0112] The molecular weight of a target peak was measured with high accuracy in the reflector mode of Autoflex. The MS/MS spectrum was acquired by lift mode measurement for obtaining the molecular weights of a target peak (parent ions) and their fragments (ions of partial peptides). The molecular weight was corrected (calibrated) by peptide calibration standard 2 (Bruker Daltonics). Based on the observed MS/MS spectrum, a peak list of the parent ions and the partial peptide sequence ions was made using BioTools (Bruker Daltonics), and the peaks were identified by MS/MS ion search of Mascot search (Matrix Science). The identification was performed using database of SwissProt.

(2) Results

[0113] Fig. 13 shows MS/MS spectra at 1738 m/z (A) and 1896 m/z (B). The peptides that agree with peak information of these spectra were screened to confirm that both the two peaks were decomposition products of C4. Table 5 shows the sequences of the peptides.

[0114]

[Table 5]
Identification of ASC marker by MS/MS ion search

m/z	Results of identification	Accession No. (SwissProt)	Sequence
1738	Complement G4-A precursor (Complement C4-B precursor)	P0C0L4 (P0C0L5)	Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile
1896	Complement C4-A precursor (Complement C4-B precursor)	P0C0L4 (P0C0L5)	Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile Arg

[0115] The results show that the 1738 m/z and 1896 m/z correspond to 1337th to 1351 st and 1337th to 1352nd amino acid sequences, respectively.

[0116] The peaks at 1738 and 1896 m/z were analyzed with the ClinProt system. Specimens were sera of 30 ASC subjects, 30 patients with chronic hepatitis (CH), 2 patients with liver cirrhosis (LC), 5 patients with hepatocellular carcinoma (HCC), 12 SS subjects, 25 NASH subjects, and 25 healthy individuals. Fig. 14 shows the results. A significant difference was uncertain between ASC and chronic hepatitis (CH), but a significant difference was found between these subjects and healthy individuals or other disease subjects.

[3] Detection of serum protein level by ELISA

[0117] It is publicly known that the complement system is activated in hepatocellular carcinoma and liver cirrhosis. However, a novel marker of an early stage liver disease may be found out by quantitative determination of a factor specific to C4a or an activating path on ASC or NAFLD (SS and NASH), which are relatively early stage liver diseases. Such finding fits to the purpose of the present invention to provide a larger number of markers of (early) liver diseases. Accordingly, quantitative determination of a factor serving as an index of activation of C4a was tried in such carriers and

liver disease patients.

(1) Material and method

5 **[0118]** The serum level of C4a was measured with a C4a enzyme immunoassay BD OptEIA Set (Becton Dickinson Japan, hereinafter abbreviated to BD). Specimens were sera of 30 ASC subjects, 30 patients with chronic hepatitis (CH), 2 patients with liver cirrhosis (LC), 5 patients with hepatocellular carcinoma (HCC), 12 SS subjects, 25 NASH subjects, and 25 healthy individuals. A C4a monoclonal antibody is immobilized to each well of the 96-well plate of this kit.

10 **[0119]** Two microliters of each serum was diluted with 600 μL of PBS(137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.68 mM KCl, and 1.47 mM KH_2PO_4). Two microliters of this diluted serum was added to 200 μL of a diluent, so that the serum was finally diluted 30000 times. One hundred microliters of the finally diluted serum was added to each well, followed by incubation at room temperature for 2 hours. Then, aspiration and washing were repeated 5 times using a washing solution (300 μL of wash buffer/well). The wash buffer composition was 100 mL of wash buffer/1900 mL of ultrapure water, 20 times dilution. Each well was filled with 100 μL of Working Detector previously prepared by mixing 12 mL of biotinylated anti-human C4a polyclonal antibody and 48 μL of 250x concentrated Streptavidin-horseradish peroxidase conjugate, followed by incubation at room temperature for 1 hour. Aspiration and washing were repeated seven times as in above. Subsequently, 100 μL of a TMB substrate was added to each well, followed by shaking for 5 seconds and then incubation (dark room) at room temperature for 30 minutes. While luminescence is observed, 50 μL of Stop Solution were added. After the color reaction, absorbance at a wavelength of 450 nm was measured. The C4a level in the serum was calculated from a calibration curve prepared using standard materials. The statistic analysis was performed by a Mann-Whitney U test, and $P < 0.05$ was determined to be statistically significant.

(2) Results

25 **[0120]** Fig. 15 shows C4a protein levels in the sera. The levels are high ($P < 0.05$) in ASCs compared to those in the healthy individuals. Therefore, the measurement of blood C4a level made it possible to discriminate ASCs from other diseases as a diagnostic marker of an ASC. These results suggest that the C4a level may be a high value marker of an ASC.

30 **[0121]** Next, the serum C4a levels were calculated, and the results thereof showed that C4a protein level decreased with aggravation of symptoms: ASCs, patients with hepatitis, patients with liver cirrhosis, and patients with hepatocellular carcinoma, in the order toward the clinical deterioration. In particular, a significant difference was also observed between two groups of ASC and hepatitis ($P < 0.05$). Evidently, peak intensities plotted for each sample are notably high in ASC (Fig. 15). In addition, as shown in Fig. 15, a significant difference is found between NASH and chronic hepatitis (CH) at $P < 0.05$, while a significant difference was found between SS and chronic hepatitis (CH) at $P < 0.05$.

[4] Detection of partial peptide derived from C4 using protein chip system

35 **[0122]** A protein chip system (Bio-Rad) consists of a protein chip and SELDI and is useful for screening serum diagnostic markers, like ClinProt. The chip surface is labeled with various functional groups and captures proteins and peptides in a serum applied thereto. Peaks are detected by measurement with the SELDI. Consequently, detection of partial peptide peaks (1738 m/z and 1896 m/z) derived from C4 were investigated by the protein chip system.

(1) Maternal and method

45 **[0123]** Specimens used were sera of ASCs and healthy individuals. The serum was applied to the protein chip as follows. Five microliters of the serum was added to 45 μL of a urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholyte). The mixture was left to stand on an ice bath for 10 minutes to denature the serum proteins and then was diluted with 450 μL of 50 mM sodium acetate at a pH of 4.5. One hundred microliters of the diluted solution was added to a cation exchange chip CM10 (Bio-Rad) equilibrated in advance. After osmotic treatment at room temperature for 30 minutes, the CM10 was washed with 50 mM sodium acetate at a pH of 4.5 three times and further with ultrapure water twice. After air drying, 0.5 μL of 50% saturated CCA were added to the spots twice to prepare a crystal mixture of the peptides and CCA. The peaks were detected with SELDI.

50 **[0124]** The peak intensities of the ASC and the healthy individual groups were investigated with biomarker Wizard software (Bio-Rad). In comparison of a plurality of mass spectra, peaks having m/z values that were in agreement with each other within an error of 0.3% were recognized as being derived from the same molecule. The significant difference between the groups was investigated by a Mann-Whitney U test, and peaks were determined to be significant at $P < 0.05$.

(2) Results

[0125] Peaks ($P < 0.05$) that significantly increase, in particular, in the ASC group were selected from the spectra of the ASC group and the healthy individual group, and peaks having a peak intensity of 5 or more were selected. Table 6 shows the results of identification of the peaks, and Fig. 16 shows typical spectral patterns of ASC and a healthy individual. Fig. 16(A) demonstrates that the peaks at 1738 m/z and 1896 m/z derived from C4 significantly increase in the ASC compared to the healthy individuals (Table 6). Fig. 16(C) evidently demonstrates that the peak intensities plotted for each sample are also remarkably high in the ASC.

[0126]

[Table 6]
Peaks with increasing expression in ASC (Protein chip)

Molecular weight	ASC (n = 20)	Healthy individual (n = 10)	
m/z	Peak intensity	Peak intensity	P values
1738	25.57±9.13	6.74±5.40	5.5×10 ⁻³
1863	10.57±3.79	4.98±2.28	1.8×10 ⁻⁴
1896	10.70±10.23	2.34±2.38	3.7×10 ⁻⁵
1817	19.75±10.34	3.42±7.48	1.1×10 ⁻⁵
1942	14.63±9.02	4.65±9.8	1.3×10 ⁻⁵
2079	8.49±4.04	5.72±6.89	1.9×10 ⁻⁵
2207	7.04±4.35	2.27±2.99	1.5×10 ⁻⁴
2858	24.588±9.80	13.78±8.02	2.4×10 ⁻³

[Example 3] (Diagnosis by ELISA using anti-kininogen antibody)

[0127] An example for discriminating between a healthy individual and a patient with NAFLD using an antibody against the kininogen as a kininogen-based marker will be described.

(1) Material and method

[0128] Sera of 11 healthy individuals, 5 NASH subjects, and 5 simple steatosis subjects were used as specimens.

[0129] An anti-human kininogen monoclonal antibody (manufactured by R&D, Catalog Number: MAB1569) was diluted with an immobilization buffer (Sumitomo Bakelite) into 1 μg/mL, and the diluted antibody solution was applied to a plate of New ELISA Plate B (Sumitomo Bakelite) in an amount of 100 μL/well, followed by leaving to stand at room temperature for 1 to 2 hours. The plate was washed with 300 μL of PBSx (PBS (phosphate buffered saline) containing 0.05% Triton-X100) three times. Then, the serum specimen was diluted 200 times with PBS and was added to each well in an amount of 100 μL/well, followed by leaving to stand at room temperature for 1 hour. The plate was washed with 300 μL of PBSx three times. An anti-KIG1 antibody (manufactured by Sigma, Catalog Number: HAP001616) diluted 1000 times with PBS was added to each well in an amount of 100 μL/well, followed by leaving to stand at room temperature for 1 hour. Then, the plate was washed with 300 μL of PBSx three times. Subsequently, a goat-anti-rabbit IgG HRP conjugate antibody (manufactured by Santa Cruz Biotechnology, Catalog Number: sc-2004) diluted 1000 times with PBS was added to each well in an amount of 100 μL/well, followed by leaving to stand at room temperature for 1 hour. Then, the plate was treated with 300 μL of PBSx (PBS containing 0.05% Triton-X100) three times for washing. Finally, Ultra-TMB-ELISA (manufactured by PIERCE, Catalog Number: 34028) was added to each well in an amount of 100 μL/well. After leaving to stand for 15 to 50 minutes, the reaction was terminated by adding 50 μL of 2 M sulfuric acid, followed by colorimetry at 450 nm.

(2) Results

[0130] From the results described above, H1 to H11, which were sera of healthy individuals, yielded an average ± standard deviation of 0.937 ± 0.117. In addition, N4 to S42, in which N means NASH and S means simple steatosis, yielded an average ± standard deviation of 0.383 ± 0.224. From Fig. 18, the kininogen of the healthy individuals is determined to be approximately 1, and that of patients with NAFLD is determined to be approximately 0.5 or less.

INDUSTRIAL APPLICABILITY

[0131] All the kininogen-based full length and the partial peptides A, B, and C of the present invention show significant differences in serum levels thereof between healthy individuals and patients with NAFLD and thus may be served as useful markers for NAFLD diagnosis. These may be used not only for diagnosis by physicians but also measurement or assay of blood or serum. Furthermore, as shown in Fig. 17, combination of the kininogen-based marker and the C4-based marker may conveniently discriminate between an ASC, a patient with hepatitis, a patient with liver cirrhosis, a patient with hepatocellular carcinoma, a patient with NAFLD, and a healthy individual. The convenient detecting method provided by the marker of the present invention enables medical examination of many subjects in pre-disease conditions and may be preferably applied to early diagnosis for discriminating between healthy individuals and patients with NAFLD, patients with CH, or ASCs and may be used for early detection of these lifestyle-related diseases or liver diseases caused therefrom.

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SEQUENCE LISTING

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55 Met Val Glu Asn Ser His Gly Leu Arg Val Arg Lys Lys Glu Val Tyr
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 Gly Gln Ile Val Phe Met Asn Arg Glu Pro Lys Arg Thr Leu Thr Ser
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 Ala Phe Tyr Tyr His Gly Asp His Pro Val Ala Asn Ser Leu Arg Val
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 Asp Val Gln Ala Gly Ala Cys Glu Gly Lys Leu Glu Leu Ser Val Asp
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 Thr Asp Ser Leu Ala Leu Val Ala Leu Gly Ala Leu Asp Thr Ala Leu
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 Tyr Ala Ala Gly Ser Lys Ser His Lys Pro Leu Asn Met Gly Lys Val
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30 Asp Ser Leu Thr Thr Trp Glu Ile His Gly Leu Ser Leu Ser Lys Thr
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35 Lys Gly Leu Cys Val Ala Thr Pro Val Gln Leu Arg Val Phe Arg Glu
820 825 830

40 Phe His Leu His Leu Arg Leu Pro Met Ser Val Arg Arg Phe Glu Gln
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45 Leu Glu Leu Arg Pro Val Leu Tyr Asn Tyr Leu Asp Lys Asn Leu Thr
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Claims

- 5 1. A biomarker for identification of a non-alcoholic fatty liver disease, the biomarker comprising a full-length high-molecular-weight kininogen and/or a partial peptide derived from the high-molecular-weight kininogen, wherein the partial peptide derived from the high-molecular-weight kininogen is any one of the following sequences A, B, and C:
- sequence A: Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln,
sequence B: His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln, and
sequence C: Lys His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln.
- 10 2. The biomarker according to Claim 1, wherein the full-length high-molecular-weight kininogen and/or the partial peptide derived from the high-molecular-weight kininogen includes a modified form thereof.
- 15 3. An antibody for identification of a non-alcoholic fatty liver disease, the antibody recognizing at least one of the biomarkers according to Claim 1 or 2 as an antigen.
- 20 4. The antibody according to Claim 3, wherein the antibody is a polyclonal antibody that is obtained by immunizing a rabbit with at least one selected from the group consisting of the full-length kininogen, the partial peptide sequence A, the partial peptide sequence B, and the partial peptide sequence C.
- 25 5. The antibody according to Claim 3, wherein the antibody is a monoclonal antibody that is obtained by immunizing a mouse with at least one selected from the group consisting of the full-length kininogen, the partial peptide sequence A, the partial peptide sequence B, and the partial peptide sequence C.
- 30 6. A diagnostic agent for identification of a non-alcoholic fatty liver disease, the agent comprising at least one selected from the group consisting of the biomarkers according to Claim 1 or 2 and the antibodies according to any of Claims 3 to 5.
- 35 7. A biomarker for identification of chronic hepatitis and an asymptomatic carrier, the biomarker being complement C4 and/or a partial peptide derived from the C4, wherein the partial peptide derived from the C4 is at least one selected from the group consisting of C4a, C4b, C4c, and the following sequences D and E:
- sequence D: Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile, and
sequence E: Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile Arg.
- 40 8. An antibody for identification of chronic hepatitis and an asymptomatic carrier, the antibody recognizing at least one of the biomarkers according to Claim 7 as an antigen.
- 45 9. The antibody according to Claim 8, wherein the antibody is a polyclonal antibody.
- 50 10. The antibody according to Claim 8, wherein the antibody is a monoclonal antibody.
- 55 11. A diagnostic agent for identification of chronic hepatitis and an asymptomatic carrier, the agent comprising at least one selected from the group consisting of the biomarker according to Claim 7 and the antibodies according to any of Claims 8 to 10.
12. A diagnostic agent for identification of a non-alcoholic fatty liver disease, chronic hepatitis, and an asymptomatic carrier, the agent comprising a combination of at least one of the biomarkers, the antibodies, and the diagnostic agent according to any of Claims 1 to 6 and at least one of the biomarker, the antibodies, and the diagnostic agent according to any of Claims 7 to 11.
13. A detecting method for identification of a non-alcoholic fatty liver disease, the method using at least one of the biomarkers, the antibodies, and the diagnostic agent according to any of Claims 1 to 6.
14. A detecting method for identification of chronic hepatitis and an asymptomatic carrier, the method using at least one of the biomarkers, the antibodies, and the diagnostic agent according to any of Claims 7 to 11.
15. A detecting method for identification of a non-alcoholic fatty liver disease, chronic hepatitis, and an asymptomatic

carrier, the method including a combination of the detecting method according to Claim 13 and the detecting method according to Claim 14.

5 16. The detecting method according to any of Claims 13 to 15, the method using an antibody that recognizes the high-molecular-weight kininogen in a sample but does not recognize any of the partial peptide sequence A, the partial peptide sequence B, and the partial peptide sequence C.

17. The detecting method according to Claim 16, wherein the detection is performed by ELISA.

10 18. The diagnostic agent according to Claim 6 or 12, wherein the antibody recognizes the high-molecular-weight kininogen in a sample but does not recognize any of the partial peptide sequence A, the partial peptide sequence B, and the partial peptide sequence C.

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Fig. 1

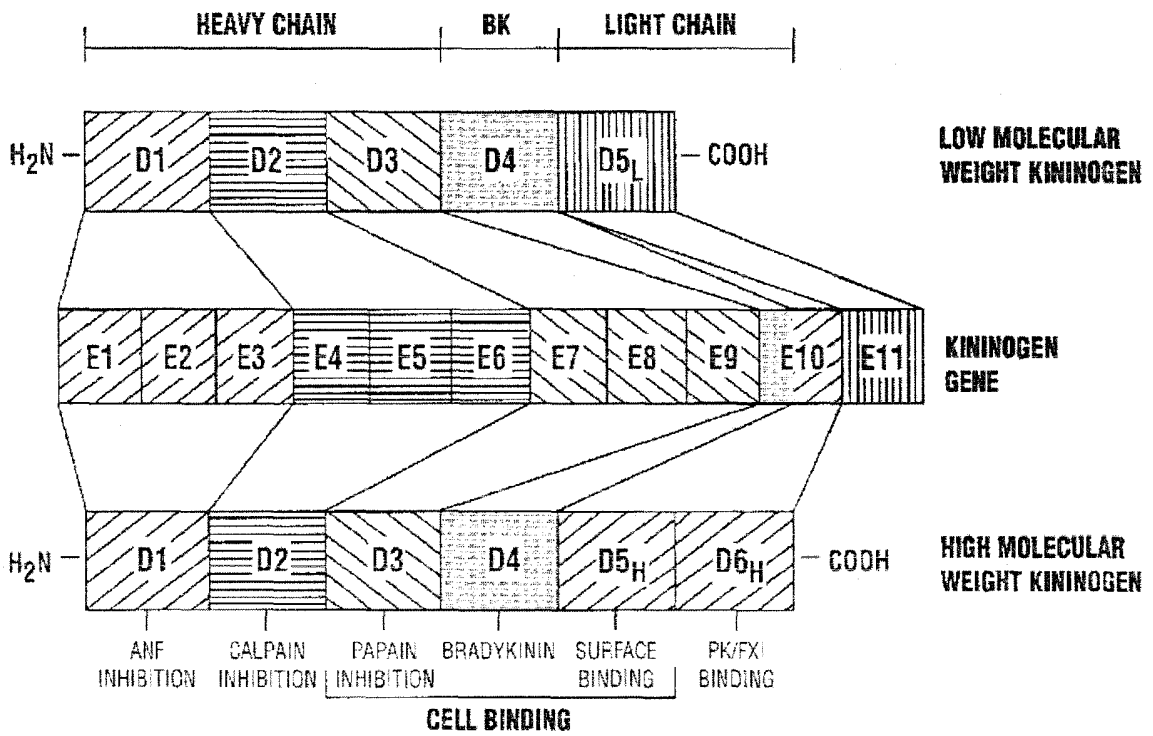


Fig. 2

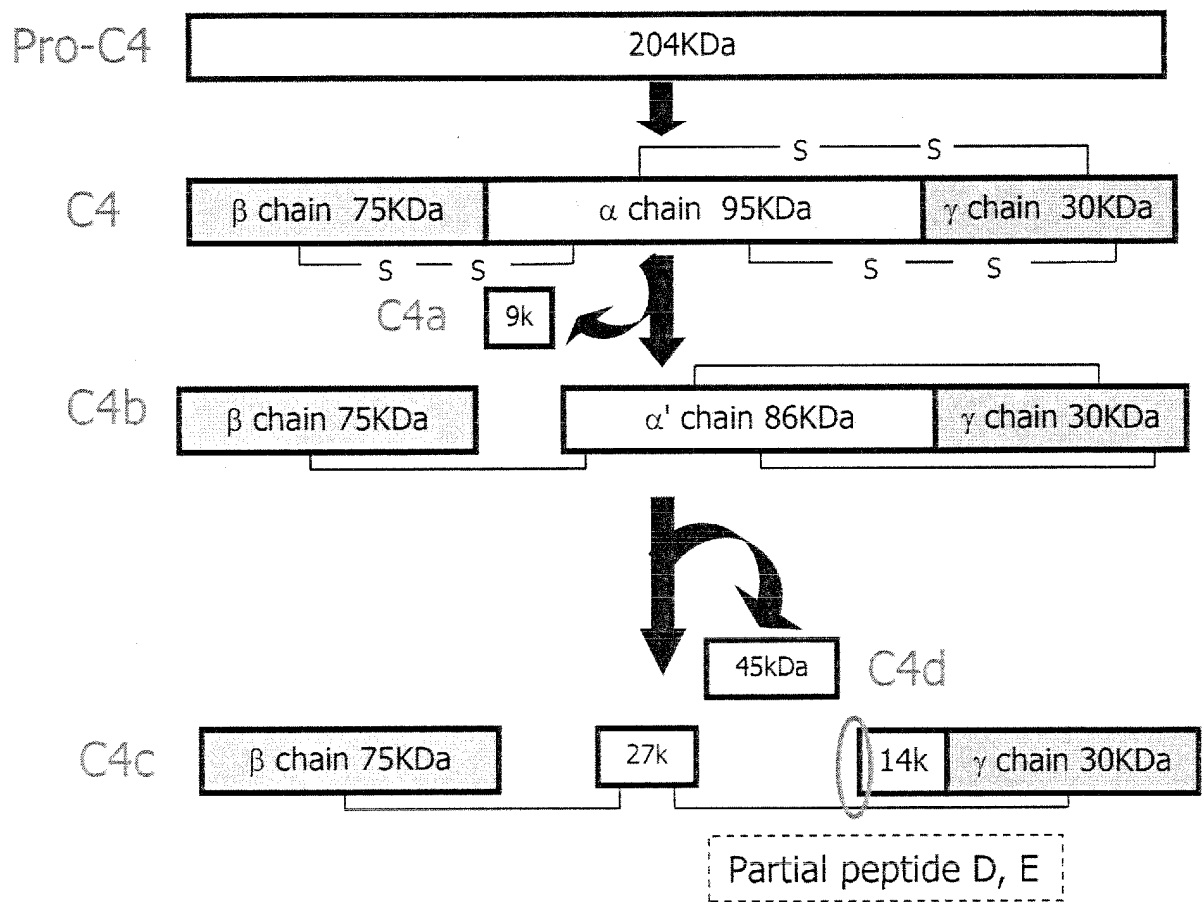


Fig. 3

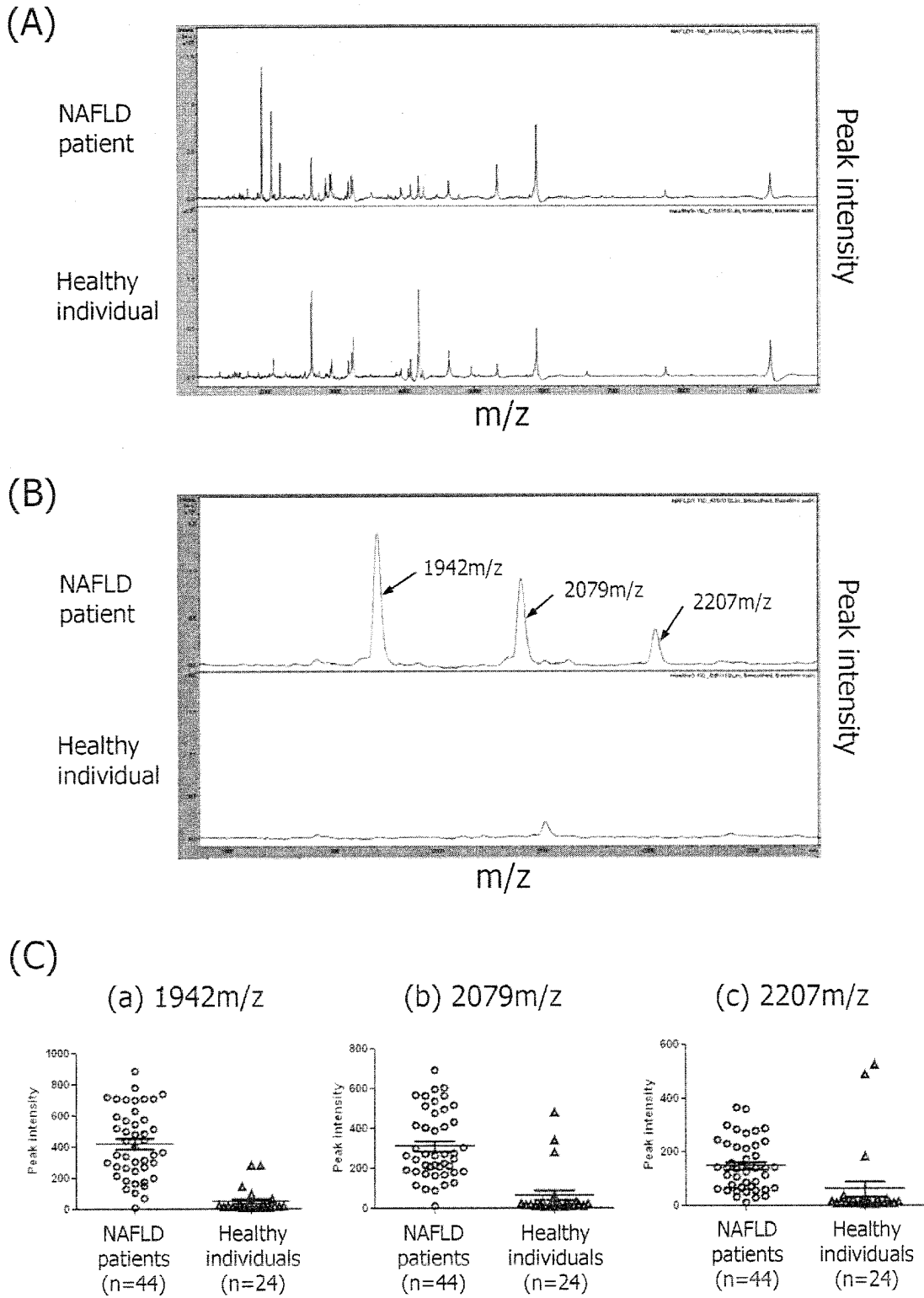
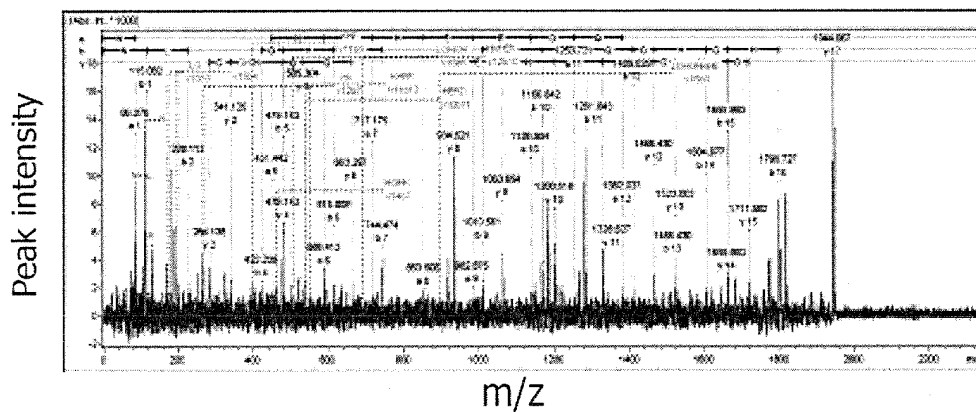
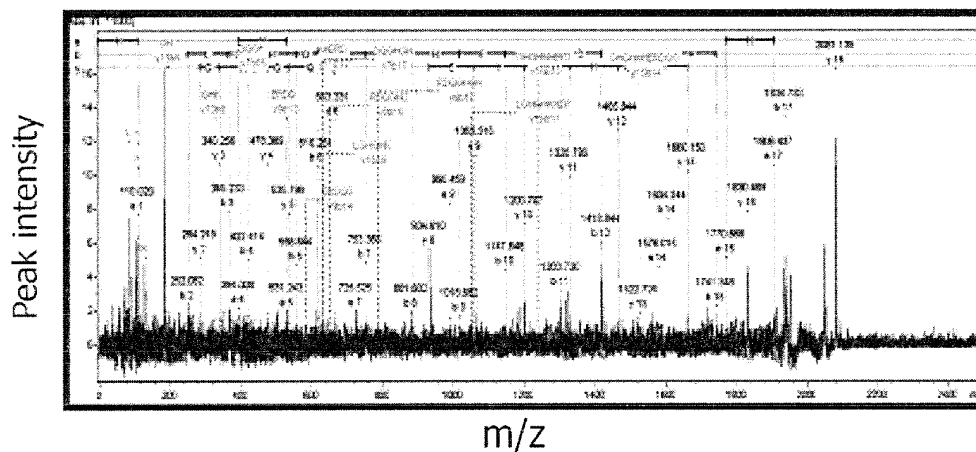


Fig. 4

(A) 1942m/z



(B) 2079m/z



(C) 2207m/z

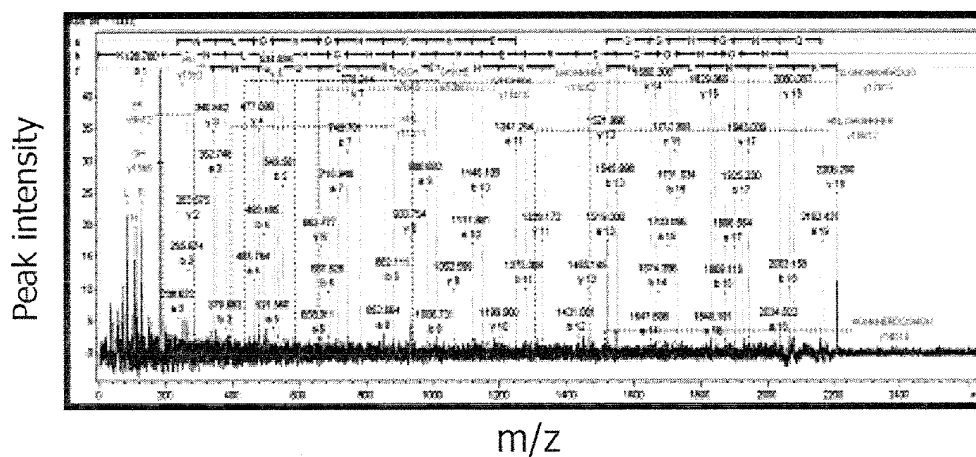


Fig. 5

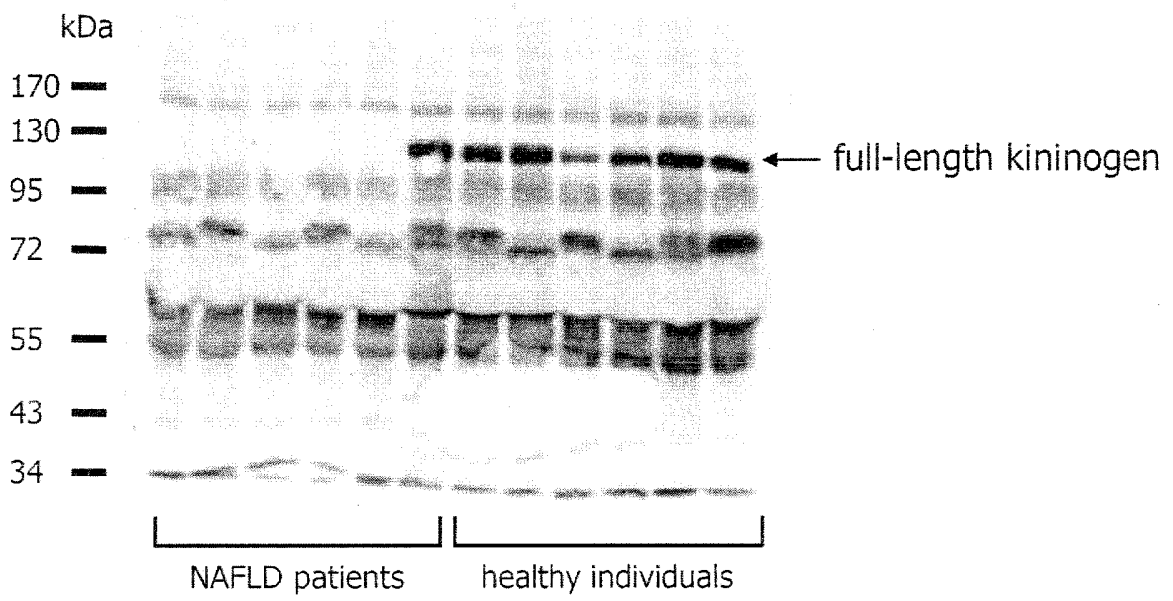


Fig. 6

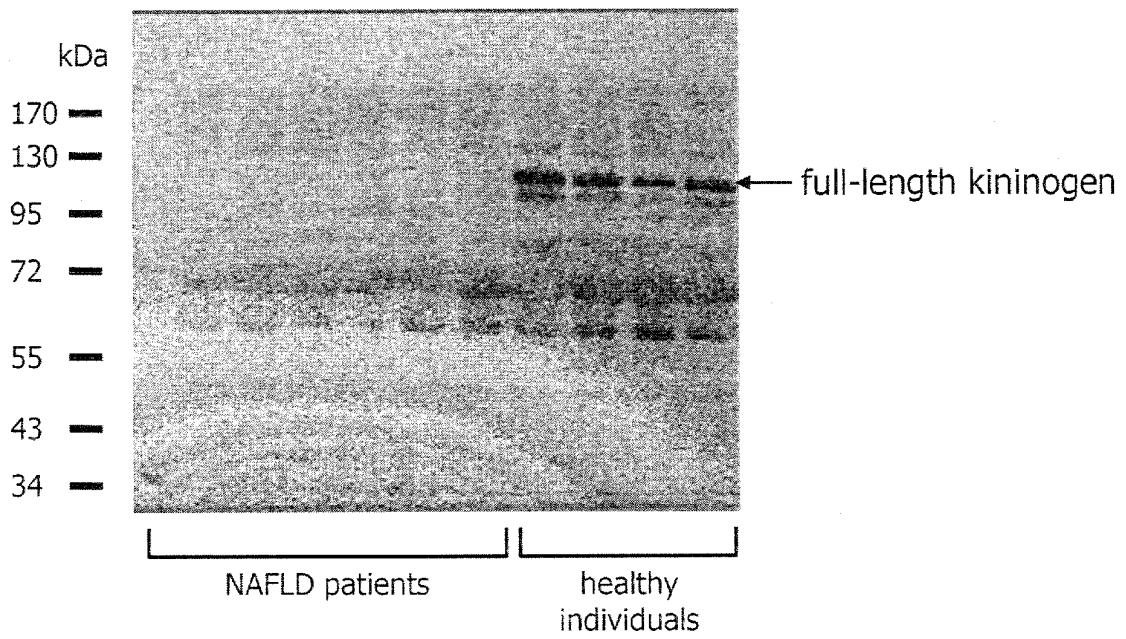


Fig. 7

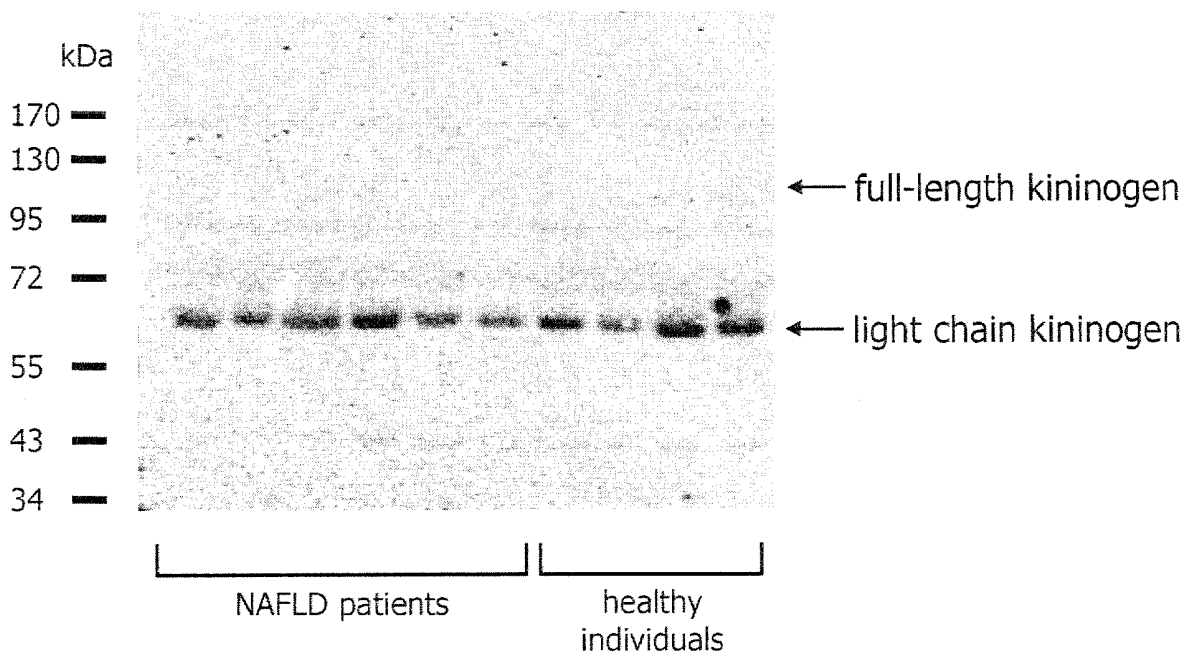


Fig. 8

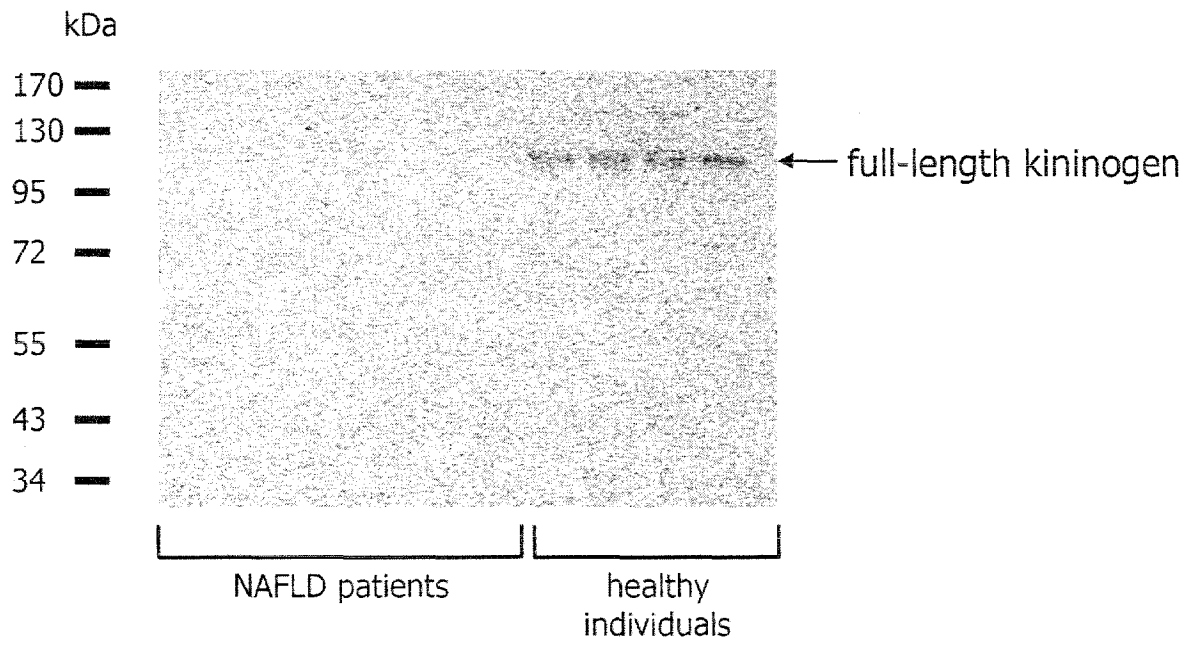


Fig. 9

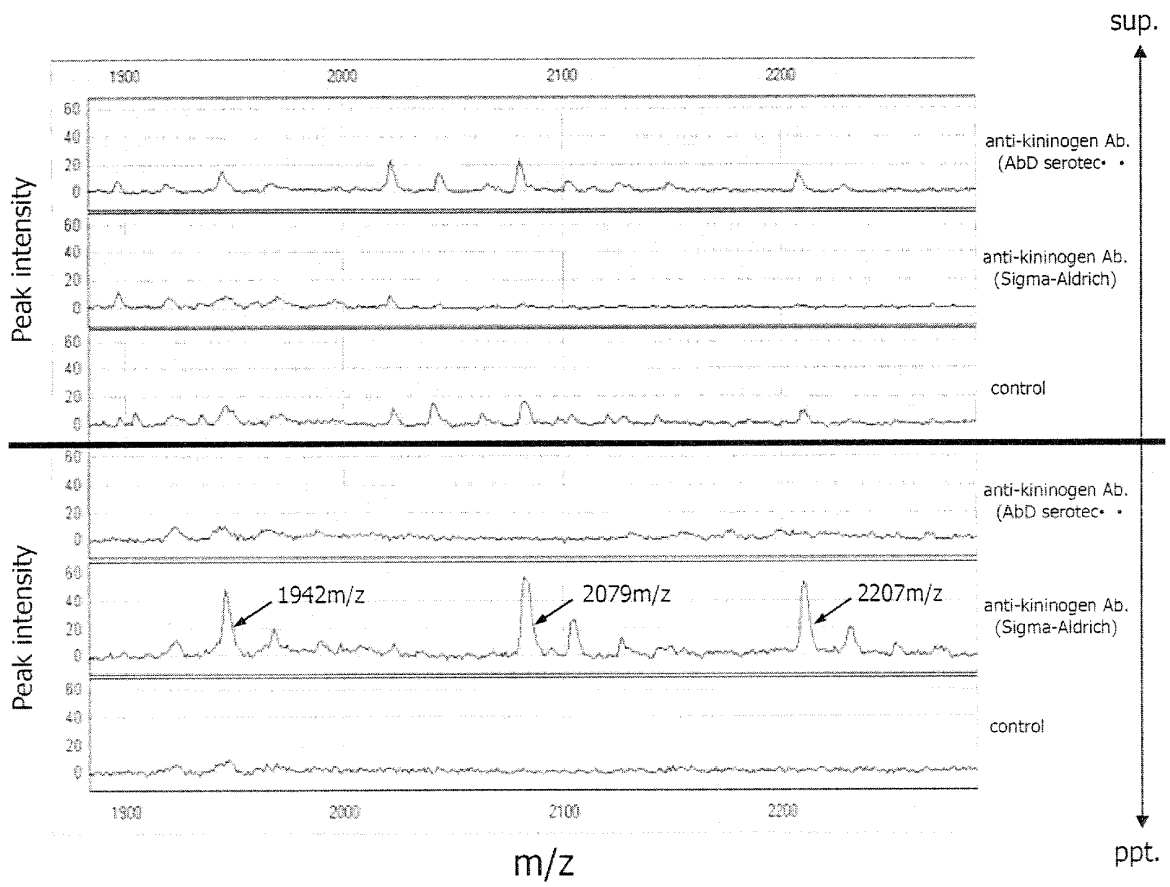


Fig. 10

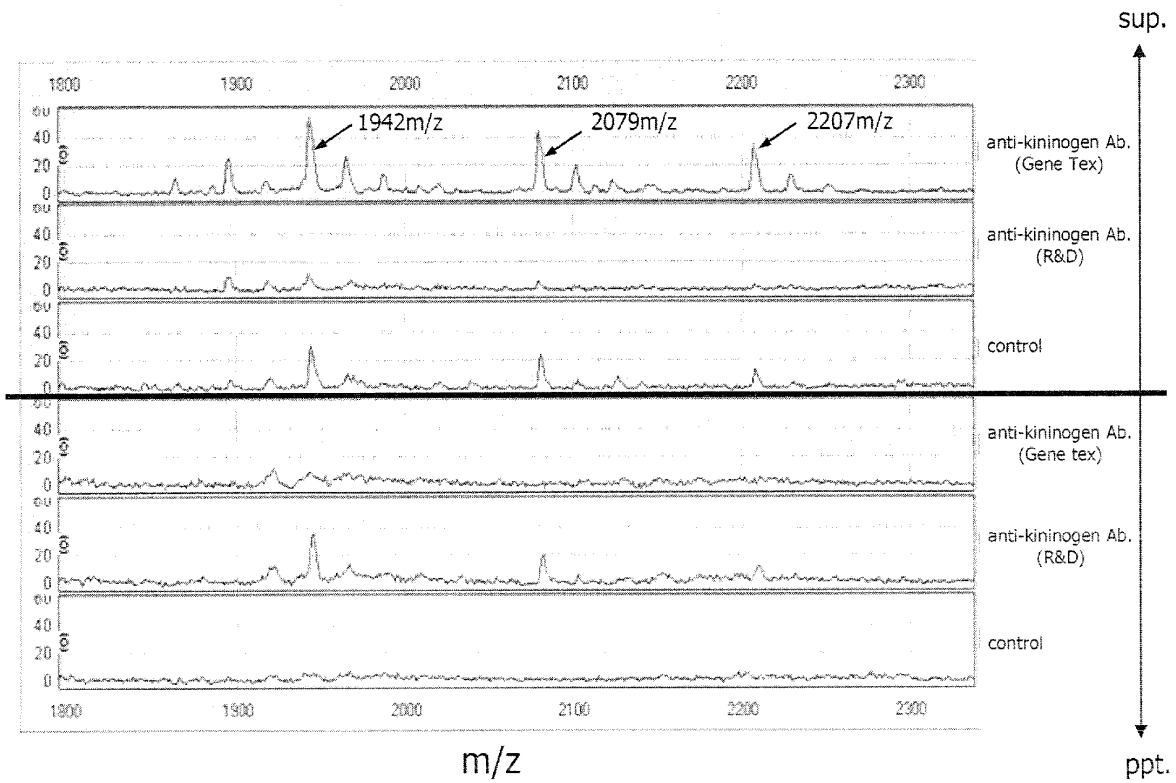


Fig. 11

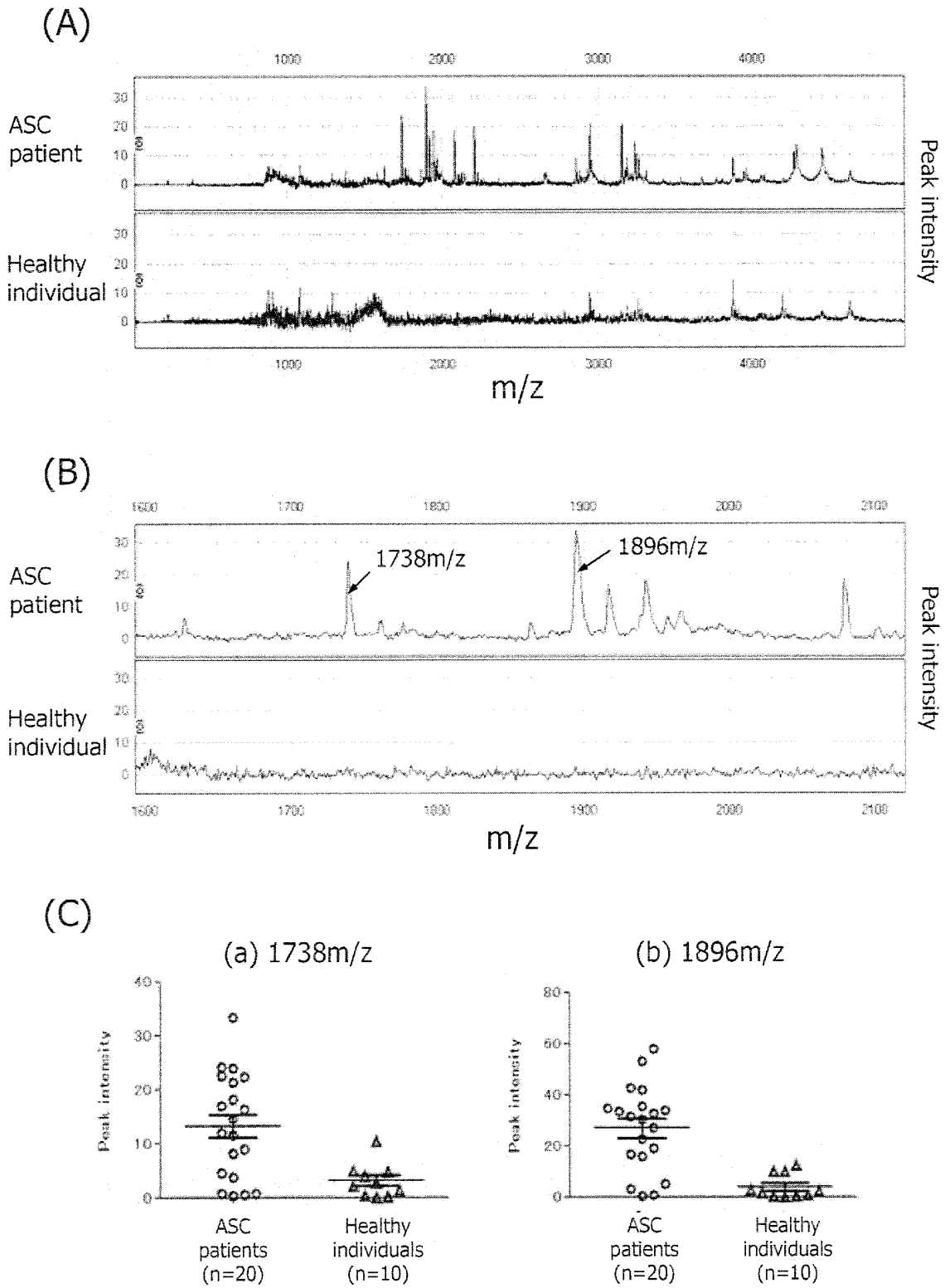


Fig. 12

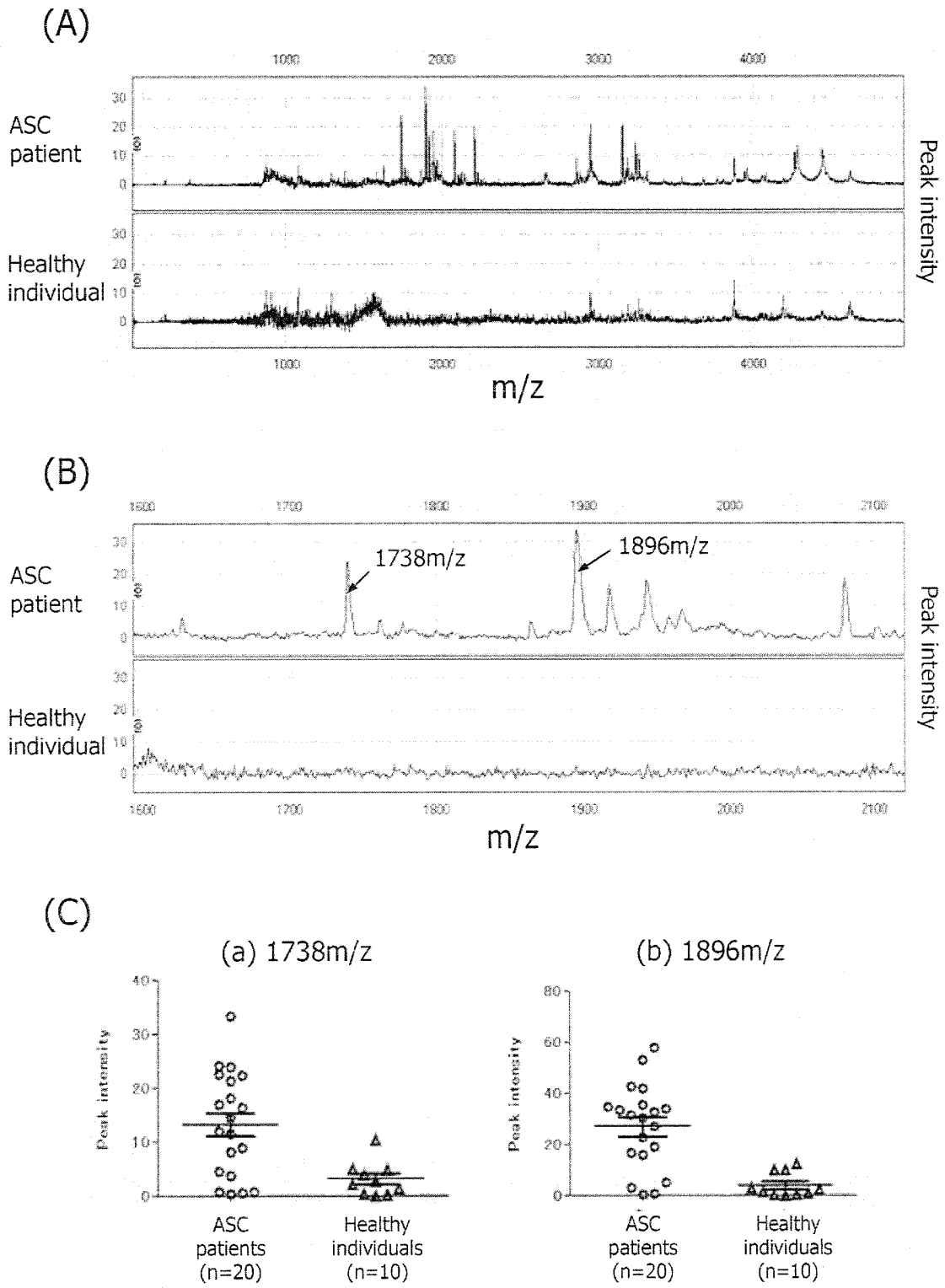
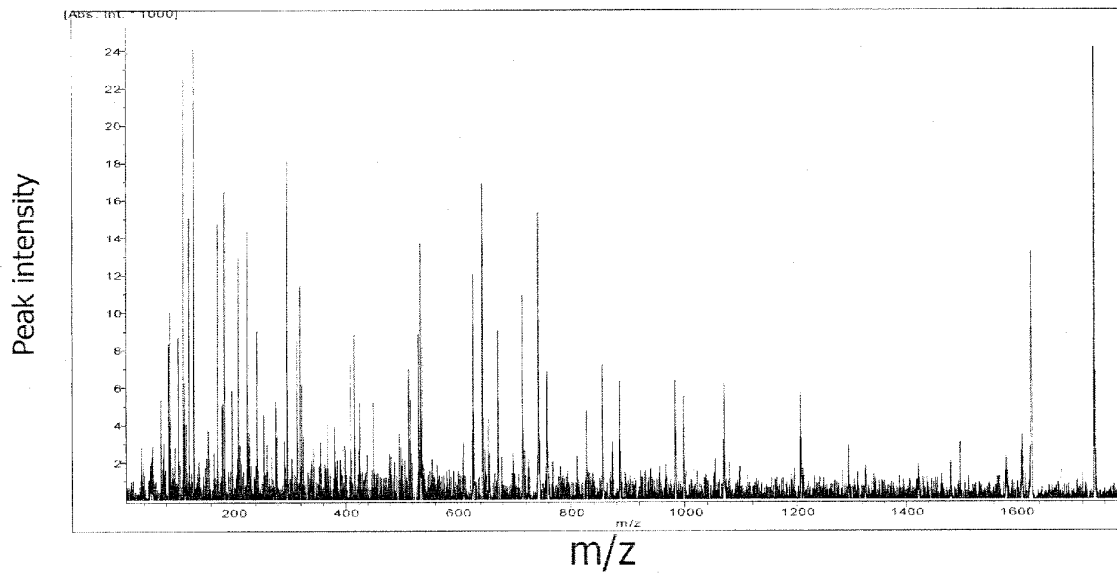


Fig. 13

(A) 1738m/z



(B) 1896m/z

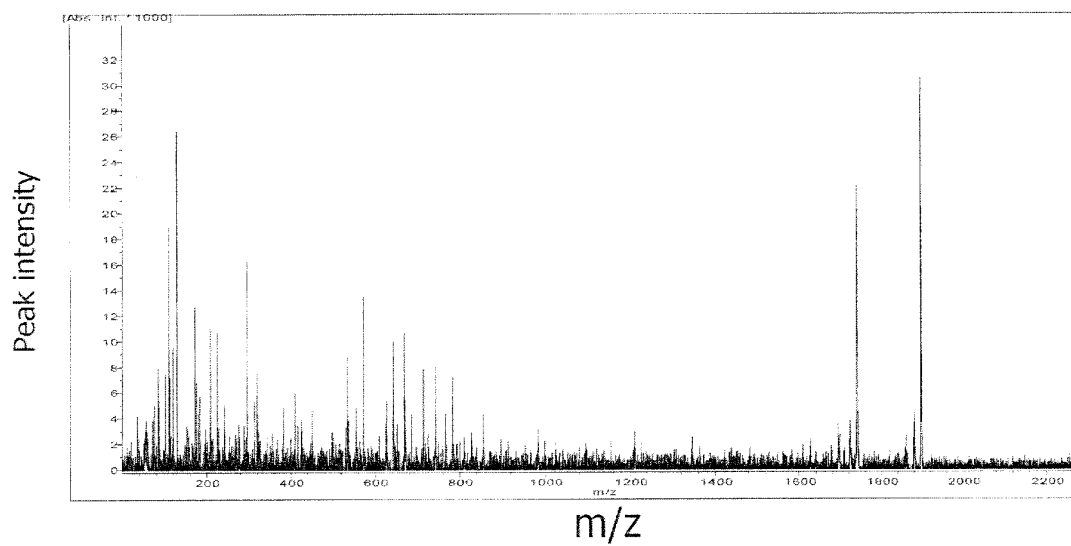
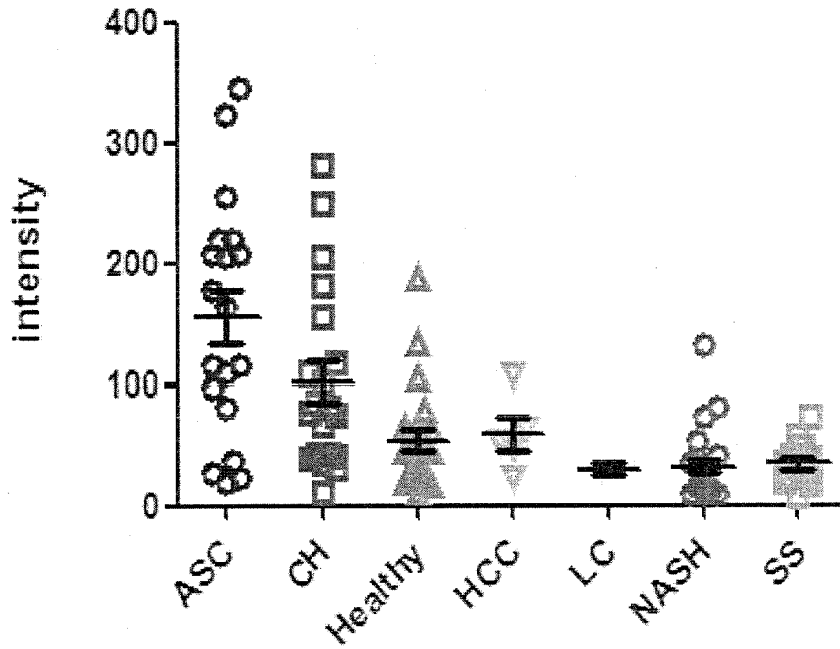


Fig. 14

(A)



(B)

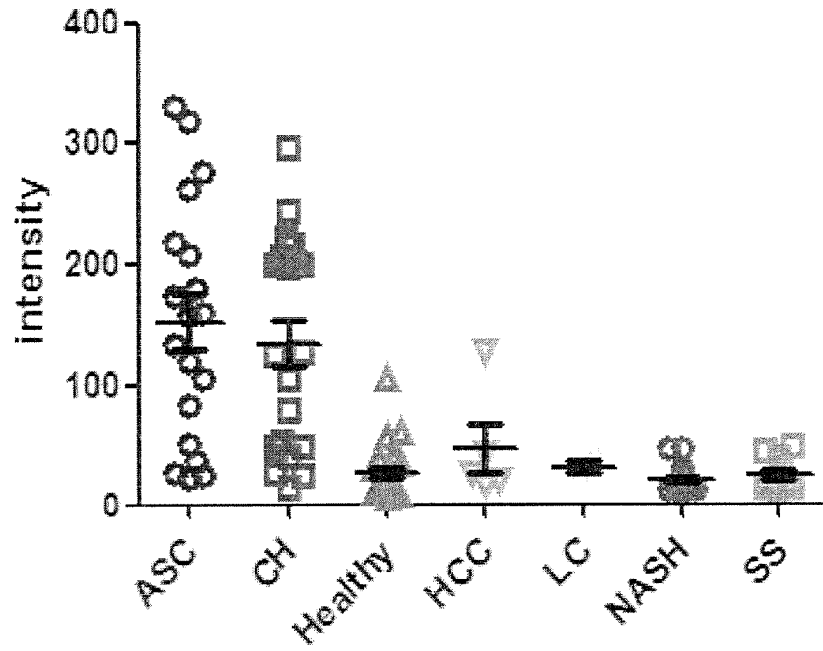


Fig. 15

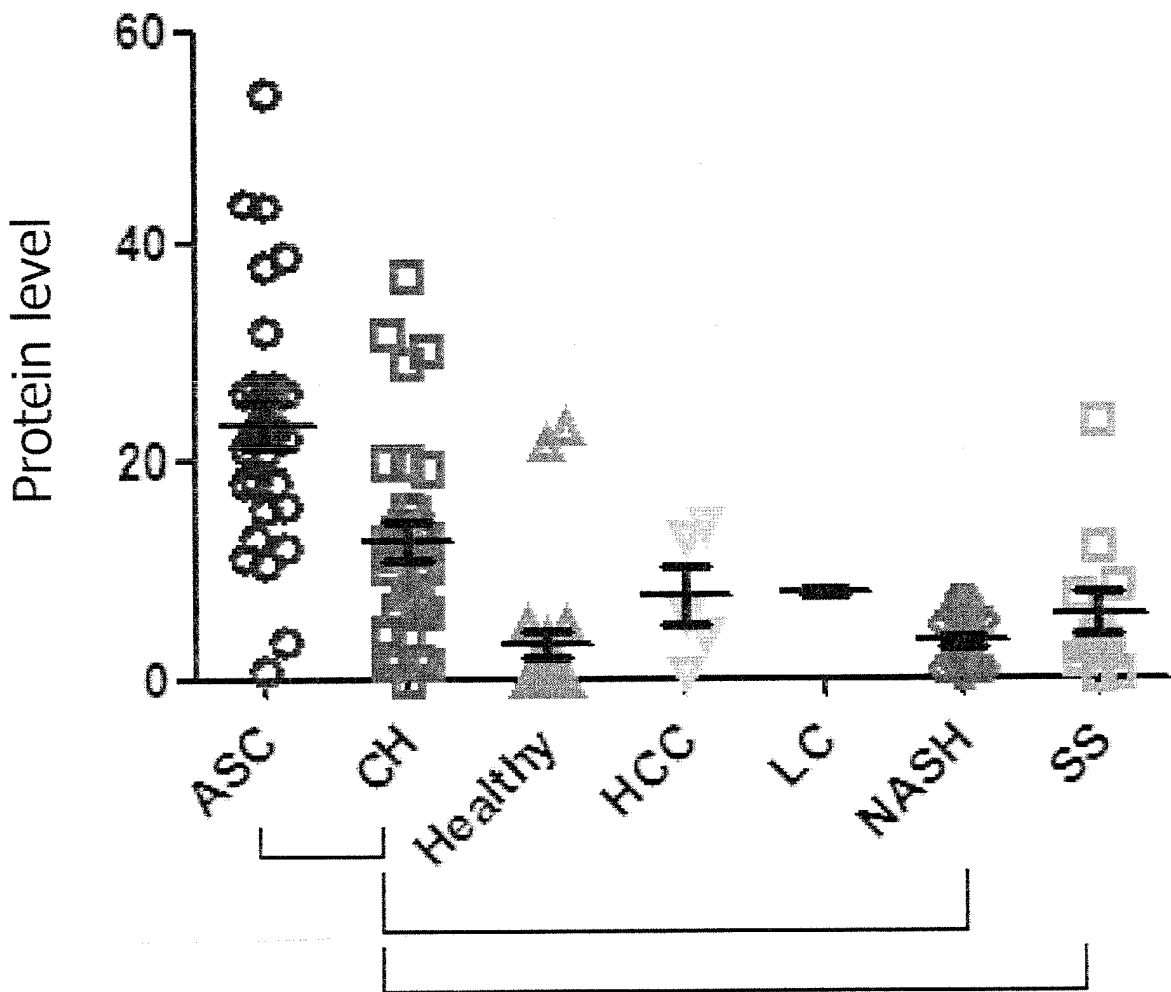


Fig. 16

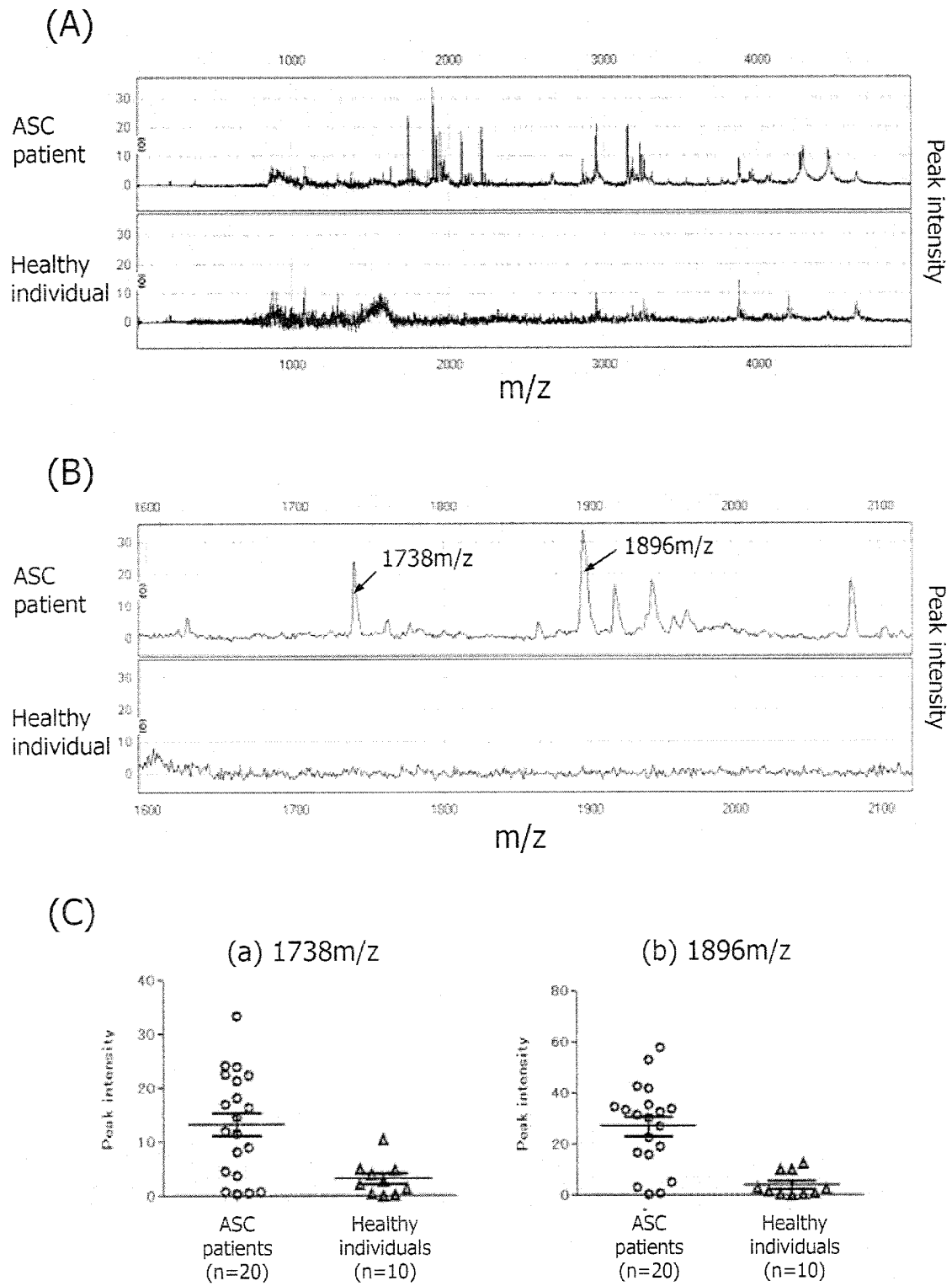


Fig. 17

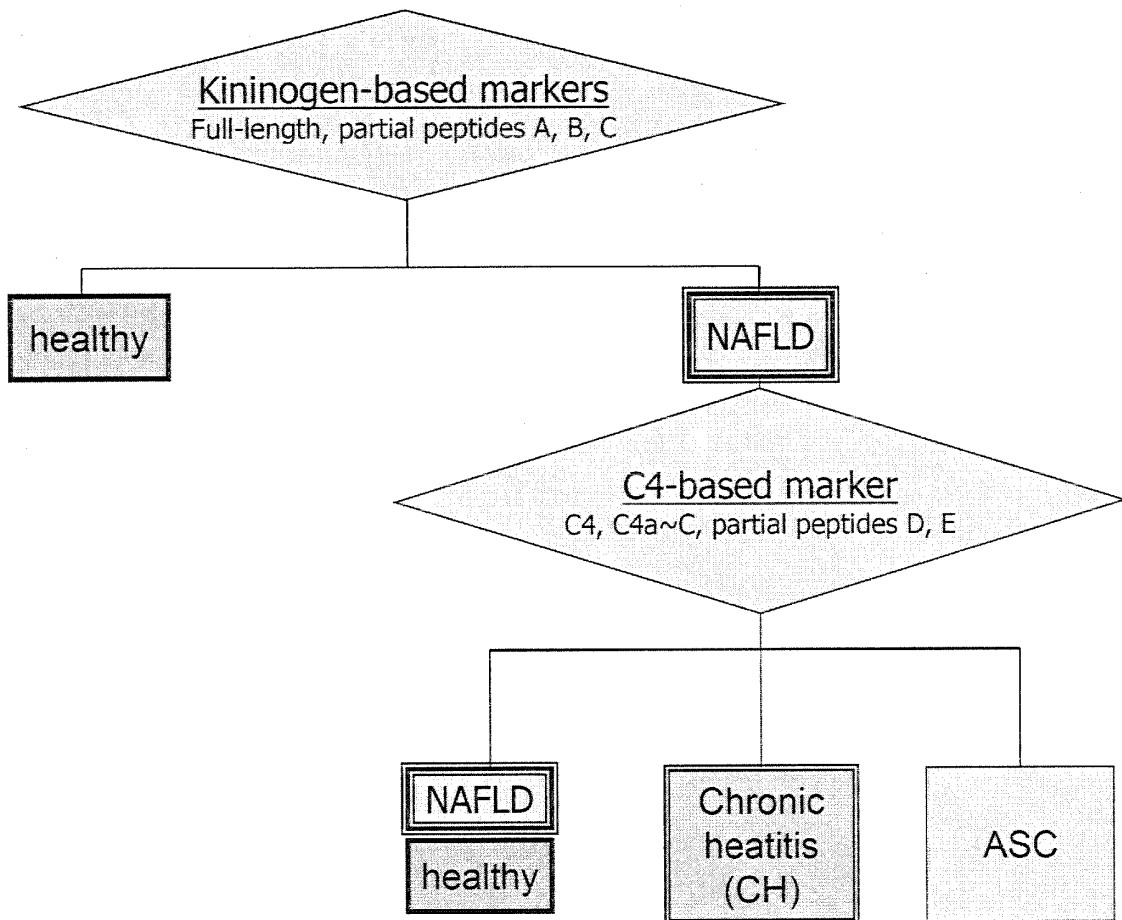
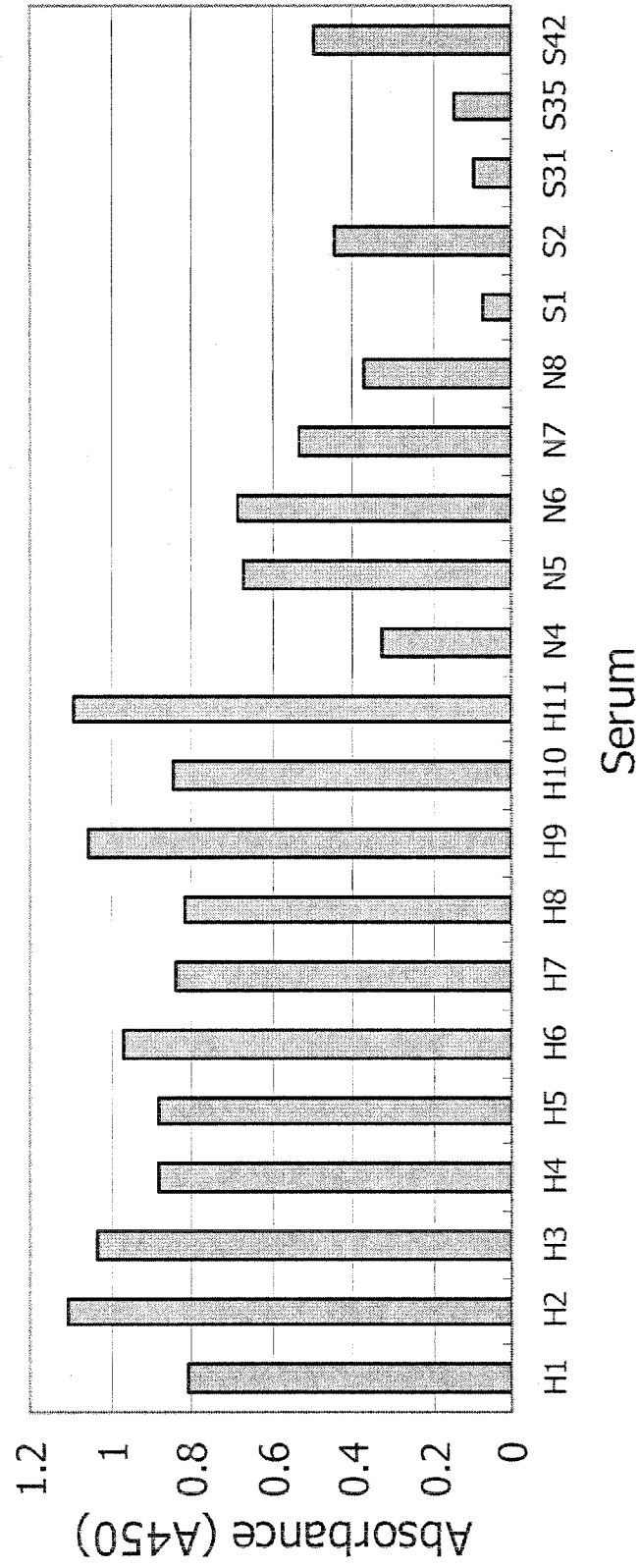


Fig. 18



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2008/068985

A. CLASSIFICATION OF SUBJECT MATTER G01N33/53 (2006.01) i, C07K16/18 (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, C07K16/18		
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 1996-2008 Kokai Jitsuyo Shinan Koho 1971-2008 Toroku Jitsuyo Shinan Koho 1994-2008		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CAplus (STN), JSTPlus (JDreamII), JMEDPlus (JDreamII), JST7580 (JDreamII)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOUHNİK J. et al., "Biochemical and physiological studies on two T-kininogen species using monoclonal antibodies.", Biochimica et Biophysica Acta, 1992.07.13, Vol.1122, No.1, P.70-76	3-5
X	JP 2006-308533 A (MCBI Inc.), 09 November, 2006 (09.11.06), (Family: none)	8-10
A	Mamoru FUJII, 'Kakushu Kan Shikkan ni Okeru Kessho Kallikrein-kinin-kei no Hendo to sono Rinshoteki Igi -Tokuni Alcohol-sei Kan Shogai o Chushin to shite-', Japanese Journal of Gastroenterological Surgery, 05 March, 1985 (05.03.85), Vol.82, No.3, pages 450 to 458	1-6, 12, 13, 15-18
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> 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	"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
"E"	earlier application or patent but published on or after the international filing date	"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
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"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	
Date of the actual completion of the international search 26 November, 2008 (26.11.08)		Date of mailing of the international search report 09 December, 2008 (09.12.08)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2008/068985

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Yuko MAEJIMA et al., 'Kan Shikkan Kanja ni Okeru Kessei Hotaika Oyobi C3, C4 Tanpaku Ryo ni tsuite no Kento', Journal of Tokyo Women's Medical University, 1981, Vol.51, No.4, pages 441 to 446	7-12, 14-18
A	Yoichiro IKEDA et al., 'Chumoku sareru Jin Kanren Tanpaku-45 C4', Kidney and dialysis, 25 December, 2006 (25.12.06), Vol.61, No.6, pages 750 to 752	7-12, 14-18
P, X	Yuko SATO et al., 'Kessei Proteome Kaiseki de Dotei shita Kininogen Danpen wa Hi-Alcohol-sei Shibokan Shikkan de Zoka suru', Dai 44 Kai The Japan Society of Hepatology Sokai Koen Yoshi, 30 April, 2008 (30.04.08), Vol.49, No.Supplement1, P.A163	1-18

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专利名称(译)	用于诊断肝病的生物标志物		
公开(公告)号	EP2216651A1	公开(公告)日	2010-08-11
申请号	EP2008840152	申请日	2008-10-20
[标]申请(专利权)人(译)	宫崎县立INDAL支持FOUND		
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[标]发明人	TSUBOUCHI HIROHITO UTO HIROFUMI OKANOUE TAKESHI ISHIDA YO ICHI SATO YUKO SUDO MASAYUKI		
发明人	TSUBOUCHI, HIROHITO UTO, HIROFUMI OKANOUE, TAKESHI ISHIDA, YO-ICHI SATO, YUKO SUDO, MASAYUKI		
IPC分类号	G01N33/53 C07K16/18		
CPC分类号	G01N33/6893 G01N2333/8139 G01N2800/085		
代理机构(译)	法思博事务所		
优先权	2007270799 2007-10-18 JP 2008145337 2008-06-03 JP		
其他公开文献	EP2216651A4		
外部链接	Espacenet		

摘要(译)

本发明公开了一种用于诊断肝脏疾病的标记物，其可以以简单的方式确定疾病;抗体指导agaist标记;诊断代理人;诊断方法;和用于血液或血清中的标记物检测的方法。蛋白质组分析显示全长激肽原和三种部分肽的数量(序列A:位置-440至位置-456,序列B:位置-439至位置-456,序列C:位置-43至位置-456)非酒精性脂肪肝患者血清与健康人血清明显不同;建立了一种可以方便地用于医学检查的非酒精性脂肪肝的诊断剂和检测方法。使用基于激肽原的标记物和基于C4的标记物(全长序列或其部分肽)的组合能够鉴定慢性肝炎和无症状病毒载体,以及非酒精性脂肪肝病。

SEQUENCE LISTING

<110> Miyazaki Prefectural Industrial Support Foundation
 Kagoshima University
 Chugai Seiyaku Kabushiki Kaisha

<120> BIOMARKER FOR DIAGNOSIS OF LIVER DISEASE

<130> S1848 EP S3

<140> ECT/JF2008/068985

<141> 2008-10-20

<150> JP 2007-270799

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