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

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530/389.1; 530/388.2

(57) **ABSTRACT**

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-438 to position-456) in sera of patients with non-alcoholic fatty liver disease are significantly 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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Fig. 1

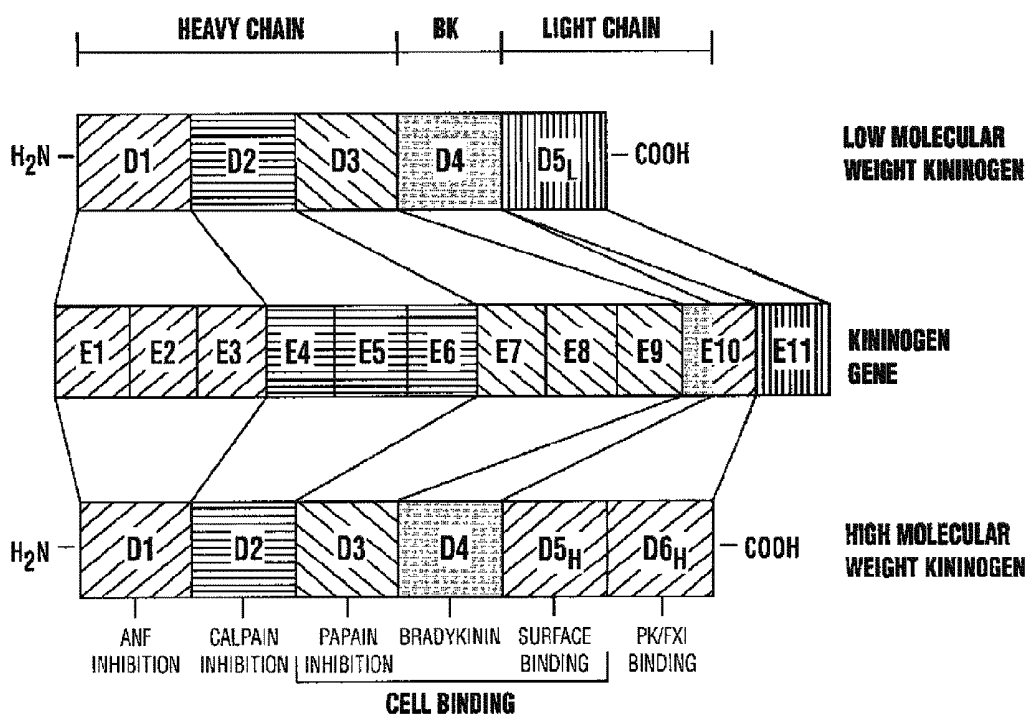


Fig. 2

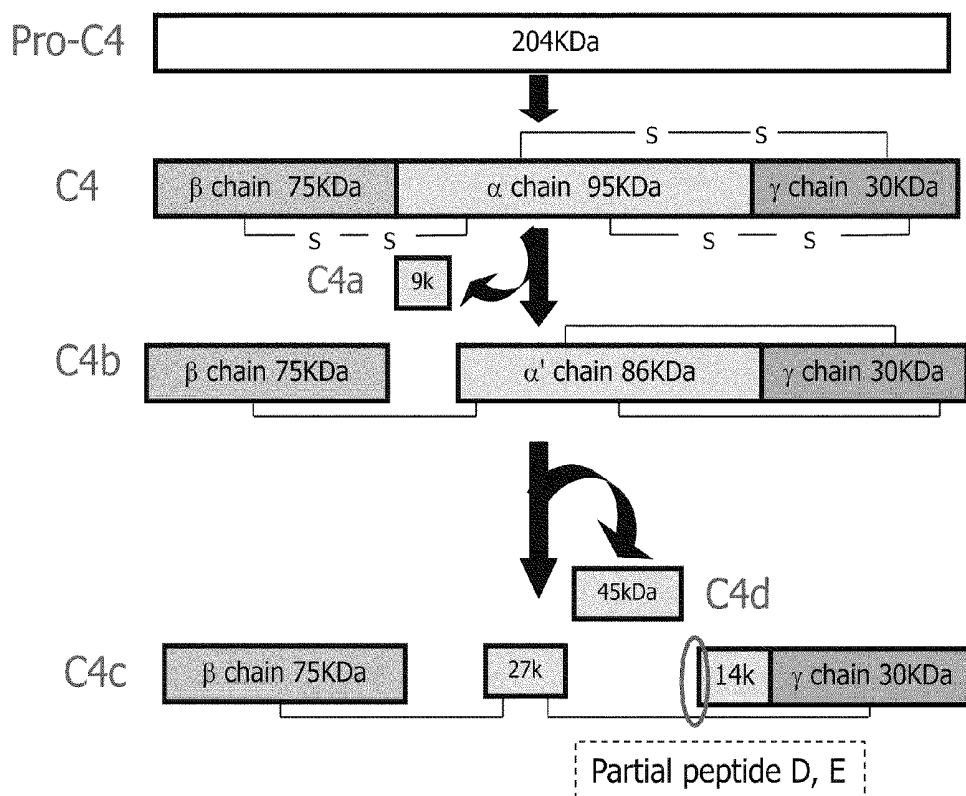


Fig. 3

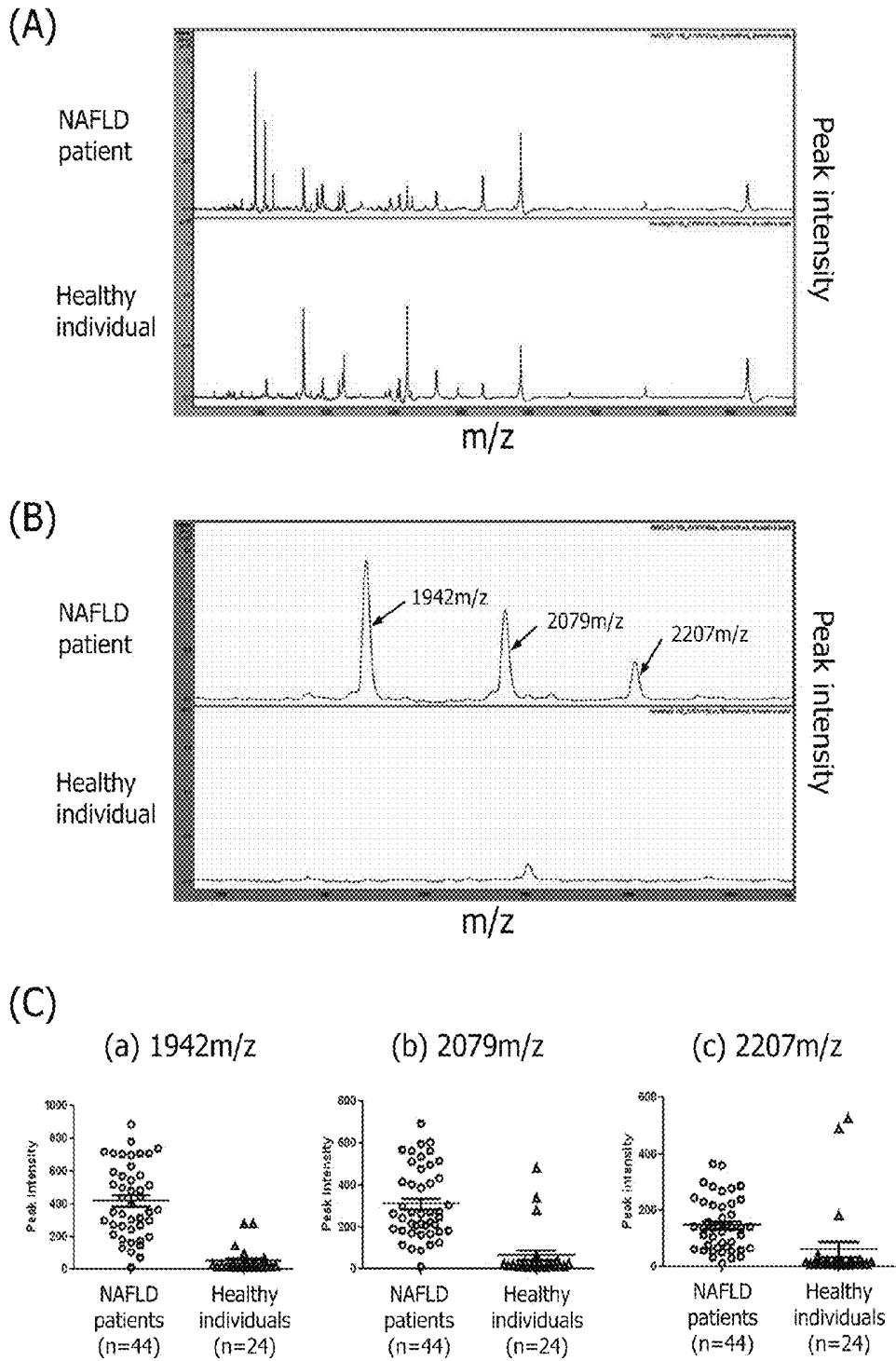
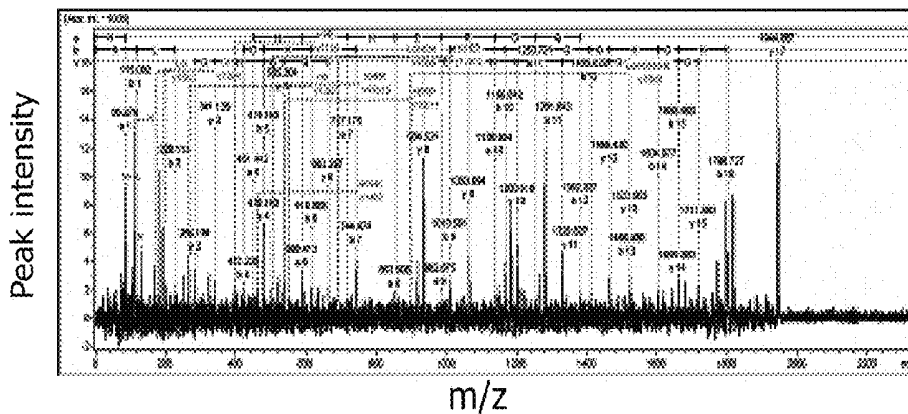
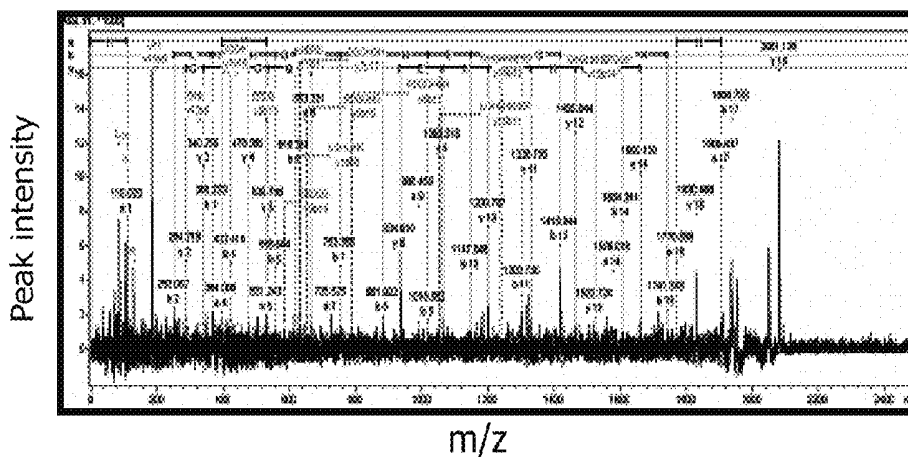


Fig. 4

(A) 1942m/z



(B) 2079m/z



(C) 2207m/z

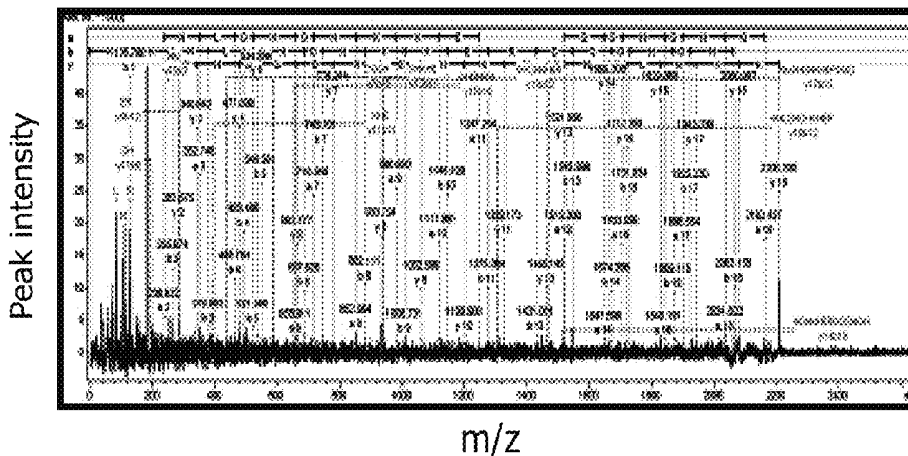


Fig. 7

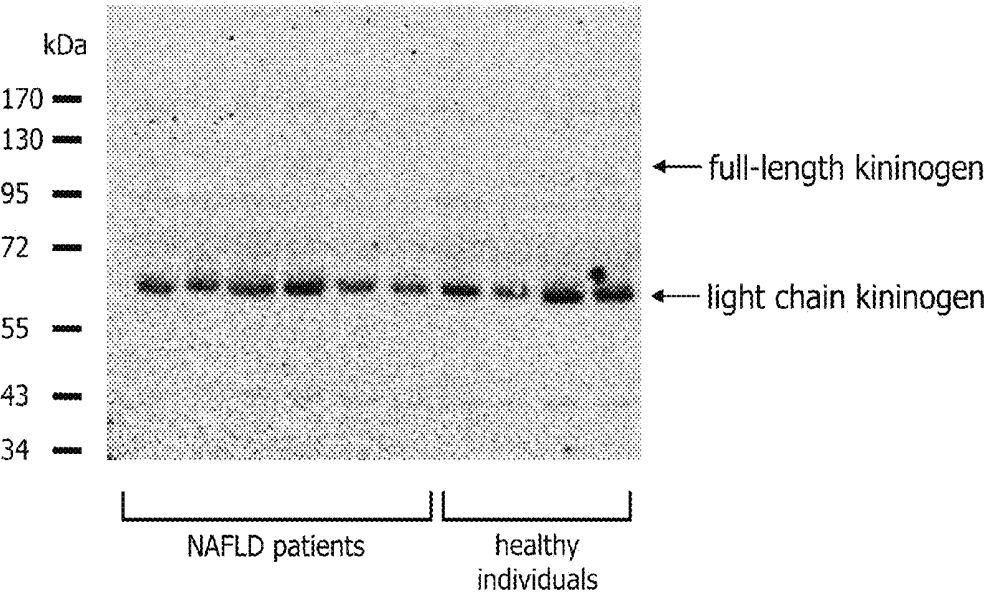


Fig. 8

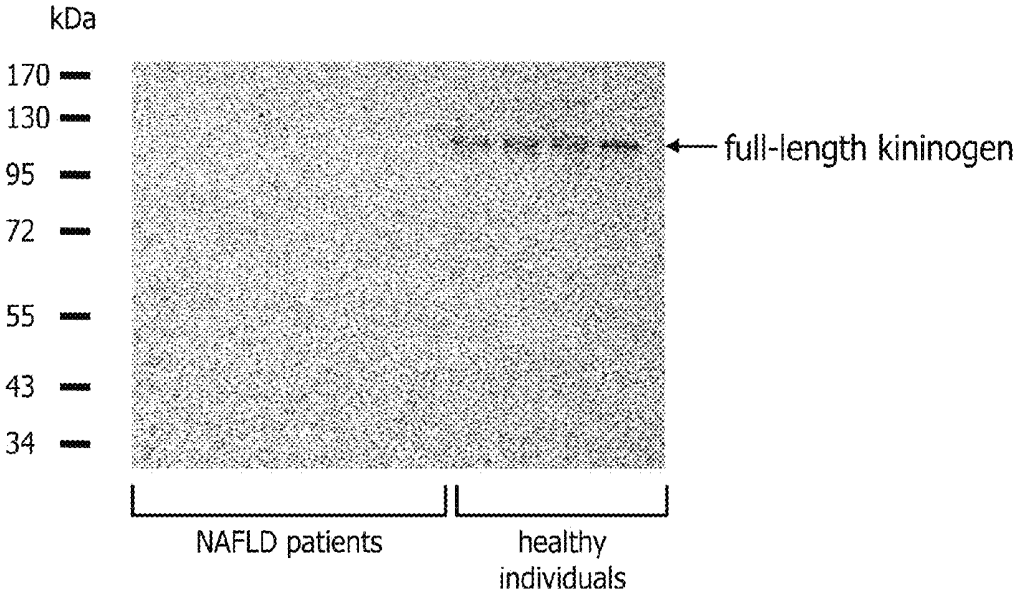


Fig. 9

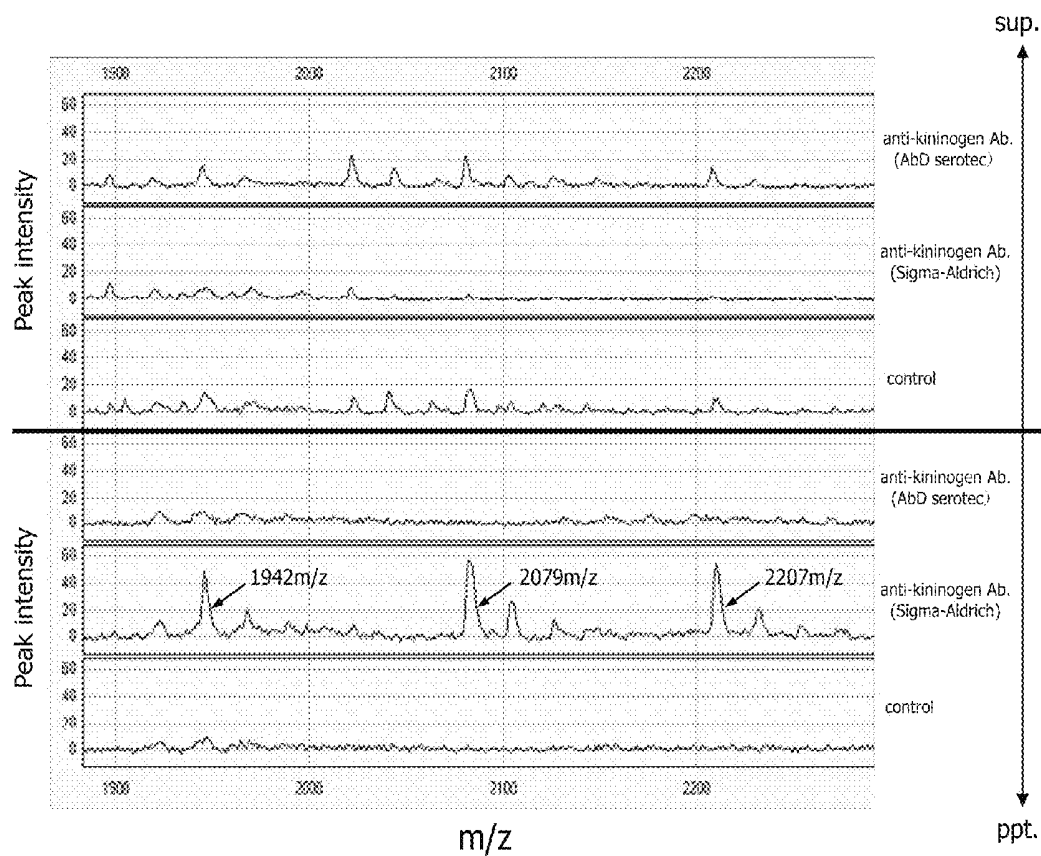


Fig. 10

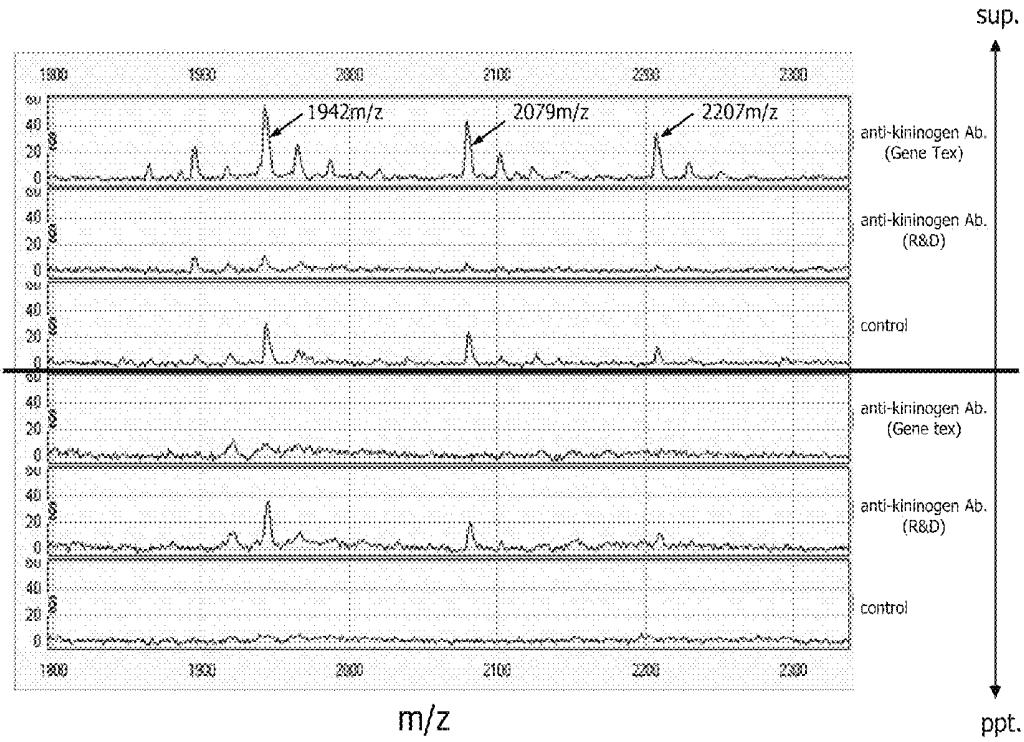


Fig. 11

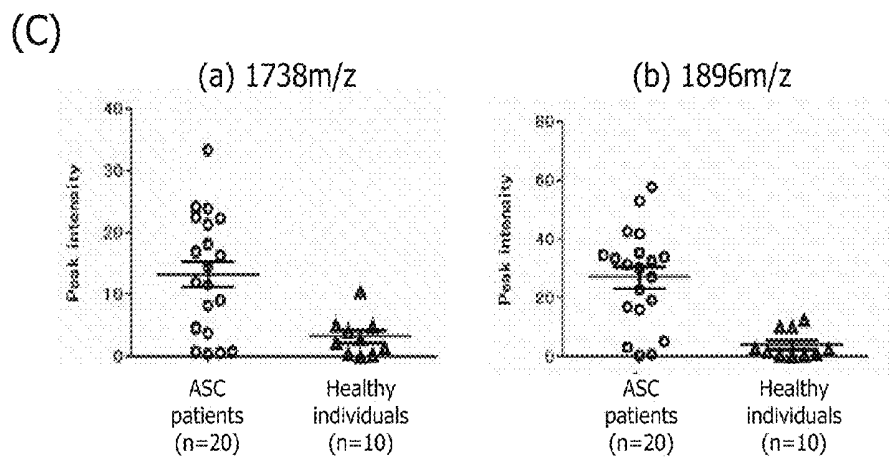
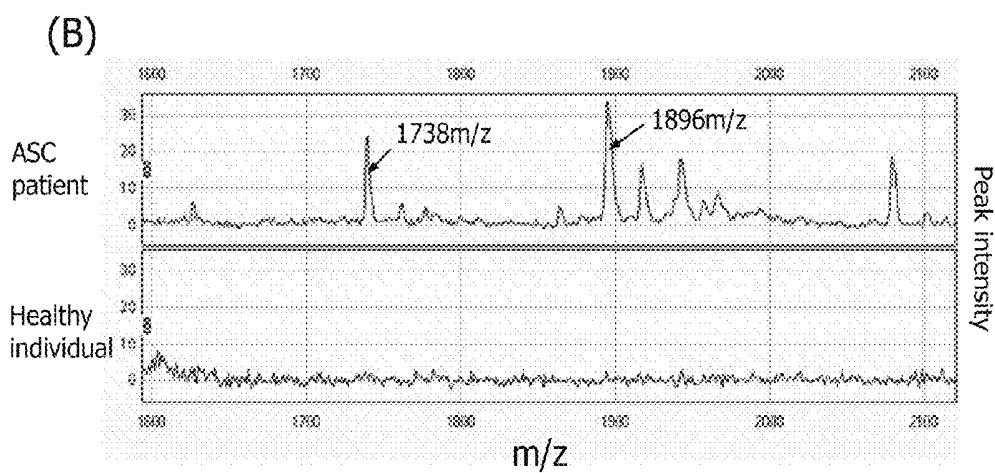
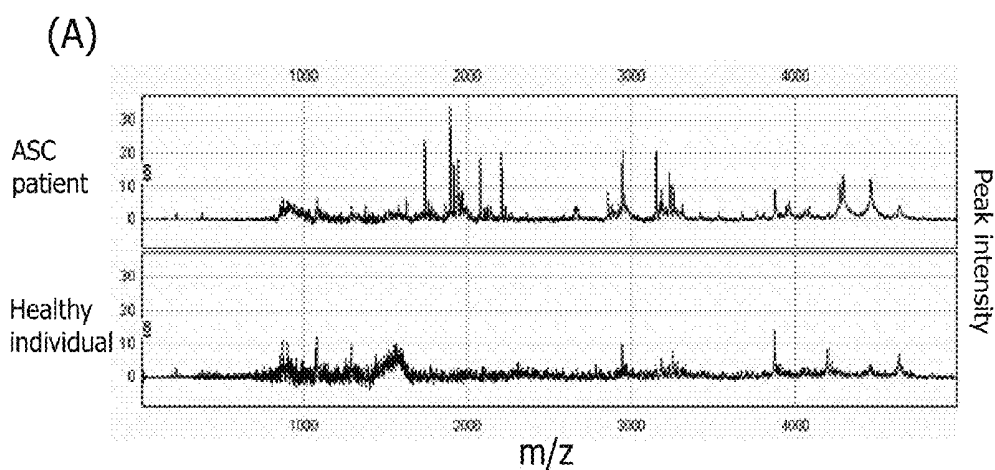


Fig. 12

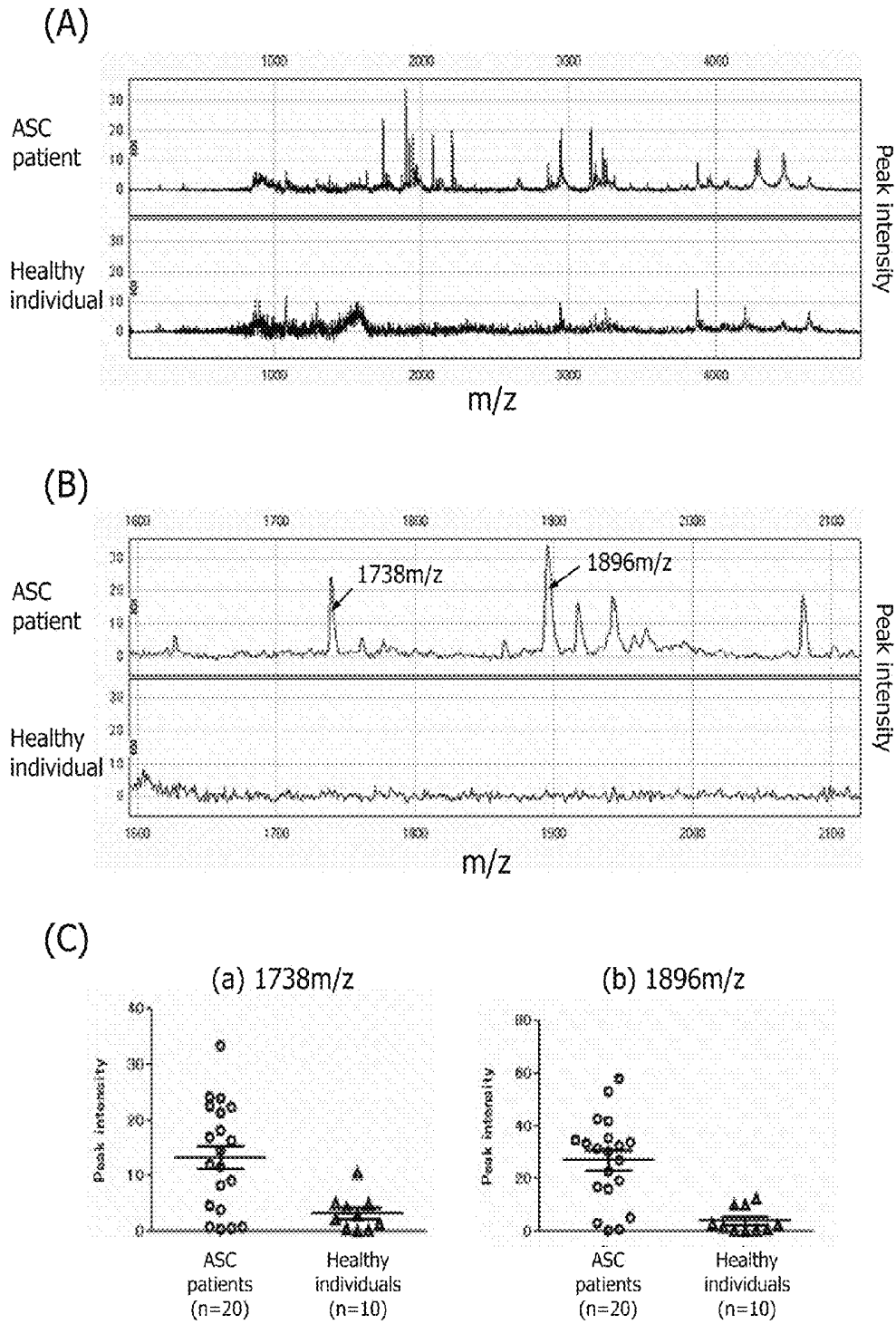
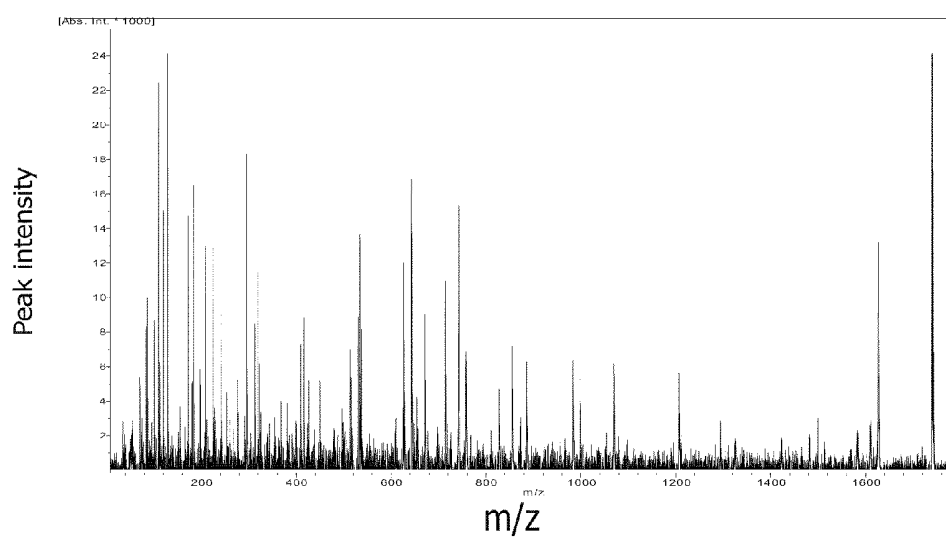


Fig. 13

(A) 1738m/z



(B) 1896m/z

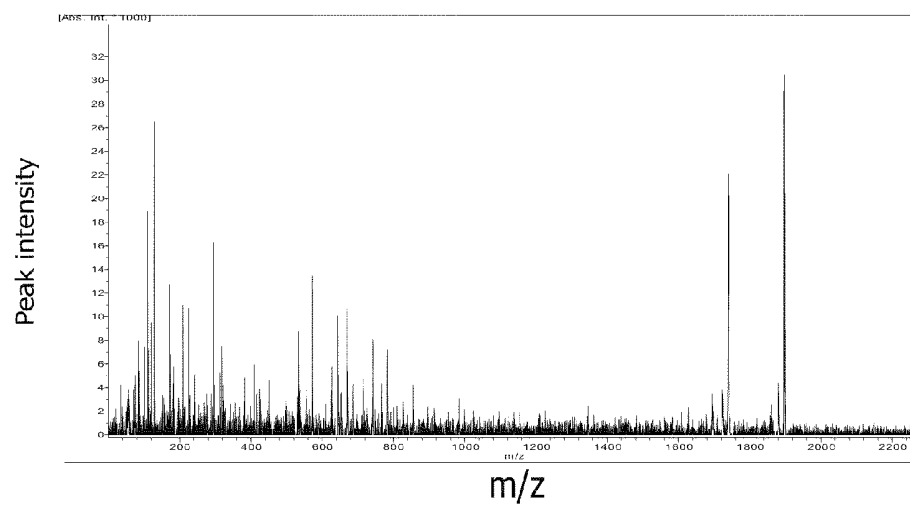


Fig. 14

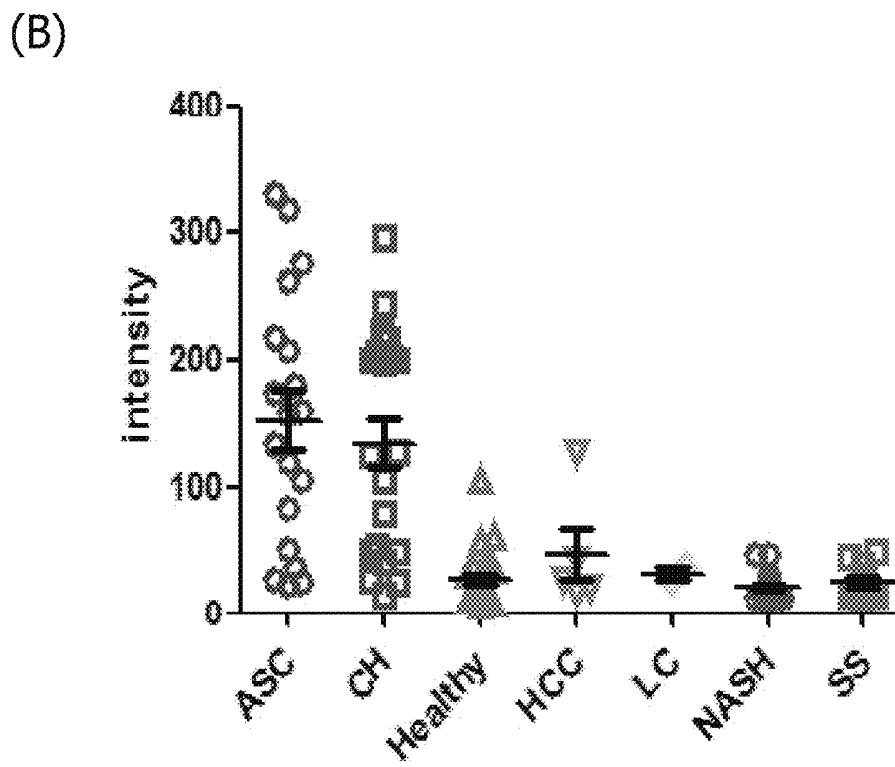
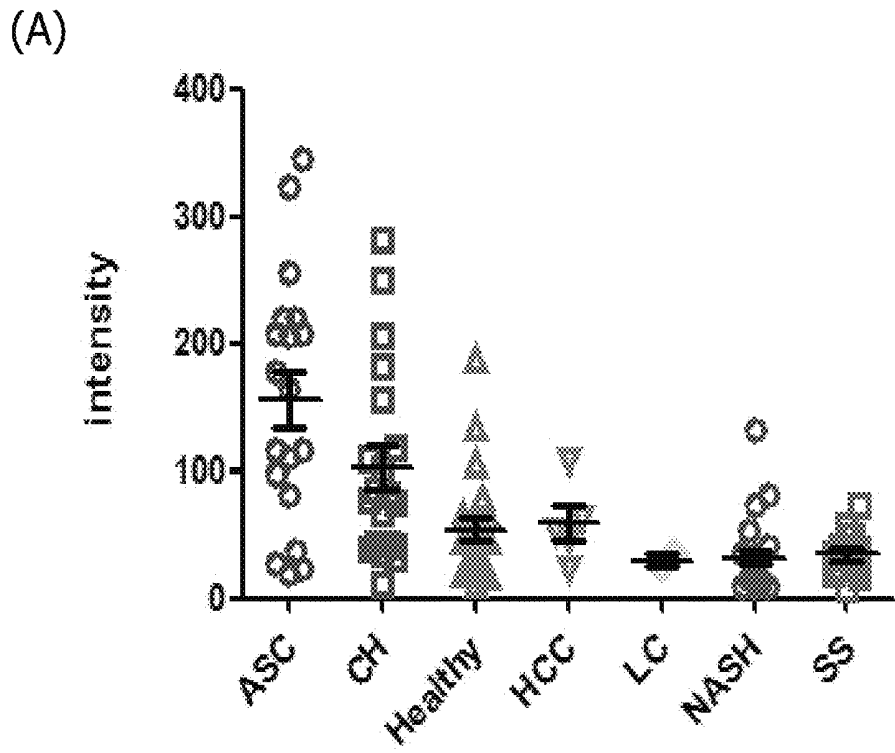


Fig. 15

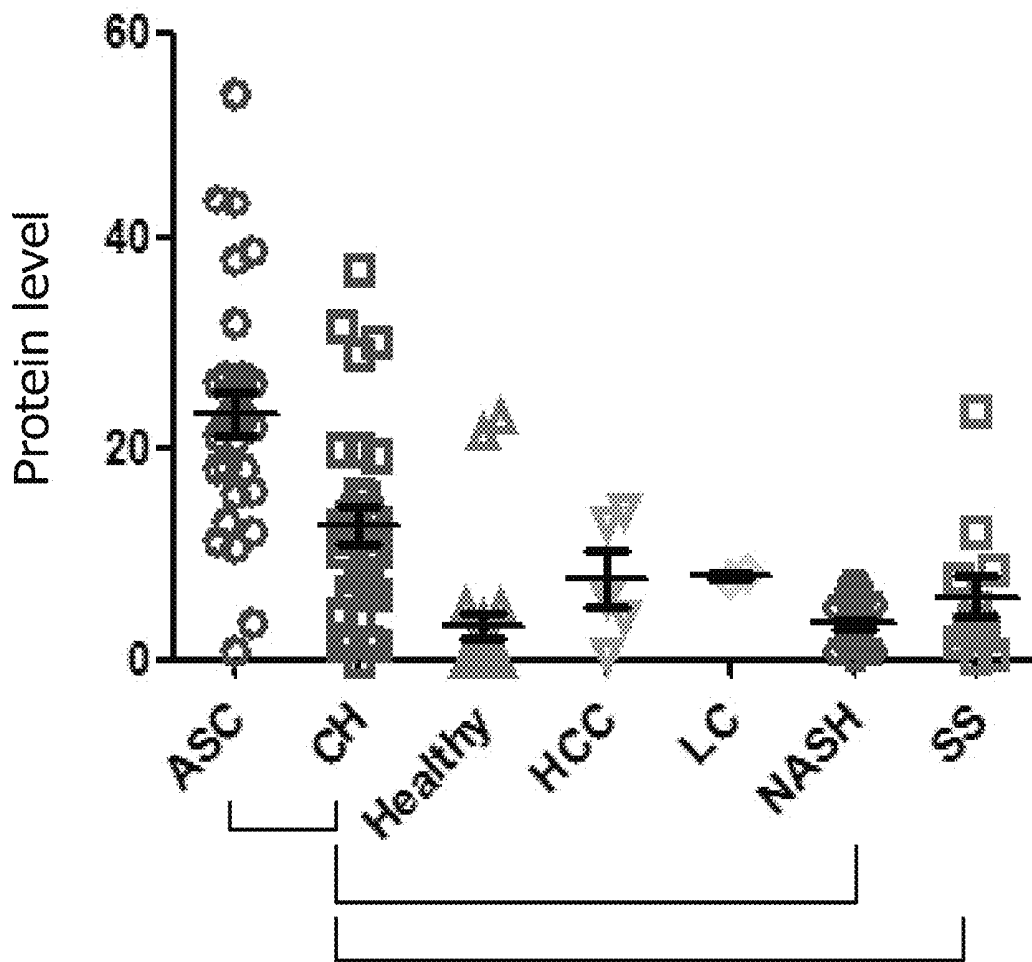


Fig. 16

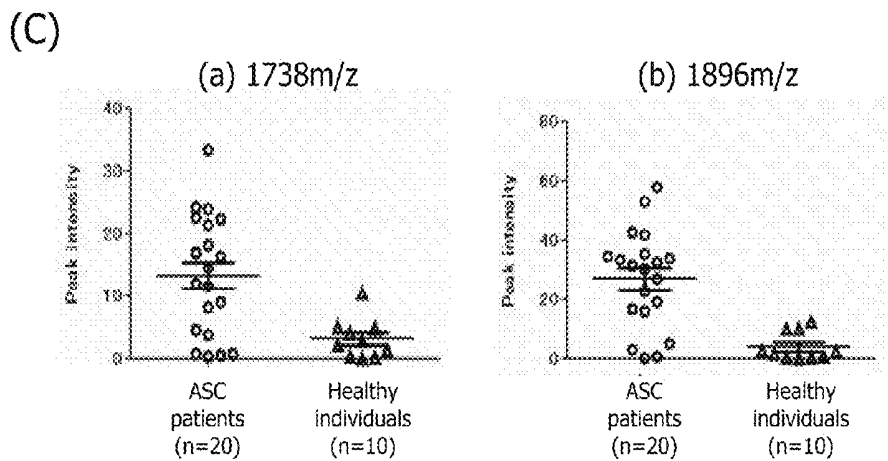
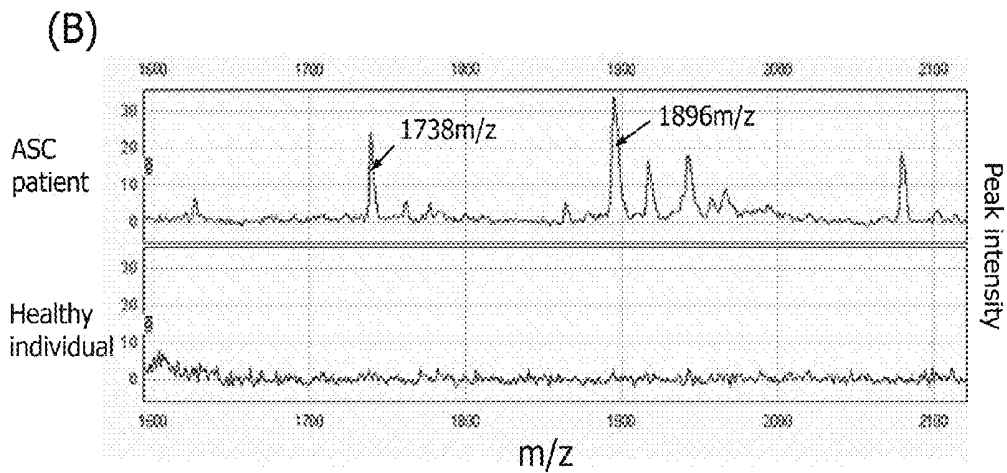
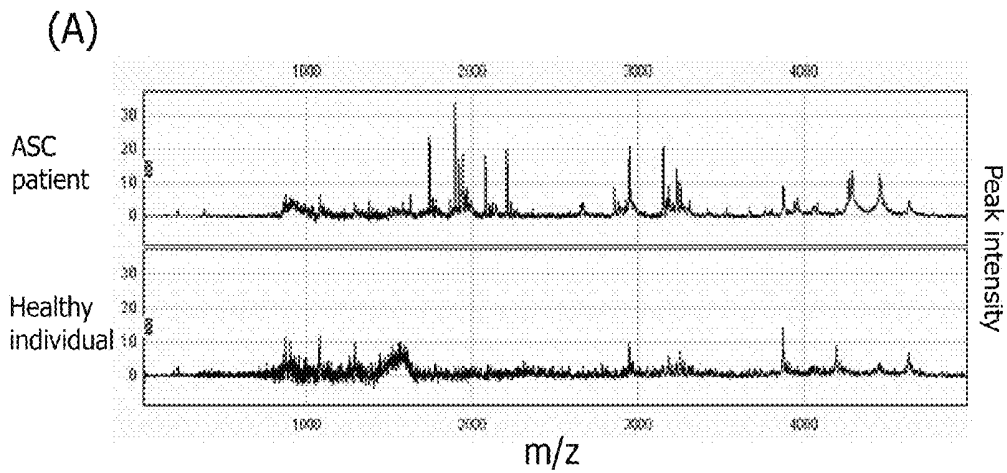


Fig. 17

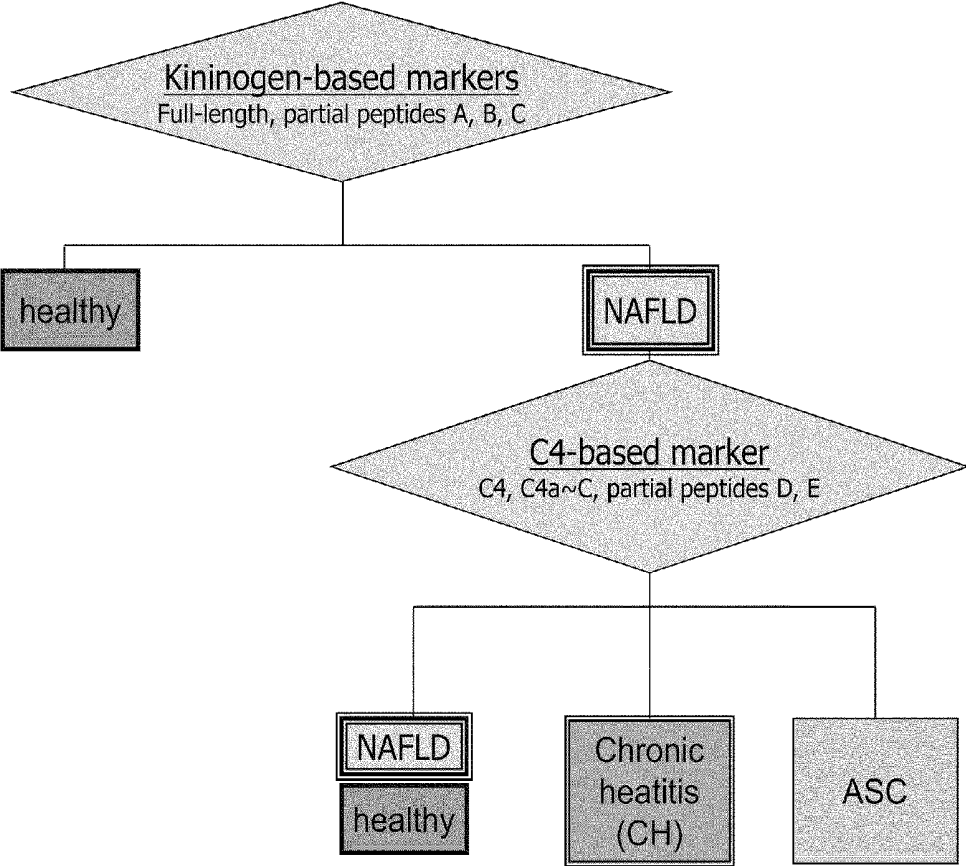
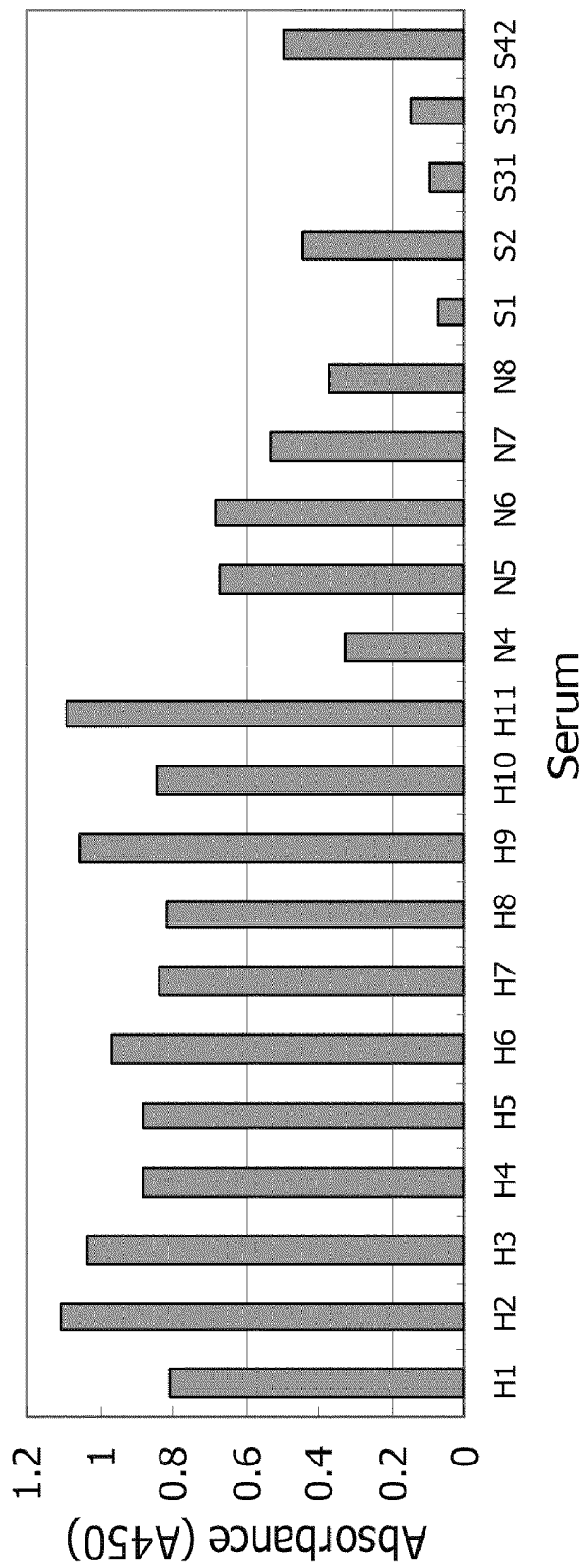


Fig. 18



BIOMARKER FOR DIAGNOSIS OF LIVER DISEASE

TECHNICAL FIELD

[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”).

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.

[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 an NAFLD diagnostic marker.

[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.

[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 indi-

viduals 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.

[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.

[0007] Patent Document 1: Japanese Unexamined Patent Application Publication No. 04-110660

[0008] Patent Document 2: Japanese Unexamined Patent Application Publication No. 2006-300689

[0009] Patent Document 3: Japanese Unexamined Patent Application Publication No. 2006-308533

[0010] 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)

[0011] 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

[0012] 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.

[0013] 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.

[0014] 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.

Means for Solving the Problems

[0015] The present invention, which achieves the above-mentioned objects, includes the following aspects:

[0016] 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

-continued

sequence C:
Lys His Asn Leu Gly His Gly His Lys His Glu

Arg Asp Gln Gly His Gly His Gln;

[0017] 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;

[0018] 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;

[0019] 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;

[0020] 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;

[0021] 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;

[0022] 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;

[0023] 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;

[0024] Aspect 9: The antibody according to Aspect 8, wherein the antibody is a polyclonal antibody;

[0025] Aspect 10: The antibody according to Aspect 8, wherein the antibody is a monoclonal antibody;

[0026] 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;

[0027] 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;

[0028] 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;

[0029] 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;

[0030] 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;

[0031] 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;

[0032] Aspect 17: The detecting method according to Aspect 16, wherein the detection is performed by ELISA; and

[0033] 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.

[0034] 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.

[0035] In addition to the above aspects, the present invention may preferably include the following embodiments:

[0036] 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;

[0037] 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;

[0038] 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;

[0039] 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;

[0040] 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;

[0041] 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;

[0042] 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;

[0043] 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

[0044] 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

[0045] The present invention is advantageous in that not only diagnosis of NAFLD but also diagnosis of progress thereafter can be expected.

[0046] (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.

[0047] (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.

[0048] (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.

[0049] (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

[0050] FIG. 1 is a schematic diagram illustrating domain structures of amino acid sequences of kininogens.

[0051] FIG. 2 is a schematic diagram illustrating domain structures of complement C4 (C4A and C4B) and its decomposition products.

[0052] 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).

[0053] 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).

[0054] FIG. 5 is a photograph showing the results of Western blotting using RABBIT ANTI HUMAN HMW-KININOGEN (anti-kininogen polyclonal antibody) manufactured by AbD Serotec.

[0055] 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.

[0056] 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.

[0057] 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.

[0058] 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.

[0059] 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.

[0060] 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).

[0061] 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).

[0062] FIG. 13 includes graphs showing MS/MS spectra of marker candidate peptides identified in ASCs using a database of SwissProt at 1738 m/z (A) and 1896 m/z (B).

[0063] FIG. 14 includes the results of analysis of peaks at 1738 m/z (a) and 1896 m/z (b) using a ClinProt system.

[0064] FIG. 15 is a graph showing the results of detection of the C4a protein level in sera by ELISA (BD OptEIA ELISA Kit).

[0065] 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).

[0066] 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.

[0067] 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

[0068] 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.

[0069] [Antibody for Detecting Markers]

[0070] 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.

[0071] 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.

[0072] 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.

[0073] 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.

[0074] 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.

[0075] Antibody-producing cells are collected after a pre-determined 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 µg per mouse in each immunization.

[0076] 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").

[0077] 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.

[0078] Examples of the myeloma cell include P3x63Ag8.653, P3x63Ag8U.1, NS-1, MPC-11, SP2/0, F0, S194, and R210.

[0079] 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.

[0080] 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).

[0081] 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.

[0082] 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.

[0083] 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.

[0084] 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.

[0085] 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-human-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 GeneTex Inc.) and (mouse-monoclonal) Anti-human-kininogen/Kininostatin antibody (manufactured by R&D Systems) specific to K438-5531 peptides.

[0086] 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.

[0087] 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, immu-

nochemical 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.

[0088] 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.

[0089] 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.

[0090] Preferably, the ClinProt system is used together with ClinProt Profiling Kit 100 MB-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.

[0091] 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.

[0092] 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.

[0093] 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.

[0094] 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.

[0095] 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 2207 m/z).

[0096] 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.

[0097] 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).

[0098] 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.

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).

[0099] 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.

[0100] In addition, in the present invention, a combination of two or more of the partial peptides A, B, and C 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.

[0101] 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.

[0102] 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.

[0103] 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.

[0104] 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 markers to obtain a protein/partial peptide profile regarding the presence or absence of each maker.

[0105] 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 multimarker, 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.

[0106] 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: Expercy) and C4B with Accession No. P0C0L5 (SwissProt: Expercy). 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 mecha-

nism (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.

[0107] 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.

[0108] 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.

[0109] 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.

[0110] 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

[0111] 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.

[0112] 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.

[0113] 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 ClinProt 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

[0114] 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).

TABLE 1

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

* data shows average \pm standard deviation

[0115] 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

[0116] 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

[0117] 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 aceton 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.

[0118] 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

[0119] 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.

TABLE 2

Identification of NAFLD marker by MS/MS ion search			
m/z	Results of identification	Accession No. (SwissProt)	Sequence
1942	Kininogen-1 precursor	P01042	Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln
2079	Kininogen-1 precursor	P01042	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

[0120] 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

[0121] 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)

[0122] 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

[0123] 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)

[0124] 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: GTX14514 (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 K438-5531 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

[0125] 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

[0126] 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

[0127] 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: MAB1569 (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

[0128] 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.

[0129] 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)

[0130] 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

[0131] 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.

[0132] 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

[0133] 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)).

TABLE 3

Peaks with increased expression in patient with NAFLD (Protein chip)			
m/z	Patient with NAFLD (n = 44) Peak intensity	Healthy individual (n = 24) Peak intensity	P value
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	4.65 \pm 9.81	1.0 $\times 10^{-6}$
2656	8.49 \pm 4.04	5.72 \pm 6.89	0.00112
2858	7.04 \pm 4.35	2.27 \pm 2.99	1.2 $\times 10^{-6}$
2949	24.58 \pm 9.80	13.78 \pm 8.0 2	5.0 $\times 10^{-5}$

Example 2

Complement C4

[0134] [1] Detection of Serum Peptide with ClinProt System

(1) Material and Method

[0135] 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.

[0136] 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 to each other. 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.

[0137] 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

[0138] 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).

TABLE 4

Peaks with increasing expression in asymptomatic carrier (ClinProt)			
m/z	ASC (n = 44) Peak intensity	Healthy individual (n = 24) Peak intensity	P value
1497.98	15.76 \pm 12.8	4.66 \pm 2.92	6.1 $\times 10^{-5}$
1738.06	100.88 \pm 63.16	57.79 \pm 44.48	0.00971
1777.32	26.12 \pm 15.93	16.27 \pm 13.49	0.0286
1864.04	50.84 \pm 35.4	21.35 \pm 21.11	7.2 $\times 10^{-4}$
1895.98	94.68 \pm 58.7	31.63 \pm 21.1	4.0 $\times 10^{-6}$
1942.69	180.35 \pm 137.12	39.8 \pm 41.5	6.3 $\times 10^{-6}$
2079.63	162.02 \pm 62.26	56.33 \pm 67.19	4.0 $\times 10^{-6}$
2207.47	124.1 \pm 70.24	46.93 \pm 89.11	0.0041

* data shows average \pm standard deviation

[0139] 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 \times (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.

[0140] 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 (specific-

ity: 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

[0141] 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

[0142] 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 aceton 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 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 μ L of 0.1% TFA.

[0143] 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

[0144] 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.

TABLE 5

Identification of ASC marker by MS/MS ion search			
m/z	Results of identification	Accession No. (SwissProt)	Sequence
1738	Complement C4-A precursor (Complement C4-B precursor)	POCOL4 (POCOL5)	Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile
1896	Complement C4-A precursor (Complement C4-B precursor)	POCOL4 (POCOL5)	Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile Arg

[0145] The results show that the 1738 m/z and 1896 m/z correspond to 1337th to 1351st and 1337th to 1352nd amino acid sequences, respectively.

[0146] The peaks at 1738 and 1896 m/z were analyzed with the ClinProt system. Specimens were sera of 30 ASC sub-

jects, 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

[0147] 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

[0148] 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.

[0149] Two microliters of each serum was diluted with 600 μ L of PBBS (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄). Two microliters of this diluted serum was added to 200 μ L 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 μ L 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 μ L of Working Detector previ-

ously prepared by mixing 12 mL of biotinylated anti-human C4a polyclonal antibody and 48 μ L of 250 \times 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 μ L 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 μ L 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 statistical analysis was performed by a Mann-Whitney U test, and $P < 0.05$ was determined to be statistically significant.

(2) Results

[0150] 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.

[0151] 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

[0152] 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) Material and Method

[0153] 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 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 was diluted with 450 μ L 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 μ L 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.

[0154] 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

[0155] 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.

TABLE 6

Peaks with increasing expression in ASC (Protein chip)			
Molecular weight m/z	ASC (n = 20) Peak intensity	Healthy individual (n = 10) Peak intensity	P value
1738	25.57 \pm 9.13	6.74 \pm 5.40	5.5 \times 10 ⁻³
1863	10.57 \pm 3.79	4.98 \pm 2.28	1.8 \times 10 ⁻⁴
1896	10.70 \pm 10.23	2.34 \pm 2.38	3.7 \times 10 ⁻⁵
1817	19.75 \pm 10.34	3.42 \pm 7.48	1.1 \times 10 ⁻⁵
1942	14.63 \pm 9.02	4.65 \pm 9.81	1.3 \times 10 ⁻⁵
2079	8.49 \pm 4.04	5.72 \pm 6.89	1.9 \times 10 ⁻⁵
2207	7.04 \pm 4.35	2.27 \pm 2.99	1.5 \times 10 ⁻⁴
2858	24.58 \pm 9.80	13.78 \pm 8.02	2.4 \times 10 ⁻³

Example 3

Diagnosis by ELISA Using Anti-Kinogen Antibody

[0156] 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

[0157] Sera of 11 healthy individuals, 5 NASH subjects, and 5 simple steatosis subjects were used as specimens.

[0158] An anti-human kininogen monoclonal antibody (manufactured by R&D, Catalog Number: MAB1569) was diluted with an immobilization buffer (Sumitomo Bakelite) into 1 μ g/mL, and the diluted antibody solution was applied to a plate of New ELISA Plate B (Sumitomo Bakelite) in an amount of 100 μ L/well, followed by leaving to stand at room temperature for 1 to 2 hours. The plate was washed with 300 μ L 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 μ L/well, followed by leaving to stand at room temperature for 1 hour. The plate was washed with 300 μ L 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 μ L/well, followed by leaving to stand at room temperature for 1 hour. Then, the plate was washed with 300 μ L 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 μ L/well, followed by leaving to stand at room temperature for 1 hour. Then, the plate was treated with 300 μ L 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 μ L/well. After leaving to stand for 15 to 50 minutes, the reaction was terminated by adding 50 μ L of 2 M sulfuric acid, followed by colorimetry at 450 nm

(2) Results

[0159] From the results described above, H1 to H11, which were sera of healthy individuals, yielded an average \pm standard deviation of 0.937 \pm 0.117. In addition, N4 to S42, in which N means NASH and S means simple steatosis, yielded an average \pm standard deviation of 0.383 \pm 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

[0160] 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.

SEQUENCE LISTING

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<211> LENGTH: 644
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

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                20           25           30

Leu Phe Lys Ala Val Asp Ala Ala Leu Lys Lys Tyr Asn Ser Gln Asn
 35           40           45

Gln Ser Asn Asn Gln Phe Val Leu Tyr Arg Ile Thr Glu Ala Thr Lys
 50           55           60

Thr Val Gly Ser Asp Thr Phe Tyr Ser Phe Lys Tyr Glu Ile Lys Glu
 65           70           75           80

Gly Asp Cys Pro Val Gln Ser Gly Lys Thr Trp Gln Asp Cys Glu Tyr
 85           90           95

Lys Asp Ala Ala Lys Ala Ala Thr Gly Glu Cys Thr Ala Thr Val Gly
 100          105          110

Lys Arg Ser Ser Thr Lys Phe Ser Val Ala Thr Gln Thr Cys Gln Ile
 115          120          125

Thr Pro Ala Glu Gly Pro Val Val Thr Ala Gln Tyr Asp Cys Leu Gly
 130          135          140

Cys Val His Pro Ile Ser Thr Gln Ser Pro Asp Leu Glu Pro Ile Leu
 145          150          155          160

Arg His Gly Ile Gln Tyr Phe Asn Asn Asn Thr Gln His Ser Ser Leu
 165          170          175

Phe Met Leu Asn Glu Val Lys Arg Ala Gln Arg Gln Val Val Ala Gly
 180          185          190

Leu Asn Phe Arg Ile Thr Tyr Ser Ile Val Gln Thr Asn Cys Ser Lys
 195          200          205
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-continued

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 245 250 255
 Val Gln Pro Pro Thr Lys Ile Cys Val Gly Cys Pro Arg Asp Ile Pro
 260 265 270
 Thr Asn Ser Pro Glu Leu Glu Glu Thr Leu Thr His Thr Ile Thr Lys
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 Arg Asp Ser Gly Lys Glu Gln Gly His Thr Arg Arg His Asp Trp Gly
 420 425 430
 His Glu Lys Gln Arg Lys His Asn Leu Gly His Gly His Lys His Glu
 435 440 445
 Arg Asp Gln Gly His Gly His Gln Arg Gly His Gly Leu Gly His Gly
 450 455 460
 His Glu Gln Gln His Gly Leu Gly His Gly His Lys Phe Lys Leu Asp
 465 470 475 480
 Asp Asp Leu Glu His Gln Gly Gly His Val Leu Asp His Gly His Lys
 485 490 495
 His Lys His Gly His Gly His Gly Lys His Lys Asn Lys Gly Lys Lys
 500 505 510
 Asn Gly Lys His Asn Gly Trp Lys Thr Glu His Leu Ala Ser Ser Ser
 515 520 525
 Glu Asp Ser Thr Thr Pro Ser Ala Gln Thr Gln Glu Lys Thr Glu Gly
 530 535 540
 Pro Thr Pro Ile Pro Ser Leu Ala Lys Pro Gly Val Thr Val Thr Phe
 545 550 555 560
 Ser Asp Phe Gln Asp Ser Asp Leu Ile Ala Thr Met Met Pro Pro Ile
 565 570 575
 Ser Pro Ala Pro Ile Gln Ser Asp Asp Asp Trp Ile Pro Asp Ile Gln
 580 585 590
 Ile Asp Pro Asn Gly Leu Ser Phe Asn Pro Ile Ser Asp Phe Pro Asp
 595 600 605

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Thr Thr Ser Pro Lys Cys Pro Gly Arg Pro Trp Lys Ser Val Ser Glu
610 615 620

Ile Asn Pro Thr Thr Gln Met Lys Glu Ser Tyr Tyr Phe Asp Leu Thr
625 630 635 640

Asp Gly Leu Ser

<210> SEQ ID NO 2
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His
1 5 10 15

Gln

<210> SEQ ID NO 3
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly
1 5 10 15

His Gln

<210> SEQ ID NO 4
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

Lys His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His
1 5 10 15

Gly His Gln

<210> SEQ ID NO 5
<211> LENGTH: 1744
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Met Arg Leu Leu Trp Gly Leu Ile Trp Ala Ser Ser Phe Phe Thr Leu
1 5 10 15

Ser Leu Gln Lys Pro Arg Leu Leu Leu Phe Ser Pro Ser Val Val His
20 25 30

Leu Gly Val Pro Leu Ser Val Gly Val Gln Leu Gln Asp Val Pro Arg
35 40 45

Gly Gln Val Val Lys Gly Ser Val Phe Leu Arg Asn Pro Ser Arg Asn
50 55 60

Asn Val Pro Cys Ser Pro Lys Val Asp Phe Thr Leu Ser Ser Glu Arg
65 70 75 80

Asp Phe Ala Leu Leu Ser Leu Gln Val Pro Leu Lys Asp Ala Lys Ser
85 90 95

Cys Gly Leu His Gln Leu Leu Arg Gly Pro Glu Val Gln Leu Val Ala
100 105 110

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His	Ser	Pro	Trp	Leu	Lys	Asp	Ser	Leu	Ser	Arg	Thr	Thr	Asn	Ile	Gln
	115						120						125		
Gly	Ile	Asn	Leu	Leu	Phe	Ser	Ser	Arg	Arg	Gly	His	Leu	Phe	Leu	Gln
	130					135					140				
Thr	Asp	Gln	Pro	Ile	Tyr	Asn	Pro	Gly	Gln	Arg	Val	Arg	Tyr	Arg	Val
145					150					155					160
Phe	Ala	Leu	Asp	Gln	Lys	Met	Arg	Pro	Ser	Thr	Asp	Thr	Ile	Thr	Val
				165					170					175	
Met	Val	Glu	Asn	Ser	His	Gly	Leu	Arg	Val	Arg	Lys	Lys	Glu	Val	Tyr
			180					185					190		
Met	Pro	Ser	Ser	Ile	Phe	Gln	Asp	Asp	Phe	Val	Ile	Pro	Asp	Ile	Ser
	195						200					205			
Glu	Pro	Gly	Thr	Trp	Lys	Ile	Ser	Ala	Arg	Phe	Ser	Asp	Gly	Leu	Glu
	210					215						220			
Ser	Asn	Ser	Ser	Thr	Gln	Phe	Glu	Val	Lys	Lys	Tyr	Val	Leu	Pro	Asn
225					230						235				240
Phe	Glu	Val	Lys	Ile	Thr	Pro	Gly	Lys	Pro	Tyr	Ile	Leu	Thr	Val	Pro
			245						250					255	
Gly	His	Leu	Asp	Glu	Met	Gln	Leu	Asp	Ile	Gln	Ala	Arg	Tyr	Ile	Tyr
		260						265					270		
Gly	Lys	Pro	Val	Gln	Gly	Val	Ala	Tyr	Val	Arg	Phe	Gly	Leu	Leu	Asp
	275						280					285			
Glu	Asp	Gly	Lys	Lys	Thr	Phe	Phe	Arg	Gly	Leu	Glu	Ser	Gln	Thr	Lys
	290					295					300				
Leu	Val	Asn	Gly	Gln	Ser	His	Ile	Ser	Leu	Ser	Lys	Ala	Glu	Phe	Gln
305					310						315				320
Asp	Ala	Leu	Glu	Lys	Leu	Asn	Met	Gly	Ile	Thr	Asp	Leu	Gln	Gly	Leu
				325					330					335	
Arg	Leu	Tyr	Val	Ala	Ala	Ala	Ile	Ile	Glu	Ser	Pro	Gly	Gly	Glu	Met
		340						345						350	
Glu	Glu	Ala	Glu	Leu	Thr	Ser	Trp	Tyr	Phe	Val	Ser	Ser	Pro	Phe	Ser
	355						360						365		
Leu	Asp	Leu	Ser	Lys	Thr	Lys	Arg	His	Leu	Val	Pro	Gly	Ala	Pro	Phe
	370					375					380				
Leu	Leu	Gln	Ala	Leu	Val	Arg	Glu	Met	Ser	Gly	Ser	Pro	Ala	Ser	Gly
385					390					395					400
Ile	Pro	Val	Lys	Val	Ser	Ala	Thr	Val	Ser	Ser	Pro	Gly	Ser	Val	Pro
			405						410					415	
Glu	Val	Gln	Asp	Ile	Gln	Gln	Asn	Thr	Asp	Gly	Ser	Gly	Gln	Val	Ser
			420						425				430		
Ile	Pro	Ile	Ile	Ile	Pro	Gln	Thr	Ile	Ser	Glu	Leu	Gln	Leu	Ser	Val
	435						440						445		
Ser	Ala	Gly	Ser	Pro	His	Pro	Ala	Ile	Ala	Arg	Leu	Thr	Val	Ala	Ala
	450					455					460				
Pro	Pro	Ser	Gly	Gly	Pro	Gly	Phe	Leu	Ser	Ile	Glu	Arg	Pro	Asp	Ser
465					470					475					480
Arg	Pro	Pro	Arg	Val	Gly	Asp	Thr	Leu	Asn	Leu	Asn	Leu	Arg	Ala	Val
				485					490					495	
Gly	Ser	Gly	Ala	Thr	Phe	Ser	His	Tyr	Tyr	Tyr	Met	Ile	Leu	Ser	Arg
			500					505					510		
Gly	Gln	Ile	Val	Phe	Met	Asn	Arg	Glu	Pro	Lys	Arg	Thr	Leu	Thr	Ser

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515				520				525							
Val	Ser	Val	Phe	Val	Asp	His	His	Leu	Ala	Pro	Ser	Phe	Tyr	Phe	Val
530						535					540				
Ala	Phe	Tyr	Tyr	His	Gly	Asp	His	Pro	Val	Ala	Asn	Ser	Leu	Arg	Val
545					550					555					560
Asp	Val	Gln	Ala	Gly	Ala	Cys	Glu	Gly	Lys	Leu	Glu	Leu	Ser	Val	Asp
				565					570					575	
Gly	Ala	Lys	Gln	Tyr	Arg	Asn	Gly	Glu	Ser	Val	Lys	Leu	His	Leu	Glu
			580					585					590		
Thr	Asp	Ser	Leu	Ala	Leu	Val	Ala	Leu	Gly	Ala	Leu	Asp	Thr	Ala	Leu
		595				600						605			
Tyr	Ala	Ala	Gly	Ser	Lys	Ser	His	Lys	Pro	Leu	Asn	Met	Gly	Lys	Val
	610					615					620				
Phe	Glu	Ala	Met	Asn	Ser	Tyr	Asp	Leu	Gly	Cys	Gly	Pro	Gly	Gly	Gly
625					630					635					640
Asp	Ser	Ala	Leu	Gln	Val	Phe	Gln	Ala	Ala	Gly	Leu	Ala	Phe	Ser	Asp
				645					650					655	
Gly	Asp	Gln	Trp	Thr	Leu	Ser	Arg	Lys	Arg	Leu	Ser	Cys	Pro	Lys	Glu
			660						665					670	
Lys	Thr	Thr	Arg	Lys	Lys	Arg	Asn	Val	Asn	Phe	Gln	Lys	Ala	Ile	Asn
		675					680						685		
Glu	Lys	Leu	Gly	Gln	Tyr	Ala	Ser	Pro	Thr	Ala	Lys	Arg	Cys	Cys	Gln
	690					695							700		
Asp	Gly	Val	Thr	Arg	Leu	Pro	Met	Met	Arg	Ser	Cys	Glu	Gln	Arg	Ala
705					710					715					720
Ala	Arg	Val	Gln	Gln	Pro	Asp	Cys	Arg	Glu	Pro	Phe	Leu	Ser	Cys	Cys
				725					730					735	
Gln	Phe	Ala	Glu	Ser	Leu	Arg	Lys	Lys	Ser	Arg	Asp	Lys	Gly	Gln	Ala
		740						745					750		
Gly	Leu	Gln	Arg	Ala	Leu	Glu	Ile	Leu	Gln	Glu	Glu	Asp	Leu	Ile	Asp
		755				760							765		
Glu	Asp	Asp	Ile	Pro	Val	Arg	Ser	Phe	Phe	Pro	Glu	Asn	Trp	Leu	Trp
	770					775					780				
Arg	Val	Glu	Thr	Val	Asp	Arg	Phe	Gln	Ile	Leu	Thr	Leu	Trp	Leu	Pro
785					790					795					800
Asp	Ser	Leu	Thr	Thr	Trp	Glu	Ile	His	Gly	Leu	Ser	Leu	Ser	Lys	Thr
				805					810					815	
Lys	Gly	Leu	Cys	Val	Ala	Thr	Pro	Val	Gln	Leu	Arg	Val	Phe	Arg	Glu
			820						825				830		
Phe	His	Leu	His	Leu	Arg	Leu	Pro	Met	Ser	Val	Arg	Arg	Phe	Glu	Gln
	835						840						845		
Leu	Glu	Leu	Arg	Pro	Val	Leu	Tyr	Asn	Tyr	Leu	Asp	Lys	Asn	Leu	Thr
	850					855					860				
Val	Ser	Val	His	Val	Ser	Pro	Val	Glu	Gly	Leu	Cys	Leu	Ala	Gly	Gly
865					870					875					880
Gly	Gly	Leu	Ala	Gln	Gln	Val	Leu	Val	Pro	Ala	Gly	Ser	Ala	Arg	Pro
				885					890					895	
Val	Ala	Phe	Ser	Val	Val	Pro	Thr	Ala	Ala	Ala	Ala	Val	Ser	Leu	Lys
			900						905				910		
Val	Val	Ala	Arg	Gly	Ser	Phe	Glu	Phe	Pro	Val	Gly	Asp	Ala	Val	Ser
		915					920						925		

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Lys Val Leu Gln Ile Glu Lys Glu Gly Ala Ile His Arg Glu Glu Leu
 930 935 940

Val Tyr Glu Leu Asn Pro Leu Asp His Arg Gly Arg Thr Leu Glu Ile
 945 950 955 960

Pro Gly Asn Ser Asp Pro Asn Met Ile Pro Asp Gly Asp Phe Asn Ser
 965 970 975

Tyr Val Arg Val Thr Ala Ser Asp Pro Leu Asp Thr Leu Gly Ser Glu
 980 985 990

Gly Ala Leu Ser Pro Gly Gly Val Ala Ser Leu Leu Arg Leu Pro Arg
 995 1000 1005

Gly Cys Gly Glu Gln Thr Met Ile Tyr Leu Ala Pro Thr Leu Ala
 1010 1015 1020

Ala Ser Arg Tyr Leu Asp Lys Thr Glu Gln Trp Ser Thr Leu Pro
 1025 1030 1035

Pro Glu Thr Lys Asp His Ala Val Asp Leu Ile Gln Lys Gly Tyr
 1040 1045 1050

Met Arg Ile Gln Gln Phe Arg Lys Ala Asp Gly Ser Tyr Ala Ala
 1055 1060 1065

Trp Leu Ser Arg Asp Ser Ser Thr Trp Leu Thr Ala Phe Val Leu
 1070 1075 1080

Lys Val Leu Ser Leu Ala Gln Glu Gln Val Gly Gly Ser Pro Glu
 1085 1090 1095

Lys Leu Gln Glu Thr Ser Asn Trp Leu Leu Ser Gln Gln Gln Ala
 1100 1105 1110

Asp Gly Ser Phe Gln Asp Pro Cys Pro Val Leu Asp Arg Ser Met
 1115 1120 1125

Gln Gly Gly Leu Val Gly Asn Asp Glu Thr Val Ala Leu Thr Ala
 1130 1135 1140

Phe Val Thr Ile Ala Leu His His Gly Leu Ala Val Phe Gln Asp
 1145 1150 1155

Glu Gly Ala Glu Pro Leu Lys Gln Arg Val Glu Ala Ser Ile Ser
 1160 1165 1170

Lys Ala Asn Ser Phe Leu Gly Glu Lys Ala Ser Ala Gly Leu Leu
 1175 1180 1185

Gly Ala His Ala Ala Ala Ile Thr Ala Tyr Ala Leu Ser Leu Thr
 1190 1195 1200

Lys Ala Pro Val Asp Leu Leu Gly Val Ala His Asn Asn Leu Met
 1205 1210 1215

Ala Met Ala Gln Glu Thr Gly Asp Asn Leu Tyr Trp Gly Ser Val
 1220 1225 1230

Thr Gly Ser Gln Ser Asn Ala Val Ser Pro Thr Pro Ala Pro Arg
 1235 1240 1245

Asn Pro Ser Asp Pro Met Pro Gln Ala Pro Ala Leu Trp Ile Glu
 1250 1255 1260

Thr Thr Ala Tyr Ala Leu Leu His Leu Leu Leu His Glu Gly Lys
 1265 1270 1275

Ala Glu Met Ala Asp Gln Ala Ser Ala Trp Leu Thr Arg Gln Gly
 1280 1285 1290

Ser Phe Gln Gly Gly Phe Arg Ser Thr Gln Asp Thr Val Ile Ala
 1295 1300 1305

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Leu Asp 1310	Ala Leu Ser Ala Tyr	Trp Ile Ala Ser His	Thr Thr Glu	1315	1320
Glu Arg 1325	Gly Leu Asn Val Thr	Leu Ser Ser Thr Gly	Arg Asn Gly	1330	1335
Phe Lys 1340	Ser His Ala Leu Gln	Leu Asn Asn Arg Gln	Ile Arg Gly	1345	1350
Leu Glu 1355	Glu Glu Leu Gln Phe	Ser Leu Gly Ser Lys	Ile Asn Val	1360	1365
Lys Val 1370	Gly Gly Asn Ser Lys	Gly Thr Leu Lys Val	Leu Arg Thr	1375	1380
Tyr Asn 1385	Val Leu Asp Met Lys	Asn Thr Thr Cys Gln	Asp Leu Gln	1390	1395
Ile Glu 1400	Val Thr Val Lys Gly	His Val Glu Tyr Thr	Met Glu Ala	1405	1410
Asn Glu 1415	Asp Tyr Glu Asp Tyr	Glu Tyr Asp Glu Leu	Pro Ala Lys	1420	1425
Asp Asp 1430	Pro Asp Ala Pro Leu	Gln Pro Val Thr Pro	Leu Gln Leu	1435	1440
Phe Glu 1445	Gly Arg Arg Asn Arg	Arg Arg Arg Glu Ala	Pro Lys Val	1450	1455
Val Glu 1460	Glu Gln Glu Ser Arg	Val His Tyr Thr Val	Cys Ile Trp	1465	1470
Arg Asn 1475	Gly Lys Val Gly Leu	Ser Gly Met Ala Ile	Ala Asp Val	1480	1485
Thr Leu 1490	Leu Ser Gly Phe His	Ala Leu Arg Ala Asp	Leu Glu Lys	1495	1500
Leu Thr 1505	Ser Leu Ser Asp Arg	Tyr Val Ser His Phe	Glu Thr Glu	1510	1515
Gly Pro 1520	His Val Leu Leu Tyr	Phe Asp Ser Val Pro	Thr Ser Arg	1525	1530
Glu Cys 1535	Val Gly Phe Glu Ala	Val Gln Glu Val Pro	Val Gly Leu	1540	1545
Val Gln 1550	Pro Ala Ser Ala Thr	Leu Tyr Asp Tyr Tyr	Asn Pro Glu	1555	1560
Arg Arg 1565	Cys Ser Val Phe Tyr	Gly Ala Pro Ser Lys	Ser Arg Leu	1570	1575
Leu Ala 1580	Thr Leu Cys Ser Ala	Glu Val Cys Gln Cys	Ala Glu Gly	1585	1590
Lys Cys 1595	Pro Arg Gln Arg Arg	Ala Leu Glu Arg Gly	Leu Gln Asp	1600	1605
Glu Asp 1610	Gly Tyr Arg Met Lys	Phe Ala Cys Tyr Tyr	Pro Arg Val	1615	1620
Glu Tyr 1625	Gly Phe Gln Val Lys	Val Leu Arg Glu Asp	Ser Arg Ala	1630	1635
Ala Phe 1640	Arg Leu Phe Glu Thr	Lys Ile Thr Gln Val	Leu His Phe	1645	1650
Thr Lys 1655	Asp Val Lys Ala Ala	Ala Asn Gln Met Arg	Asn Phe Leu	1660	1665
Val Arg 1670	Ala Ser Cys Arg Leu	Arg Leu Glu Pro Gly	Lys Glu Tyr	1675	1680
Leu Ile	Met Gly Leu Asp Gly	Ala Thr Tyr Asp Leu	Glu Gly His		

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1685		1690		1695											
Pro	Gln	Tyr	Leu	Leu	Asp	Ser	Asn	Ser	Trp	Ile	Glu	Glu	Met	Pro	
	1700						1705					1710			
Ser	Glu	Arg	Leu	Cys	Arg	Ser	Thr	Arg	Gln	Arg	Ala	Ala	Cys	Ala	
	1715						1720					1725			
Gln	Leu	Asn	Asp	Phe	Leu	Gln	Glu	Tyr	Gly	Thr	Gln	Gly	Cys	Gln	
	1730						1735					1740			
Val															
<210> SEQ ID NO 6															
<211> LENGTH: 1744															
<212> TYPE: PRT															
<213> ORGANISM: Homo sapiens															
<400> SEQUENCE: 6															
Met	Arg	Leu	Leu	Trp	Gly	Leu	Ile	Trp	Ala	Ser	Ser	Phe	Phe	Thr	Leu
1			5						10					15	
Ser	Leu	Gln	Lys	Pro	Arg	Leu	Leu	Leu	Phe	Ser	Pro	Ser	Val	Val	His
			20					25					30		
Leu	Gly	Val	Pro	Leu	Ser	Val	Gly	Val	Gln	Leu	Gln	Asp	Val	Pro	Arg
		35					40					45			
Gly	Gln	Val	Val	Lys	Gly	Ser	Val	Phe	Leu	Arg	Asn	Pro	Ser	Arg	Asn
		50				55					60				
Asn	Val	Pro	Cys	Ser	Pro	Lys	Val	Asp	Phe	Thr	Leu	Ser	Ser	Glu	Arg
	65				70					75					80
Asp	Phe	Ala	Leu	Leu	Ser	Leu	Gln	Val	Pro	Leu	Lys	Asp	Ala	Lys	Ser
			85						90					95	
Cys	Gly	Leu	His	Gln	Leu	Leu	Arg	Gly	Pro	Glu	Val	Gln	Leu	Val	Ala
			100					105					110		
His	Ser	Pro	Trp	Leu	Lys	Asp	Ser	Leu	Ser	Arg	Thr	Thr	Asn	Ile	Gln
		115					120						125		
Gly	Ile	Asn	Leu	Leu	Phe	Ser	Ser	Arg	Arg	Gly	His	Leu	Phe	Leu	Gln
		130				135					140				
Thr	Asp	Gln	Pro	Ile	Tyr	Asn	Pro	Gly	Gln	Arg	Val	Arg	Tyr	Arg	Val
	145				150					155					160
Phe	Ala	Leu	Asp	Gln	Lys	Met	Arg	Pro	Ser	Thr	Asp	Thr	Ile	Thr	Val
			165						170					175	
Met	Val	Glu	Asn	Ser	His	Gly	Leu	Arg	Val	Arg	Lys	Lys	Glu	Val	Tyr
			180					185					190		
Met	Pro	Ser	Ser	Ile	Phe	Gln	Asp	Asp	Phe	Val	Ile	Pro	Asp	Ile	Ser
		195					200						205		
Glu	Pro	Gly	Thr	Trp	Lys	Ile	Ser	Ala	Arg	Phe	Ser	Asp	Gly	Leu	Glu
		210				215						220			
Ser	Asn	Ser	Ser	Thr	Gln	Phe	Glu	Val	Lys	Lys	Tyr	Val	Leu	Pro	Asn
	225				230					235					240
Phe	Glu	Val	Lys	Ile	Thr	Pro	Gly	Lys	Pro	Tyr	Ile	Leu	Thr	Val	Pro
			245						250					255	
Gly	His	Leu	Asp	Glu	Met	Gln	Leu	Asp	Ile	Gln	Ala	Arg	Tyr	Ile	Tyr
			260					265					270		
Gly	Lys	Pro	Val	Gln	Gly	Val	Ala	Tyr	Val	Arg	Phe	Gly	Leu	Leu	Asp
		275					280					285			
Glu	Asp	Gly	Lys	Lys	Thr	Phe	Phe	Arg	Gly	Leu	Glu	Ser	Gln	Thr	Lys

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290	295	300
Leu Val Asn Gly Gln Ser His Ile Ser Leu Ser Lys Ala Glu Phe Gln 305	310	315
Asp Ala Leu Glu Lys Leu Asn Met Gly Ile Thr Asp Leu Gln Gly Leu 325	330	335
Arg Leu Tyr Val Ala Ala Ala Ile Ile Glu Ser Pro Gly Gly Glu Met 340	345	350
Glu Glu Ala Glu Leu Thr Ser Trp Tyr Phe Val Ser Ser Pro Phe Ser 355	360	365
Leu Asp Leu Ser Lys Thr Lys Arg His Leu Val Pro Gly Ala Pro Phe 370	375	380
Leu Leu Gln Ala Leu Val Arg Glu Met Ser Gly Ser Pro Ala Ser Gly 385	390	395
Ile Pro Val Lys Val Ser Ala Thr Val Ser Ser Pro Gly Ser Val Pro 405	410	415
Glu Val Gln Asp Ile Gln Gln Asn Thr Asp Gly Ser Gly Gln Val Ser 420	425	430
Ile Pro Ile Ile Ile Pro Gln Thr Ile Ser Glu Leu Gln Leu Ser Val 435	440	445
Ser Ala Gly Ser Pro His Pro Ala Ile Ala Arg Leu Thr Val Ala Ala 450	455	460
Pro Pro Ser Gly Gly Pro Gly Phe Leu Ser Ile Glu Arg Pro Asp Ser 465	470	475
Arg Pro Pro Arg Val Gly Asp Thr Leu Asn Leu Asn Leu Arg Ala Val 485	490	495
Gly Ser Gly Ala Thr Phe Ser His Tyr Tyr Tyr Met Ile Leu Ser Arg 500	505	510
Gly Gln Ile Val Phe Met Asn Arg Glu Pro Lys Arg Thr Leu Thr Ser 515	520	525
Val Ser Val Phe Val Asp His His Leu Ala Pro Ser Phe Tyr Phe Val 530	535	540
Ala Phe Tyr Tyr His Gly Asp His Pro Val Ala Asn Ser Leu Arg Val 545	550	555
Asp Val Gln Ala Gly Ala Cys Glu Gly Lys Leu Glu Leu Ser Val Asp 565	570	575
Gly Ala Lys Gln Tyr Arg Asn Gly Glu Ser Val Lys Leu His Leu Glu 580	585	590
Thr Asp Ser Leu Ala Leu Val Ala Leu Gly Ala Leu Asp Thr Ala Leu 595	600	605
Tyr Ala Ala Gly Ser Lys Ser His Lys Pro Leu Asn Met Gly Lys Val 610	615	620
Phe Glu Ala Met Asn Ser Tyr Asp Leu Gly Cys Gly Pro Gly Gly Gly 625	630	635
Asp Ser Ala Leu Gln Val Phe Gln Ala Ala Gly Leu Ala Phe Ser Asp 645	650	655
Gly Asp Gln Trp Thr Leu Ser Arg Lys Arg Leu Ser Cys Pro Lys Glu 660	665	670
Lys Thr Thr Arg Lys Lys Arg Asn Val Asn Phe Gln Lys Ala Ile Asn 675	680	685
Glu Lys Leu Gly Gln Tyr Ala Ser Pro Thr Ala Lys Arg Cys Cys Gln 690	695	700

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Asp Gly Val Thr Arg Leu Pro Met Met Arg Ser Cys Glu Gln Arg Ala
 705 710 715 720
 Ala Arg Val Gln Gln Pro Asp Cys Arg Glu Pro Phe Leu Ser Cys Cys
 725 730 735
 Gln Phe Ala Glu Ser Leu Arg Lys Lys Ser Arg Asp Lys Gly Gln Ala
 740 745 750
 Gly Leu Gln Arg Ala Leu Glu Ile Leu Gln Glu Glu Asp Leu Ile Asp
 755 760 765
 Glu Asp Asp Ile Pro Val Arg Ser Phe Phe Pro Glu Asn Trp Leu Trp
 770 775 780
 Arg Val Glu Thr Val Asp Arg Phe Gln Ile Leu Thr Leu Trp Leu Pro
 785 790 795 800
 Asp Ser Leu Thr Thr Trp Glu Ile His Gly Leu Ser Leu Ser Lys Thr
 805 810 815
 Lys Gly Leu Cys Val Ala Thr Pro Val Gln Leu Arg Val Phe Arg Glu
 820 825 830
 Phe His Leu His Leu Arg Leu Pro Met Ser Val Arg Arg Phe Glu Gln
 835 840 845
 Leu Glu Leu Arg Pro Val Leu Tyr Asn Tyr Leu Asp Lys Asn Leu Thr
 850 855 860
 Val Ser Val His Val Ser Pro Val Glu Gly Leu Cys Leu Ala Gly Gly
 865 870 875 880
 Gly Gly Leu Ala Gln Gln Val Leu Val Pro Ala Gly Ser Ala Arg Pro
 885 890 895
 Val Ala Phe Ser Val Val Pro Thr Ala Ala Ala Ala Val Ser Leu Lys
 900 905 910
 Val Val Ala Arg Gly Ser Phe Glu Phe Pro Val Gly Asp Ala Val Ser
 915 920 925
 Lys Val Leu Gln Ile Glu Lys Glu Gly Ala Ile His Arg Glu Glu Leu
 930 935 940
 Val Tyr Glu Leu Asn Pro Leu Asp His Arg Gly Arg Thr Leu Glu Ile
 945 950 955 960
 Pro Gly Asn Ser Asp Pro Asn Met Ile Pro Asp Gly Asp Phe Asn Ser
 965 970 975
 Tyr Val Arg Val Thr Ala Ser Asp Pro Leu Asp Thr Leu Gly Ser Glu
 980 985 990
 Gly Ala Leu Ser Pro Gly Gly Val Ala Ser Leu Leu Arg Leu Pro Arg
 995 1000 1005
 Gly Cys Gly Glu Gln Thr Met Ile Tyr Leu Ala Pro Thr Leu Ala
 1010 1015 1020
 Ala Ser Arg Tyr Leu Asp Lys Thr Glu Gln Trp Ser Thr Leu Pro
 1025 1030 1035
 Pro Glu Thr Lys Asp His Ala Val Asp Leu Ile Gln Lys Gly Tyr
 1040 1045 1050
 Met Arg Ile Gln Gln Phe Arg Lys Ala Asp Gly Ser Tyr Ala Ala
 1055 1060 1065
 Trp Leu Ser Arg Asp Ser Ser Thr Trp Leu Thr Ala Phe Val Leu
 1070 1075 1080
 Lys Val Leu Ser Leu Ala Gln Glu Gln Val Gly Gly Ser Pro Glu
 1085 1090 1095

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Lys 1100	Leu	Gln	Glu	Thr	Ser	Asn 1105	Trp	Leu	Leu	Ser	Gln 1110	Gln	Gln	Ala
Asp 1115	Gly	Ser	Phe	Gln	Asp	Leu 1120	Ser	Pro	Val	Ile	His 1125	Arg	Ser	Met
Gln 1130	Gly	Gly	Leu	Val	Gly	Asn 1135	Asp	Glu	Thr	Val	Ala 1140	Leu	Thr	Ala
Phe 1145	Val	Thr	Ile	Ala	Leu	His 1150	His	Gly	Leu	Ala	Val 1155	Phe	Gln	Asp
Glu 1160	Gly	Ala	Glu	Pro	Leu	Lys 1165	Gln	Arg	Val	Glu	Ala 1170	Ser	Ile	Ser
Lys 1175	Ala	Asn	Ser	Phe	Leu	Gly 1180	Glu	Lys	Ala	Ser	Ala 1185	Gly	Leu	Leu
Gly 1190	Ala	His	Ala	Ala	Ala	Ile 1195	Thr	Ala	Tyr	Ala	Leu 1200	Ser	Leu	Thr
Lys 1205	Ala	Pro	Val	Asp	Leu	Leu 1210	Gly	Val	Ala	His	Asn 1215	Asn	Leu	Met
Ala 1220	Met	Ala	Gln	Glu	Thr	Gly 1225	Asp	Asn	Leu	Tyr	Trp 1230	Gly	Ser	Val
Thr 1235	Gly	Ser	Gln	Ser	Asn	Ala 1240	Val	Ser	Pro	Thr	Pro 1245	Ala	Pro	Arg
Asn 1250	Pro	Ser	Asp	Pro	Met	Pro 1255	Gln	Ala	Pro	Ala	Leu 1260	Trp	Ile	Glu
Thr 1265	Thr	Ala	Tyr	Ala	Leu	Leu 1270	His	Leu	Leu	Leu	His 1275	Glu	Gly	Lys
Ala 1280	Glu	Met	Ala	Asp	Gln	Ala 1285	Ser	Ala	Trp	Leu	Thr 1290	Arg	Gln	Gly
Ser 1295	Phe	Gln	Gly	Gly	Phe	Arg 1300	Ser	Thr	Gln	Asp	Thr 1305	Val	Ile	Ala
Leu 1310	Asp	Ala	Leu	Ser	Ala	Tyr 1315	Trp	Ile	Ala	Ser	His 1320	Thr	Thr	Glu
Glu 1325	Arg	Gly	Leu	Asn	Val	Thr 1330	Leu	Ser	Ser	Thr	Gly 1335	Arg	Asn	Gly
Phe 1340	Lys	Ser	His	Ala	Leu	Gln 1345	Leu	Asn	Asn	Arg	Gln 1350	Ile	Arg	Gly
Leu 1355	Glu	Glu	Glu	Leu	Gln	Phe 1360	Ser	Leu	Gly	Ser	Lys 1365	Ile	Asn	Val
Lys 1370	Val	Gly	Gly	Asn	Ser	Lys 1375	Gly	Thr	Leu	Lys	Val 1380	Leu	Arg	Thr
Tyr 1385	Asn	Val	Leu	Asp	Met	Lys 1390	Asn	Thr	Thr	Cys	Gln 1395	Asp	Leu	Gln
Ile 1400	Glu	Val	Thr	Val	Lys	Gly 1405	His	Val	Glu	Tyr	Thr 1410	Met	Glu	Ala
Asn 1415	Glu	Asp	Tyr	Glu	Asp	Tyr 1420	Glu	Tyr	Asp	Glu	Leu 1425	Pro	Ala	Lys
Asp 1430	Asp	Pro	Asp	Ala	Pro	Leu 1435	Gln	Pro	Val	Thr	Pro 1440	Leu	Gln	Leu
Phe 1445	Glu	Gly	Arg	Arg	Asn	Arg 1450	Arg	Arg	Arg	Glu	Ala 1455	Pro	Lys	Val
Val 1460	Glu	Glu	Gln	Glu	Ser	Arg 1465	Val	His	Tyr	Thr	Val 1470	Cys	Ile	Trp
Arg	Asn	Gly	Lys	Val	Gly	Leu	Ser	Gly	Met	Ala	Ile	Ala	Asp	Val

-continued

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1490	1495	1500
Leu Thr Ser Leu Ser Asp Arg Tyr Val Ser His Phe Glu Thr Glu		
1505	1510	1515
Gly Pro His Val Leu Leu Tyr Phe Asp Ser Val Pro Thr Ser Arg		
1520	1525	1530
Glu Cys Val Gly Phe Glu Ala Val Gln Glu Val Pro Val Gly Leu		
1535	1540	1545
Val Gln Pro Ala Ser Ala Thr Leu Tyr Asp Tyr Tyr Asn Pro Glu		
1550	1555	1560
Arg Arg Cys Ser Val Phe Tyr Gly Ala Pro Ser Lys Ser Arg Leu		
1565	1570	1575
Leu Ala Thr Leu Cys Ser Ala Glu Val Cys Gln Cys Ala Glu Gly		
1580	1585	1590
Lys Cys Pro Arg Gln Arg Arg Ala Leu Glu Arg Gly Leu Gln Asp		
1595	1600	1605
Glu Asp Gly Tyr Arg Met Lys Phe Ala Cys Tyr Tyr Pro Arg Val		
1610	1615	1620
Glu Tyr Gly Phe Gln Val Lys Val Leu Arg Glu Asp Ser Arg Ala		
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Ala Phe Arg Leu Phe Glu Thr Lys Ile Thr Gln Val Leu His Phe		
1640	1645	1650
Thr Lys Asp Val Lys Ala Ala Ala Asn Gln Met Arg Asn Phe Leu		
1655	1660	1665
Val Arg Ala Ser Cys Arg Leu Arg Leu Glu Pro Gly Lys Glu Tyr		
1670	1675	1680
Leu Ile Met Gly Leu Asp Gly Ala Thr Tyr Asp Leu Glu Gly His		
1685	1690	1695
Pro Gln Tyr Leu Leu Asp Ser Asn Ser Trp Ile Glu Glu Met Pro		
1700	1705	1710
Ser Glu Arg Leu Cys Arg Ser Thr Arg Gln Arg Ala Ala Cys Ala		
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Val

<210> SEQ ID NO 7

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

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<210> SEQ ID NO 8
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile Arg
1           5           10           15

```

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.

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.

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 as an antigen.

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.

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.

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 and antibodies recognizing at least one of said biomarkers.

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.

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.

9. The antibody according to claim 8, wherein the antibody is a polyclonal antibody.

10. The antibody according to claim 8, wherein the antibody is a monoclonal antibody.

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 antibodies recognizing at least one of said biomarkers as an antigen.

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 according to claim 1, at least one biomarker that is 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, and antibodies recognizing at least one of said biomarkers as an antigen.

13. A detecting method for identification of a non-alcoholic fatty liver disease, the method using at least one of the diagnostic agents of claim 6.

14. A detecting method for identification of chronic hepatitis and an asymptomatic carrier, the method using at least one of the diagnostic agents of claim 11.

15. A detecting method for identification of a non-alcoholic fatty liver disease, chronic hepatitis, and an asymptomatic carrier, the method using a combination of the diagnostic agents of claim 12.

16. The detecting method according to claim 13, 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.

18. The diagnostic agent according to claim 6, 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.

19. The detecting method according to claim 14, 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.

20. The detecting method according to claim 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.

21. The diagnostic agent according to claim 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.

* * * * *

专利名称(译)	用于诊断肝病的生物标志物		
公开(公告)号	US20110129859A1	公开(公告)日	2011-06-02
申请号	US12/738684	申请日	2008-10-20
[标]申请(专利权)人(译)	壶内日本天皇裕仁 反对派弘文 OKANOUE TAKESHI 石田YO ICHI SATO YUKO 须藤MASAYUKI		
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IPC分类号	G01N33/53 C07K7/08 C07K14/435 C07K16/18		
CPC分类号	G01N33/6893 G01N2800/085 G01N2333/8139		
优先权	2007270799 2007-10-18 JP		
其他公开文献	US8263347		
外部链接	Espacenet USPTO		

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;针对标记物的抗体;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及其三个部分肽的量(序列A:位置-440至位置-456,序列B:位置-

439至位置-456，以及序列C：位置-438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。

Fig. 1

