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

(11) EP 2 216 651 A1

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

(43) Date of publication:
11.08.2010 Bulletin 2010/32

(21) Application number: **08840152.6**

(22) Date of filing: **20.10.2008**

(51) Int Cl.:
G01N 33/53 *(2006.01)* ***C07K 16/18*** *(2006.01)*

(86) International application number:
PCT/JP2008/068985

(87) International publication number:
WO 2009/051259 (23.04.2009 Gazette 2009/17)

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

(30) Priority: **18.10.2007 JP 2007270799**
03.06.2008 JP 2008145337

(71) Applicants:
- **Miyazaki Prefectural Industrial Support Foundation**
  Miyazaki 880-0303 (JP)
- **Kagoshima University**
  Kagoshima-shi, Kagoshima 890-8580 (JP)
- **Chugai Seiyaku Kabushiki Kaisha**
  Kita-ku
  Tokyo 115-8543 (JP)

(72) Inventors:
- **TSUBOUCHI, Hirohito**
  Kagoshima-shi
  Kagoshima 890-8580 (JP)
- **UTO, Hirofumi**
  Kagoshima-shi
  Kagoshima 890-8580 (JP)
- **OKANOUE, Takeshi**
  Kyoto-shi
  Kyoto 606-0022 (JP)
- **ISHIDA, Yo-ichi**
  Miyazaki-shi
  Miyazaki 880-0303 (JP)
- **SATO, Yuko**
  Miyazaki-shi
  Miyazaki 880-0303 (JP)
- **SUDO, Masayuki**
  Kamakura-shi
  Kanagawa 247-8530 (JP)

(74) Representative: **Vossius & Partner**
Siebertstrasse 4
81675 München (DE)

(54) BIOMARKER FOR DIAGNOSIS OF LIVER DISEASE

(57) Disclosed are: a marker for the diagnosis of a liver disease, which can determine the disease in a simple manner; an antibody directed agaist 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 therof (sequence A: postion-440 to position-456, sequence B: postion-439 to postion-456, and sequence C: position-43 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.

EP 2 216 651 A1

Description

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 I 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 individuals and liver cancer patients and are therefore useful as markers for discriminating patients with liver caner 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.

Patent Document 1: Japanese Unexamined Patent Application Publication No. 04-110660
Patent Document 2: Japanese Unexamined Patent Application Publication No. 2006-300689
Patent Document 3: Japanese Unexamined Patent Application Publication No. 2006-308533
Non Patent Document 1: C Cordova, et al., "Hageman factor, high molecular weight kininogen, and prekallikrein in chronic liver disease"', J Clin Pathol, 39, 1003-1005, (1986)
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

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

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

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

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

MEANS FOR SOLVING THE PROBLEMS

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

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

sequence A: Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln,
sequence B: His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln, and
sequence C: Lys His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln;
Aspect 2: The biomarker according to Aspect 1, wherein the fun-length high-molecular-weight kininogen and/or the partial peptide derived from the high-molecular-weight kininogen include a modified form thereof;

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

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

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

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

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

sequence D: Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile, and
sequence E: Asn Gly Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile Arg;
Aspect 8: An antibody for identification of chronic hepatitis and an ASC, the antibody recognizing at least one of the biomarkers according to Aspect 7 as an antigen;

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

ADVANTAGES OF THE INVENTION

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

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

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

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

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

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

BRIEF DESCRIPTION OF THE DRAWINGS

[0034]

Fig. 1 is a schematic diagram illustrating domain structures of amino acid sequences of kininogens.
Fig. 2 is a schematic diagram illustrating domain structures of complement C4 (C4A and C4B) and its decomposition products.
Fig. 3 includes graphs showing spectral comparison of patients with NAFLD and healthy individuals using ClinProt; Graph (A) shows typical spectral patterns of a patient with NAFLD and a healthy individual measured by a linear mode of Autoflex; Graph (B) shows an enlarged view of the region near 1800 to 2300 m/z in Graph (A), and three peaks observed in the patient with NAFLD are peaks at 1942 m/z, 2079 m/z, and 2207 m/z from the left; the vertical axis represents peak intensity, and the horizontal axis represents molecular weight in Graphs (A) and (B); and Graph (C) plots peak intensities of the patients with NAFLD and the healthy individuals at 1942 m/z (a). 2079 m/z (b), and 2207 m/z (c).
Fig. 4 includes graphs showing MS/MS spectra of marker candidate peptides at 1942 m/z Graph (A), 2079 m/z Graph (B), and 2207 m/z Graph (C).
Fig. 5 is a photograph showing the results of Western blotting using RABBIT ANTI HUMAN HMW-KINNOGEN (anti-kininogen polyclonal antibody) manufactured by AbD Serotec.
Fig. 6 is a photograph showing the results of Western blotting using Rabbit-anti-human-kininogen (anti-kininogen polyclonal antibody) manufactured by Sigma-Aldrich Japan.
Fig. 7 is a photograph showing the results of Western blotting using HMW Kininogen Light Chain antibody [1.B.709] (anti-kininogen monoclonal antibody) manufactured by GeneTex.
Fig. 8 is a photograph showing the results of Western blotting using Anti-human-kininogen/Kininostatin Antibody (anti-kininogen polyclonal antibody) manufactured by R&D Systems.
Fig. 9 is a graph showing the results of measurement of 1942 m/z (a). 2079 m/z (b), and 2207 m/z (c) peaks by immunoprecipitation using anti-kininogen polyclonal antibodies (an AbD Serotec product and a Sigma-Aldrich contract product), the vertical axis represents peak intensity, and the horizontal axis represents molecular weight.
Fig. 10 is a graph showing the results of measurement of 1942 m/z (a), 2079 m/z (b), and 2207 m/z (c) peaks by immunoprecipitation using anti-kininogen monoclonal antibodies (products of GeneTex and R&D Systems), the vertical axis represents peak intensity, and the horizontal axis represents molecular weight.
Fig. 11 includes graphs showing spectral comparison of patients with NAFLD and healthy individuals using a protein chip; Graph (A) shows typical spectral patterns of a patient with NAFLD and a healthy individual measured by SELDI; Graph (B) shows an enlarged view of a region near 1800 to 2300 m/z in Graph (A), and three peaks observed in the patient with NAFLD are peaks at 1942 m/z, 2079 m/z, and 2207 m/z from the left; and Graph (C) plots peak intensities of the patients with NAFLD and the healthy individuals at 1942 m/z (a), 2079 m/z (b), and 2207 m/z (c).
Fig. 12 includes graphs showing spectral comparison of ASCs and healthy individuals using a protein chip; Graph (A) shows typical spectral patterns of an ASC and a healthy individual measured by SELDI; Graph (B) shows an enlarged view of the region near 1600 to 2100 m/z in Graph (A), and two peaks observed in the ASC are peaks at 1738 m/z and 1896 m/z from the left; the vertical axis represents peak intensity, and the horizontal axis represents molecular weight in Graphs (A) and (B); and Graph (C) plots peak intensities of the ASCs and the healthy individuals at 1738 m/z (a) and 1896 m/z (b).
Fig. 13 includes graphs showing MS/MS spectra of marker candidate peptides identified in ASCs using a database of Sw-issProt at 1738 m/z (A) and 1896 m/z (B).
Fig. 14 includes the results of analysis of peaks at 1738 m/z (a) and 1896 m/z (b) using a ClinProt system.
Fig. 15 is a graph showing the results of detection of the C4a protein level in sera by ELISA (BD OptEIA ELISA Kit).
Fig. 16 includes graphs showing spectral comparison of ASCs and healthy individuals using ClinProt; Graph (A) shows typical spectral patterns of an ASC and a healthy individual measured by a linear mode of Autoflex; Graph (B) shows an enlarged view of the region near 1600 to 2000 m/z in Graph (A), and two peaks observed in the ASC are peaks at 1738 m/z and 1896 m/z from the left; the vertical axis represents peak intensity; and Graph (C) plots peak intensities of the ASCs and the healthy individuals at 1738 m/z (a) and 1896 m/z (b).
Fig. 17 is a schematic diagram (multimarker system) of a flow for identification of liver diseases using kininogen-based and C4-based markers of the present invention.
Fig. 18 is a graph showing discrimination between patients with NAFLD and healthy individuals by kininogen-based ELISA.

BEST MODE FOR CARRYING OUT THE INVENTION

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

[Antibody for detecting markers]

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

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

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

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

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

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

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

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

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

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

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

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

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

[0048] The screening of the hybridoma is performed by, for example, a limiting dilution method or a fluorescence-activated cell sorting method, in order to obtain a monoclonal antibody-producing hybridoma finally. Examples of the method for harvesting the monoclonal antibody from the resulting hybridoma include usual cell culturing and ascitic fluid formation. In the cell culturing, the hybridoma is cultured, for example, in a medium for culturing animal cells, such as RPMI-1640 or MEM containing 10 to 20% fetal bovine serum, or a serum-free medium, under usual culture conditions (for example, at 37°C, 5% CO_2) 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.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

[0062] In the present invention, the kininogen that is a candidate of these markers is identified by, for example, MS/MS analysis. The test specimen for the identification may be prepared by a thin film technique. For example, a saturated acetone solution of CCA is applied to the anchor surface of an anchor chip in advance to form a thin film therefrom. Then, about 1 μL 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 μL of 0.1% trifluoroacetic acid (hereinafter referred to as "TFA") around three times for desalination.

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

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

[0065]

Sequence A: Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln (440th to 456th).
Sequence B: His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln (439th to 456th), and
Sequence C: Lys His Asn Leu Gly His Gly His Lys His Glu Arg Asp Gln Gly His Gly His Gln (438th to 456th).

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

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

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

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

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

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

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

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

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

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

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

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

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

EXAMPLES

[Example 1] (Kininogen)

[1] Detection serum peptide by ClinProt system

(1) Material and method

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

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

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

(2) Results

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

[0081]

[Table 1]

Peaks increased in expression in patients with NAFLD (ClinProt)

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

* data shows average ± standard deviation

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

[2] Identification of kininogen as NAFLD marker

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

(1) Material and method

[0084] An MS/MS spectrum was acquired as follows: First, crystals of the peptides and a matrix were prepared by a thin film technique. A saturated 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 μL 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 μL of a 0.1 % TFA three times.

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

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

(2) Results

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

[0087]

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

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

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

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

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

(1) Material and method (polyclonal antibody)

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

(2) Results

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

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

(3) Material and method (monoclonal antibody)

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

(4) Results

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

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

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

(1) Material and method

[0095] The specific detection of kininogen partial peptides was performed by immunoprecipitation using anti-kininogen antibodies. Ten microliters of an NAFLD patient serum was mixed with 40 μL of PBS, followed by addition of 10 μL of RABBIT ANTI HUMAN HMW-KININOGEN (manufactured by AbD Serotec) or rabbit-anti-human-kininogen (Sigma-Aldrich Japan contract product) as the polyclonal antibody or HMW Kininogen Light Chain antibody [1.B.709] (manufactured by GeneTex Inc.) or Anti-human-kininogen/Kininostatin Antibody: Catalog Number: MAB 1569 (manufactured by R&D Systems) as the monoclonal antibody. The mixture was left to stand on ice bath (4°C) for 1 hour for a reaction of the antigen in the serum and the antibody. After 30 μL 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 μL of PBS to remove unadsorbed proteins and peptides, and the proteins and peptides adsorbed onto the beads were eluted by addition of 30 μL of 50% acetonitrile and centrifugation. Twenty microliters of the eluate was diluted with 80 μL 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 μL of 20% saturated CCA/50% acetonitrile/0.5% TFA twice. The identified kininogen partial peptides were detected by SELDI measurement.

(2) Results

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

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

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

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

(1) Material and method

[0099] The serum was applied to the protein chip as follows. Five microliters of the serum were added to 45 μL of a urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholyte). The mixture was left to stand on an ice bath for 10 minutes to denature the serum proteins and then diluted with 450 μL 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 μL of 50 mM sodium acetate at a pH of 4.5 three times and then with ultrapure water twice. After air drying, 0.5 μL of a 50% saturated CCA were added to the spot twice to prepare a crystal mixture of the peptides and CCA. The peaks were detected with SELDI.

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

(2) Results

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

[0102]

[Table 3]

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

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

(continued)

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

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

[Example 2] (Complement C4)

[1] Detection of serum peptide with ClinProt system

(1) Material and method

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

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

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

(2) Result

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

[0107]

[Table 4]

Peaks with increasing expression in asymptomatic carrier (ClinProt)

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

* data shows average ± standard deviation

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

healthy individuals were subjected to diagnosis.

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

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

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

(1) Material and method

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

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

(2) Results

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

[0114]

[Table 5]

Identification of ASC marker by MS/MS ion search

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

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

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

[3] Detection of serum protein level by ELISA

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

liver disease patients.

(1) Material and method

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

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

(2) Results

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

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

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

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

(1) Maternal and method

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

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

(2) Results

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

[0126]

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

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

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

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

(1) Material and method

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

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

(2) Results

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

INDUSTRIAL APPLICABILITY

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

SEQUENCE LISTING

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

<120> BIOMARKER FOR DIAGNOSIS OF LIVER DISEASE

<130> S1848 EP S3

<140> PCT/JP2008/068985
<141> 2008-10-20

<150> JP 2007-270799
<151> 2007-10-18

<150> JP 2008-145337
<151> 2008-06-03

<160> 8

<170> PatentIn version 3.1

<210> 1
<211> 644
<212> PRT
<213> Homo sapiens

<400> 1

Met Lys Leu Ile Thr Ile Leu Phe Leu Cys Ser Arg Leu Leu Leu Ser
1 5 10 15

Leu Thr Gln Glu Ser Gln Ser Glu Glu Ile Asp Cys Asn Asp Lys Asp
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

Glu Asn Phe Leu Phe Leu Thr Pro Asp Cys Lys Ser Leu Trp Asn Gly
    210                 215                 220

Asp Thr Gly Glu Cys Thr Asp Asn Ala Tyr Ile Asp Ile Gln Leu Arg
225                 230                 235                 240

Ile Ala Ser Phe Ser Gln Asn Cys Asp Ile Tyr Pro Gly Lys Asp Phe
                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
        275                 280                 285

Leu Asn Ala Glu Asn Asn Ala Thr Phe Tyr Phe Lys Ile Asp Asn Val
    290                 295                 300

Lys Lys Ala Arg Val Gln Val Val Ala Gly Lys Lys Tyr Phe Ile Asp
305                 310                 315                 320

Phe Val Ala Arg Glu Thr Thr Cys Ser Lys Glu Ser Asn Glu Glu Leu
                325                 330                 335

Thr Glu Ser Cys Glu Thr Lys Lys Leu Gly Gln Ser Leu Asp Cys Asn
            340                 345                 350
```

```
Ala Glu Val Tyr Val Val Pro Trp Glu Lys Lys Ile Tyr Pro Thr Val
        355                 360                 365

Asn Cys Gln Pro Leu Gly Met Ile Ser Leu Met Lys Arg Pro Pro Gly
    370                 375                 380

Phe Ser Pro Phe Arg Ser Ser Arg Ile Gly Glu Ile Lys Glu Glu Thr
385                 390                 395                 400

Thr Val Ser Pro Pro His Thr Ser Met Ala Pro Ala Gln Asp Glu Glu
                405                 410                 415

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

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> 2
<211> 17
<212> PRT
<213> Homo sapiens

<400> 2

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

Gln

<210> 3
<211> 18
<212> PRT
<213> Homo sapiens

<400> 3

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

His Gln

<210> 4
<211> 19
<212> PRT
<213> Homo sapiens

<400> 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> 5
<211> 1744
<212> PRT
<213> Homo sapiens

<400> 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

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
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

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

Leu Asp  Ala Leu Ser Ala Tyr  Trp Ile Ala Ser His  Thr Thr Glu
    1310                 1315                 1320

Glu Arg  Gly Leu Asn Val Thr  Leu Ser Ser Thr Gly  Arg Asn Gly
    1325                 1330                 1335

Phe Lys  Ser His Ala Leu Gln  Leu Asn Asn Arg Gln  Ile Arg Gly
    1340                 1345                 1350
```

```
Leu Glu  Glu Glu Leu Gln Phe  Ser Leu Gly Ser Lys  Ile Asn Val
    1355                1360                1365

Lys Val  Gly Gly Asn Ser Lys  Gly Thr Leu Lys Val  Leu Arg Thr
    1370                1375                1380

Tyr Asn  Val Leu Asp Met Lys  Asn Thr Thr Cys Gln  Asp Leu Gln
    1385                1390                1395

Ile Glu  Val Thr Val Lys Gly  His Val Glu Tyr Thr  Met Glu Ala
    1400                1405                1410

Asn Glu  Asp Tyr Glu Asp Tyr  Glu Tyr Asp Glu Leu  Pro Ala Lys
    1415                1420                1425

Asp Asp  Pro Asp Ala Pro Leu  Gln Pro Val Thr Pro  Leu Gln Leu
    1430                1435                1440

Phe Glu  Gly Arg Arg Asn Arg  Arg Arg Arg Glu Ala  Pro Lys Val
    1445                1450                1455

Val Glu  Glu Gln Glu Ser Arg  Val His Tyr Thr Val  Cys Ile Trp
    1460                1465                1470

Arg Asn  Gly Lys Val Gly Leu  Ser Gly Met Ala Ile  Ala Asp Val
    1475                1480                1485

Thr Leu  Leu Ser Gly Phe His  Ala Leu Arg Ala Asp  Leu Glu Lys
    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
1625 1630 1635

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
1715 1720 1725

Gln Leu Asn Asp Phe Leu Gln Glu Tyr Gly Thr Gln Gly Cys Gln
1730 1735 1740

Val

<210> 6
<211> 1744
<212> PRT
<213> Homo sapiens

<400> 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
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
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

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 Leu Ser Pro Val Ile His 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

Leu Asp Ala Leu Ser Ala Tyr Trp Ile Ala Ser His Thr Thr Glu
1310 1315 1320

Glu Arg Gly Leu Asn Val Thr Leu Ser Ser Thr Gly Arg Asn Gly
1325 1330 1335

Phe Lys Ser His Ala Leu Gln Leu Asn Asn Arg Gln Ile Arg Gly
1340 1345 1350

Leu Glu Glu Glu Leu Gln Phe Ser Leu Gly Ser Lys Ile Asn Val
1355 1360 1365

Lys Val Gly Gly Asn Ser Lys Gly Thr Leu Lys Val Leu Arg Thr
1370 1375 1380

Tyr Asn Val Leu Asp Met Lys Asn Thr Thr Cys Gln Asp Leu Gln
1385 1390 1395

```
Ile Glu  Val Thr Val Lys Gly  His Val Glu Tyr Thr  Met Glu Ala
    1400                1405                1410

Asn Glu  Asp Tyr Glu Asp Tyr  Glu Tyr Asp Glu Leu  Pro Ala Lys
    1415                1420                1425

Asp Asp  Pro Asp Ala Pro Leu  Gln Pro Val Thr Pro  Leu Gln Leu
    1430                1435                1440

Phe Glu  Gly Arg Arg Asn Arg  Arg Arg Arg Glu Ala  Pro Lys Val
    1445                1450                1455

Val Glu  Glu Gln Glu Ser Arg  Val His Tyr Thr Val  Cys Ile Trp
    1460                1465                1470

Arg Asn  Gly Lys Val Gly Leu  Ser Gly Met Ala Ile  Ala Asp Val
    1475                1480                1485

Thr Leu  Leu Ser Gly Phe His  Ala Leu Arg Ala Asp  Leu Glu Lys
    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
1625 1630 1635

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
1715 1720 1725

Gln Leu Asn Asp Phe Leu Gln Glu Tyr Gly Thr Gln Gly Cys Gln
1730 1735 1740

Val

<210> 7
<211> 15
<212> PRT
<213> Homo sapiens

<400> 7

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

<210> 8
<211> 16
<212> PRT
<213> Homo sapiens

<400> 8

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

Claims

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 or 2 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 or 2 and the antibodies according to any of Claims 3 to 5.

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 the antibodies according to any of Claims 8 to 10.

12. A diagnostic agent for identification of a non-alcoholic fatty liver disease, chronic hepatitis, and an asymptomatic carrier, the agent comprising a combination of at least one of the biomarkers, the antibodies, and the diagnostic agent according to any of Claims 1 to 6 and at least one of the biomarker, the antibodies, and the diagnostic agent according to any of Claims 7 to 11.

13. A detecting method for identification of a non-alcoholic fatty liver disease, the method using at least one of the biomarkers, the antibodies, and the diagnostic agent according to any of Claims 1 to 6.

14. A detecting method for identification of chronic hepatitis and an asymptomatic carrier, the method using at least one of the biomarkers, the antibodies, and the diagnostic agent according to any of Claims 7 to 11.

15. A detecting method for identification of a non-alcoholic fatty liver disease, chronic hepatitis, and an asymptomatic

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

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

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

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

Fig. 1

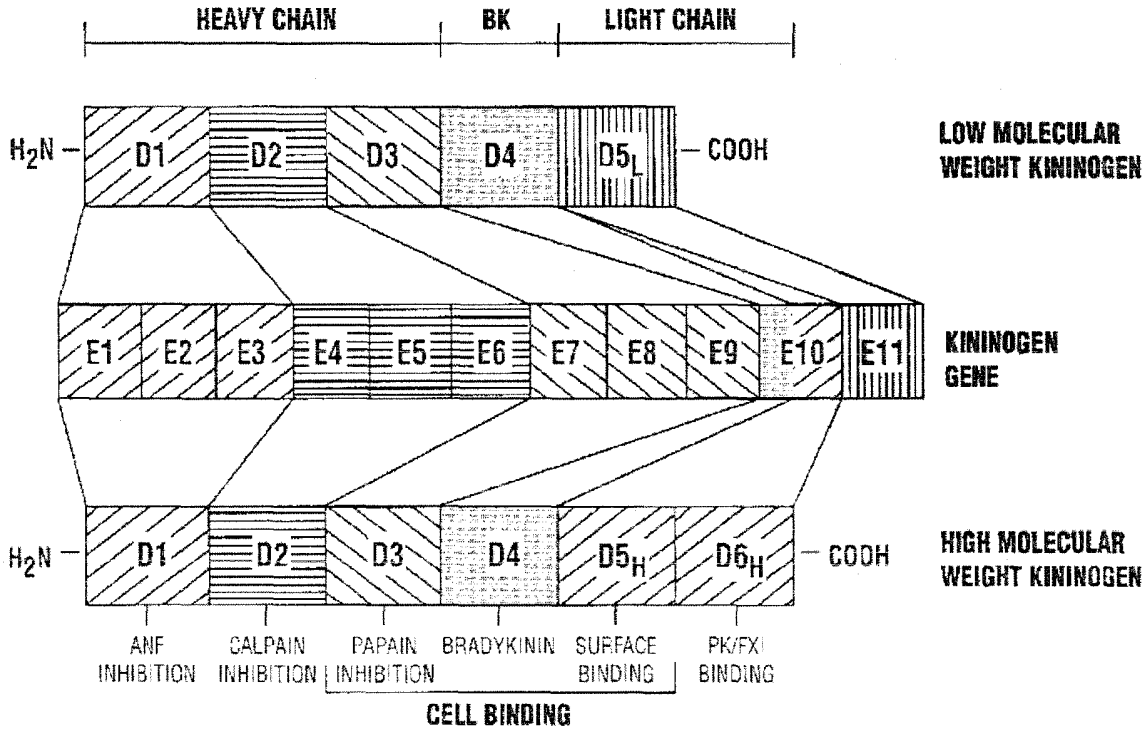

HEAVY CHAIN
BK
LIGHT CHAIN
H2N
D1
D2
D3
D4
D5L
COOH
LOW MOLECULAR WEIGHT KININOGEN
E1
E2
E3
E4
E5
E6
E7
E8
E9
E10
E11
KININOGEN GENE
H2N
D1
D2
D3
D4
D5H
D6H
COOH
HIGH MOLECULAR WEIGHT KININOGEN
ANF INHIBITION
CALPAIN INHIBITION
PAPAIN INHIBITION
BRADYKININ
SURFACE BINDING
PK/FXI BINDING
CELL BINDING

Fig. 2

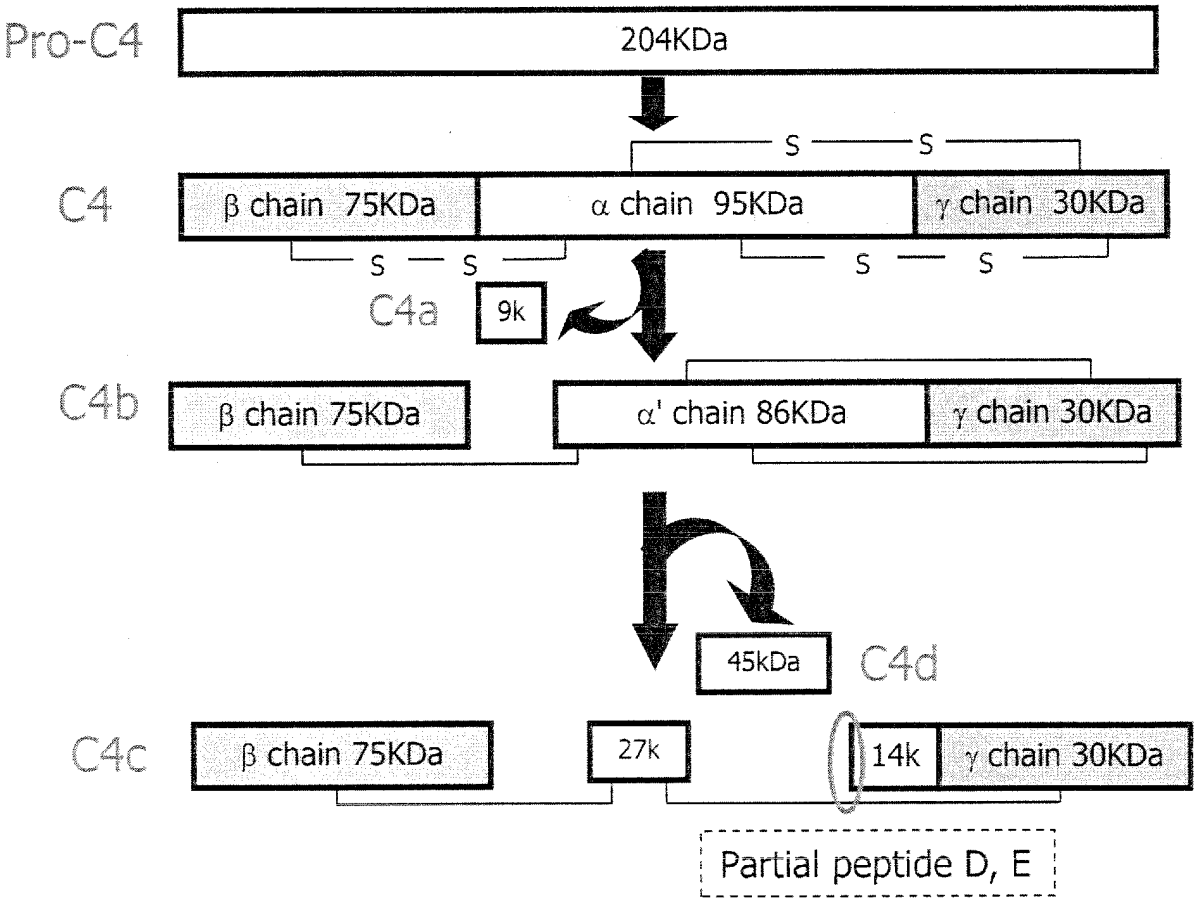

Pro-C4
204KDa
S S
C4
β chain 75KDa
α chain 95KDa
γ chain 30KDa
S S
S S
C4a
9k
C4b
β chain 75KDa
α' chain 86KDa
γ chain 30KDa
45kDa
C4d
C4c
β chain 75KDa
27k
14k
γ chain 30KDa
Partial peptide D, E

Fig. 3

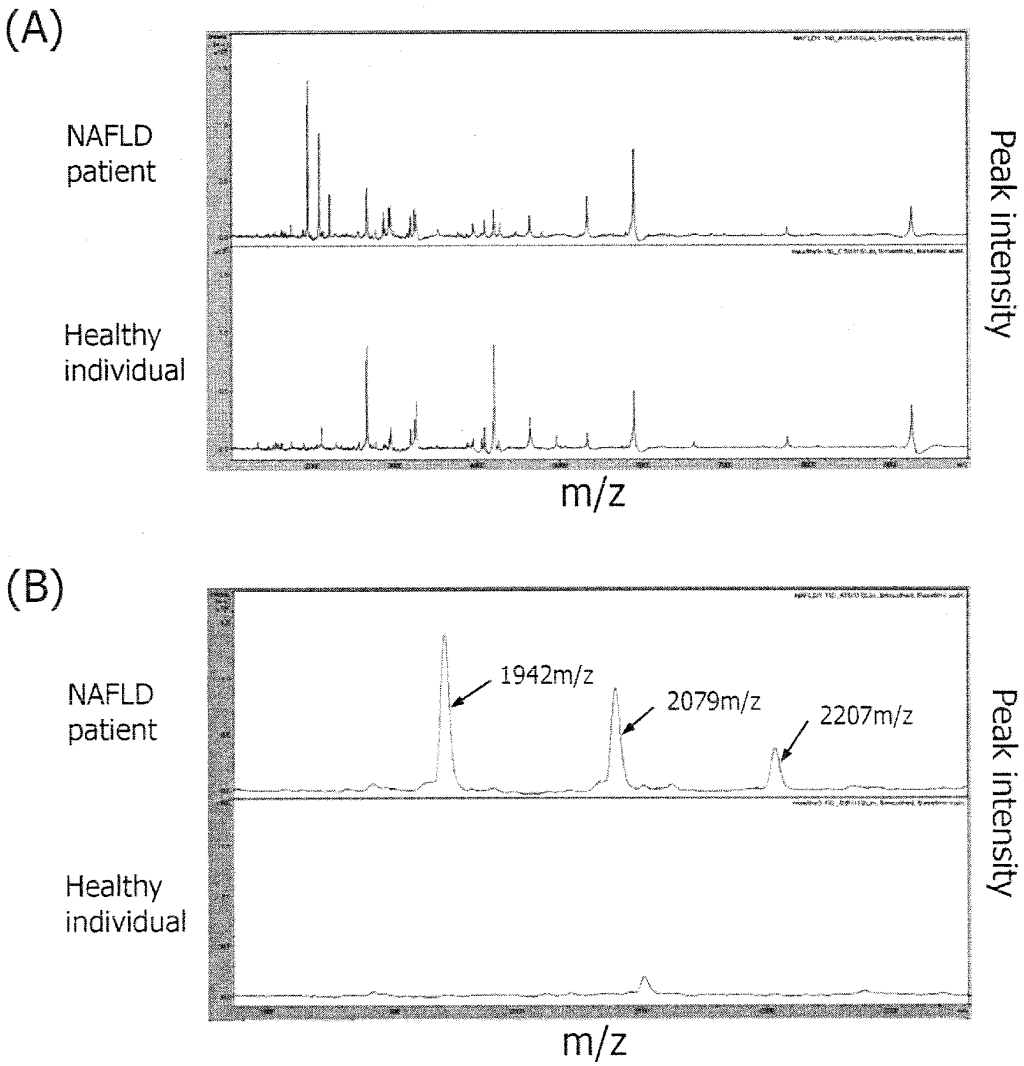
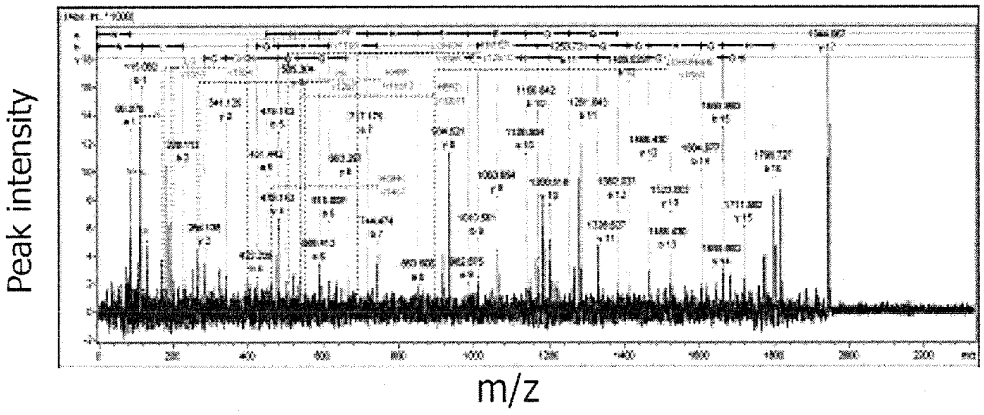
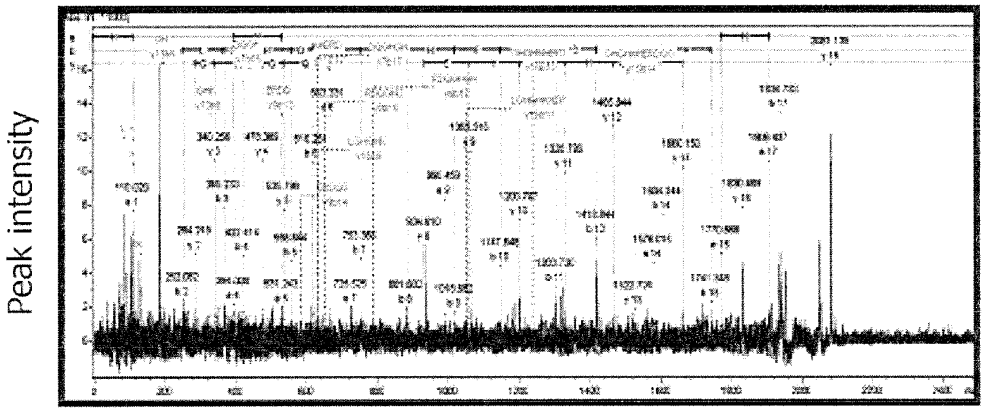

(A)
NAFLD patient
Healthy individual
Peak intensity
m/z
(B)
NAFLD patient
Healthy individual
1942m/z
2079m/z
2207m/z
Peak intensity
m/z
(C)
(a) 1942m/z
(b) 2079m/z
(c) 2207m/z
Peak intensity
NAFLD patients (n=44)
Healthy individuals (n=24)

Fig. 4

(A) 1942m/z



(B) 2079m/z



(C) 2207m/z

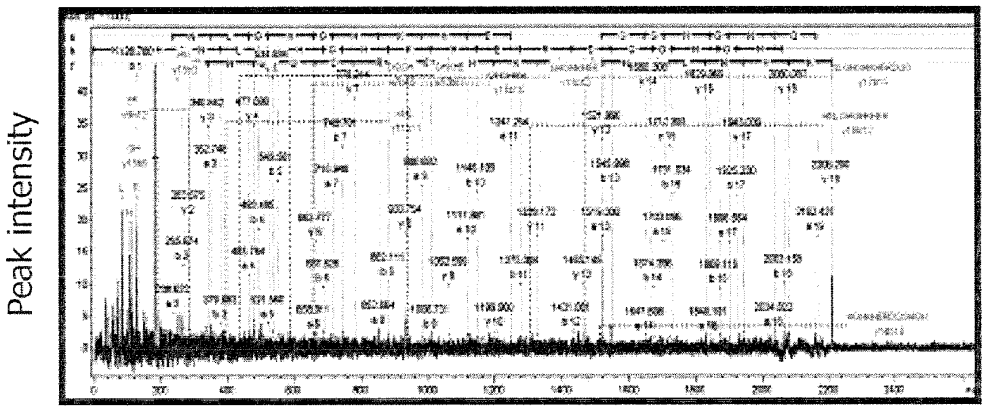


Fig. 5

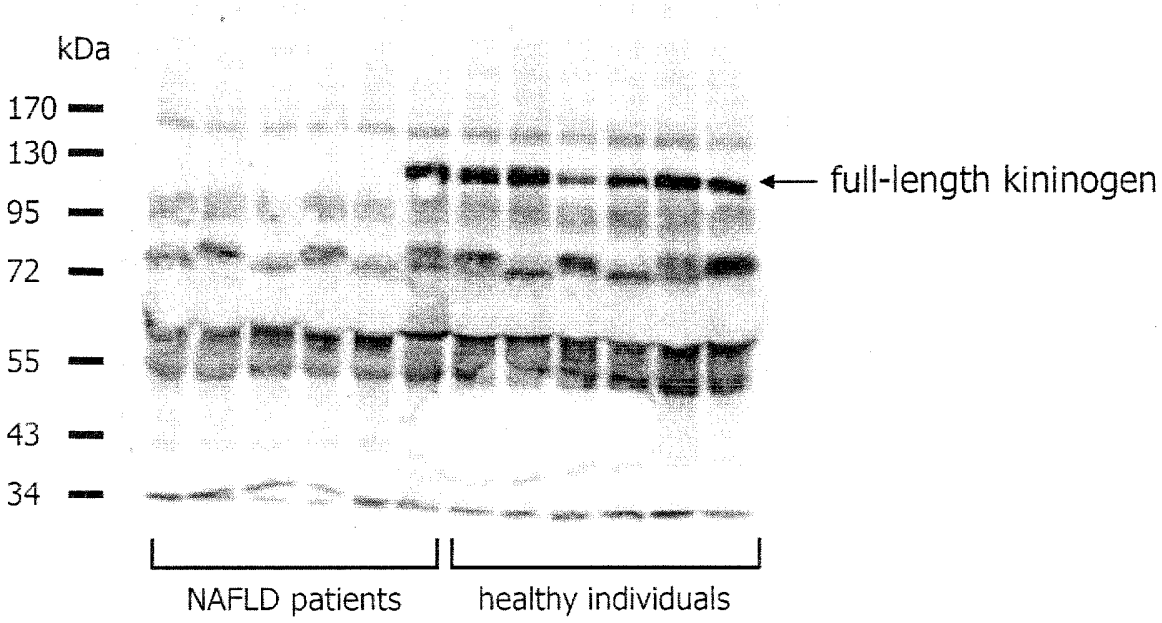

kDa
170
130
95
72
55
43
34
full-length kininogen
NAFLD patients
healthy individuals

Fig. 6

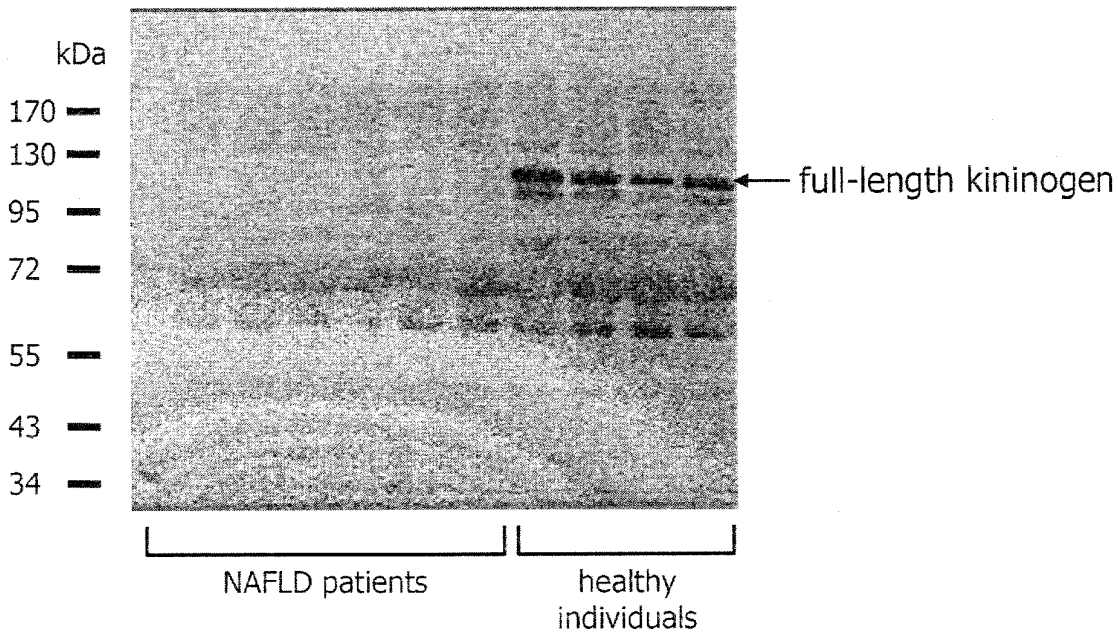

kDa
170
130
95
72
55
43
34
full-length kininogen
NAFLD patients
healthy individuals

Fig. 7

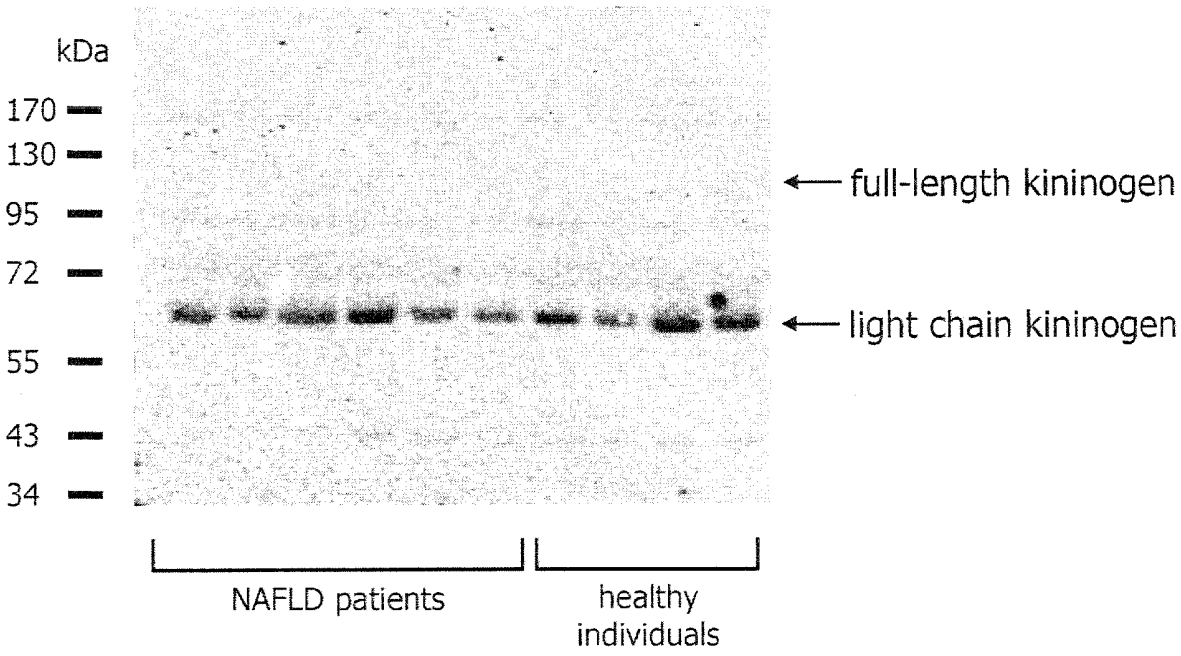

kDa
170
130
95
72
55
43
34
full-length kininogen
light chain kininogen
NAFLD patients
healthy individuals

Fig. 8

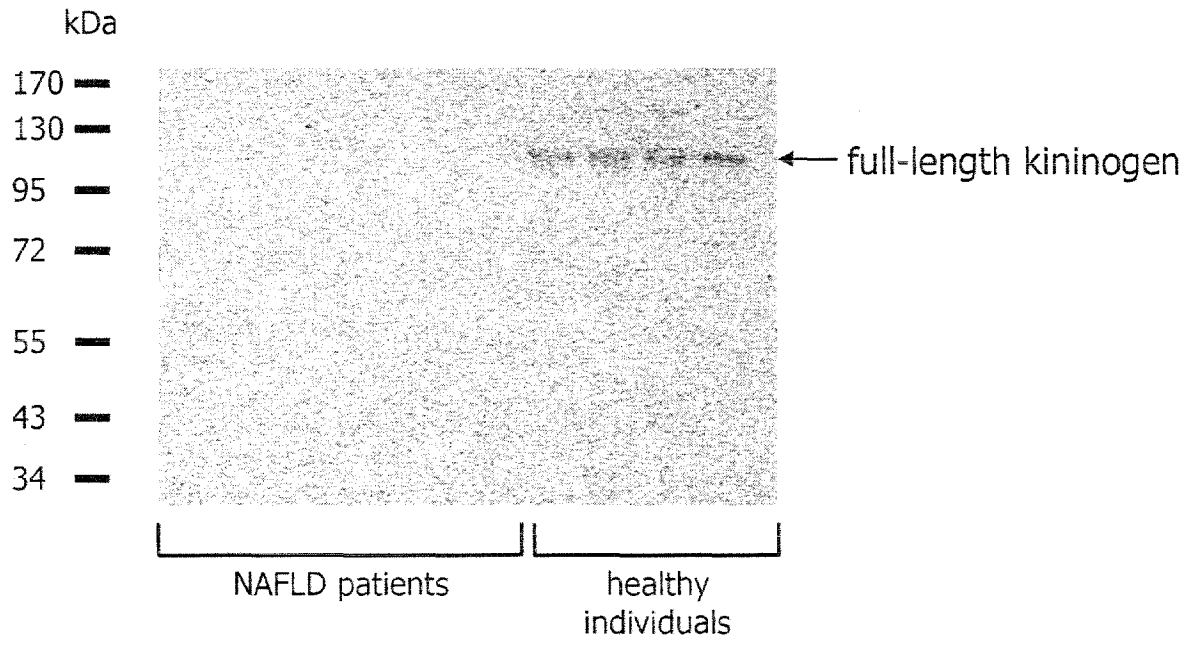

kDa
170
130
95
72
55
43
34
full-length kininogen
NAFLD patients
healthy individuals

Fig. 9

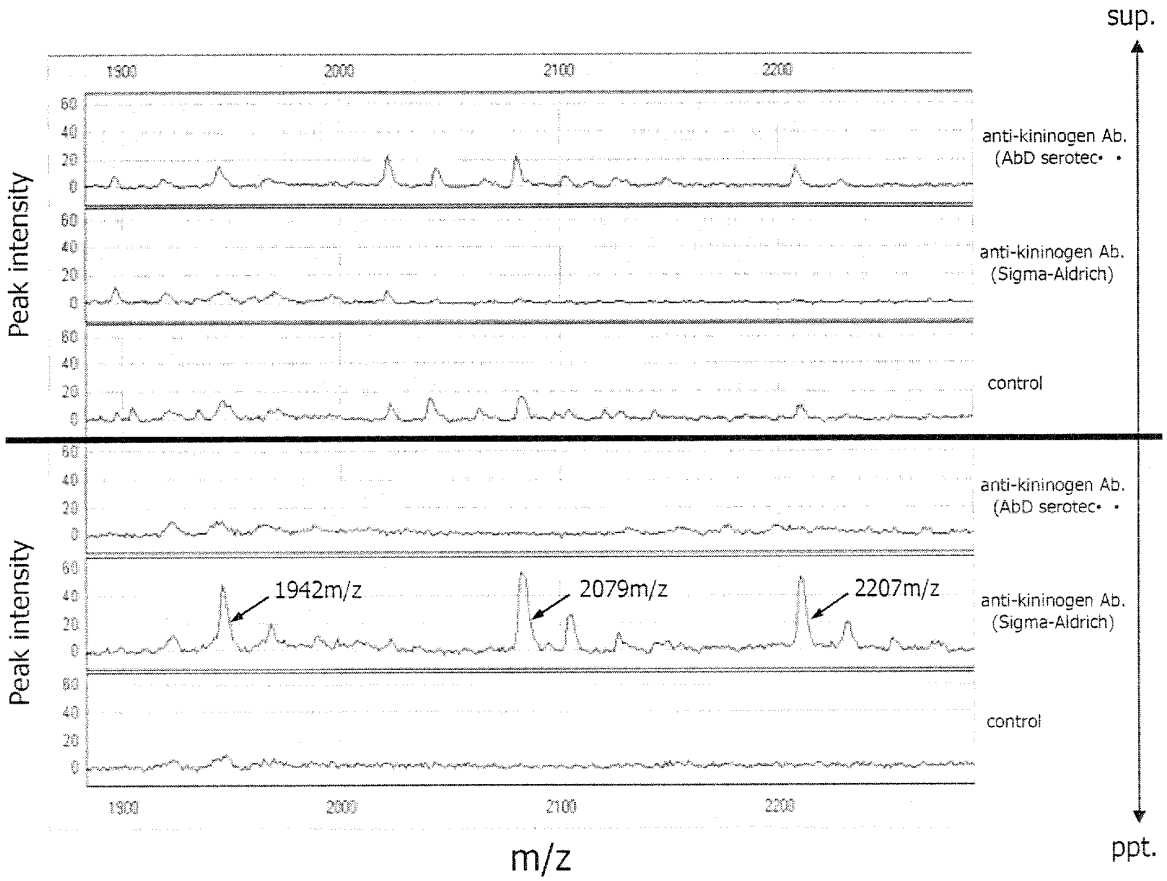

sup.
ppt.
Peak intensity
Peak intensity
1900
2000
2100
2200
m/z
anti-kininogen Ab. (AbD serotec• •
anti-kininogen Ab. (Sigma-Aldrich)
control
anti-kininogen Ab. (AbD serotec• •
anti-kininogen Ab. (Sigma-Aldrich)
control
1942m/z
2079m/z
2207m/z

Fig. 10

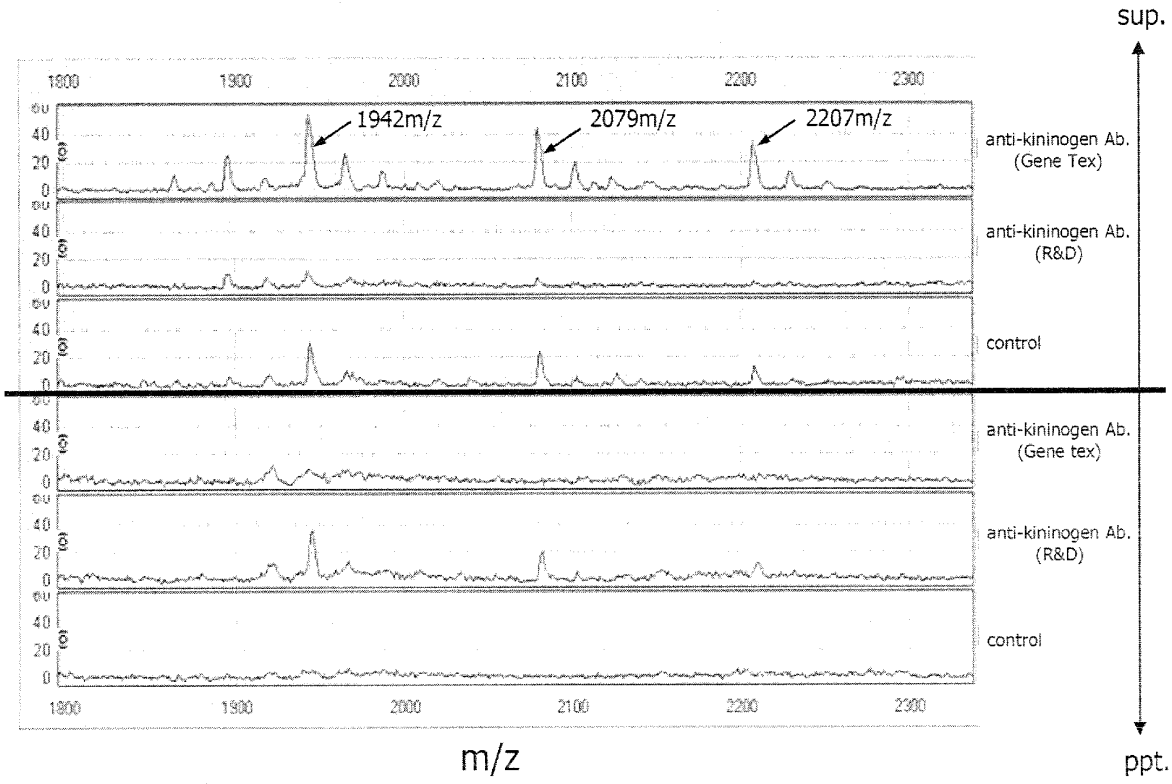

sup.
1800
1900
2000
2100
2200
2300
1942m/z
2079m/z
2207m/z
anti-kininogen Ab. (Gene Tex)
anti-kininogen Ab. (R&D)
control
anti-kininogen Ab. (Gene tex)
anti-kininogen Ab. (R&D)
control
m/z
ppt.

Fig. 11

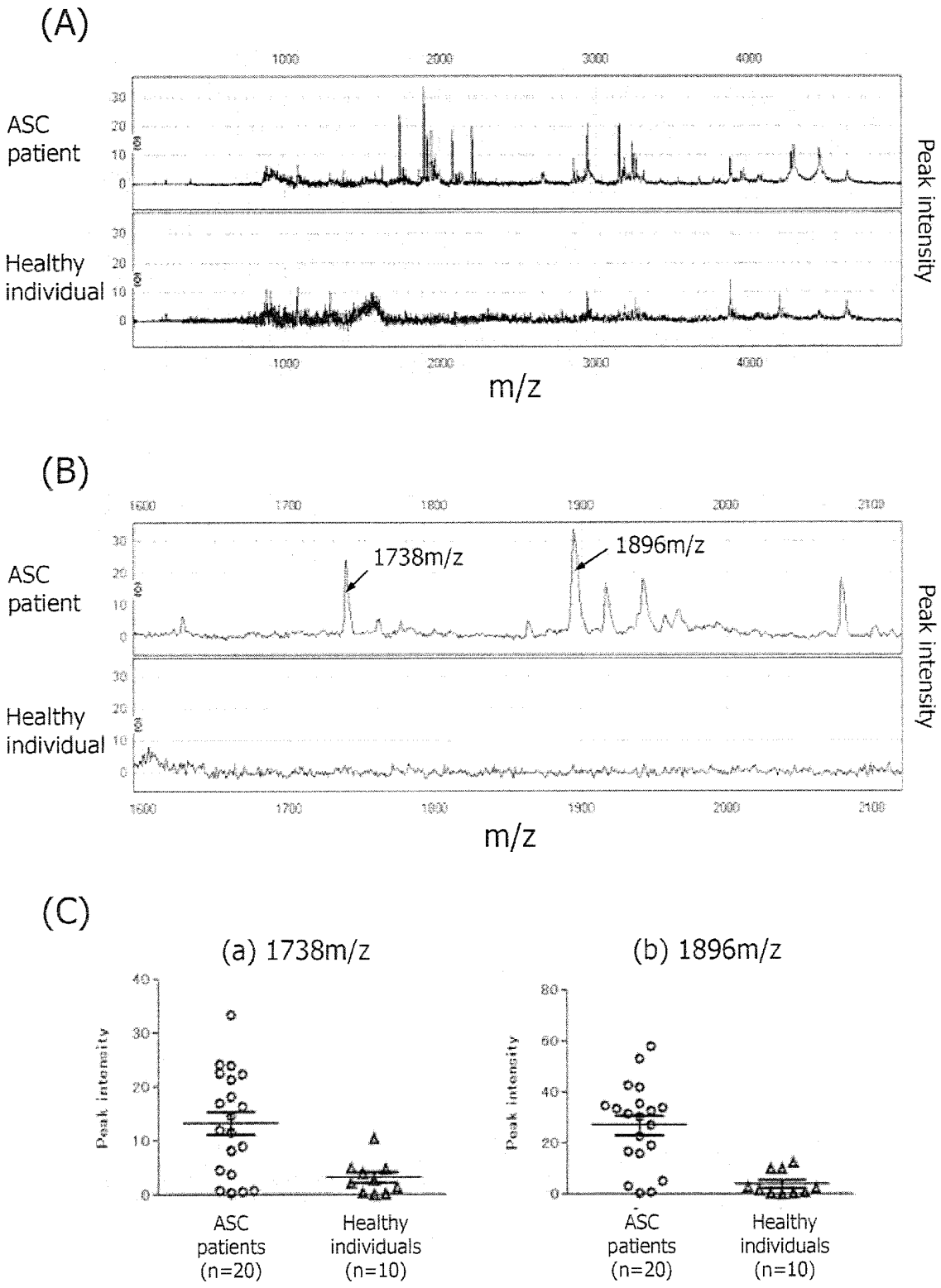

(A)
ASC patient
Healthy individual
Peak intensity
m/z
(B)
ASC patient
Healthy individual
1738m/z
1896m/z
Peak intensity
m/z
(C)
(a) 1738m/z
(b) 1896m/z
Peak intensity
ASC patients (n=20)
Healthy individuals (n=10)
ASC patients (n=20)
Healthy individuals (n=10)

Fig. 12

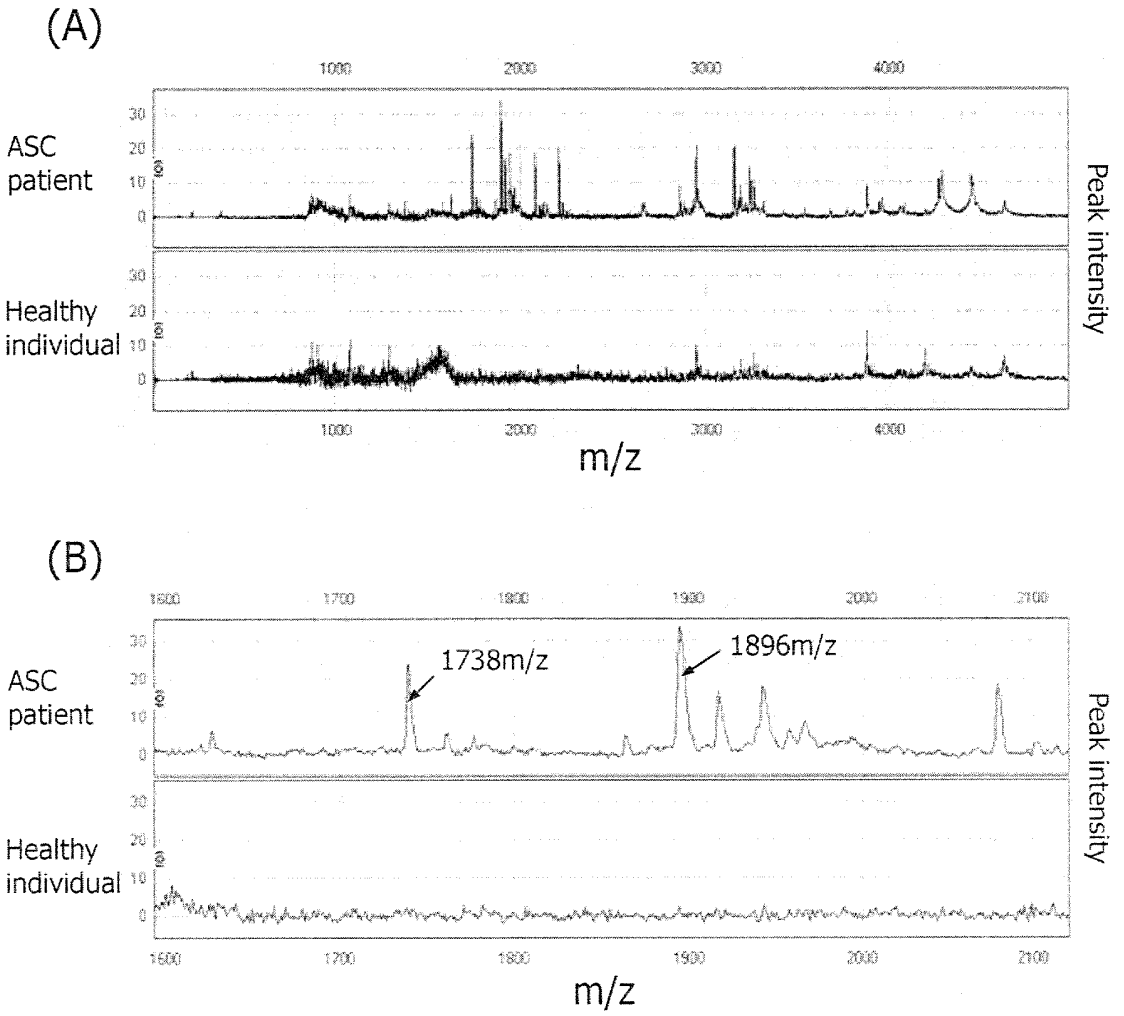
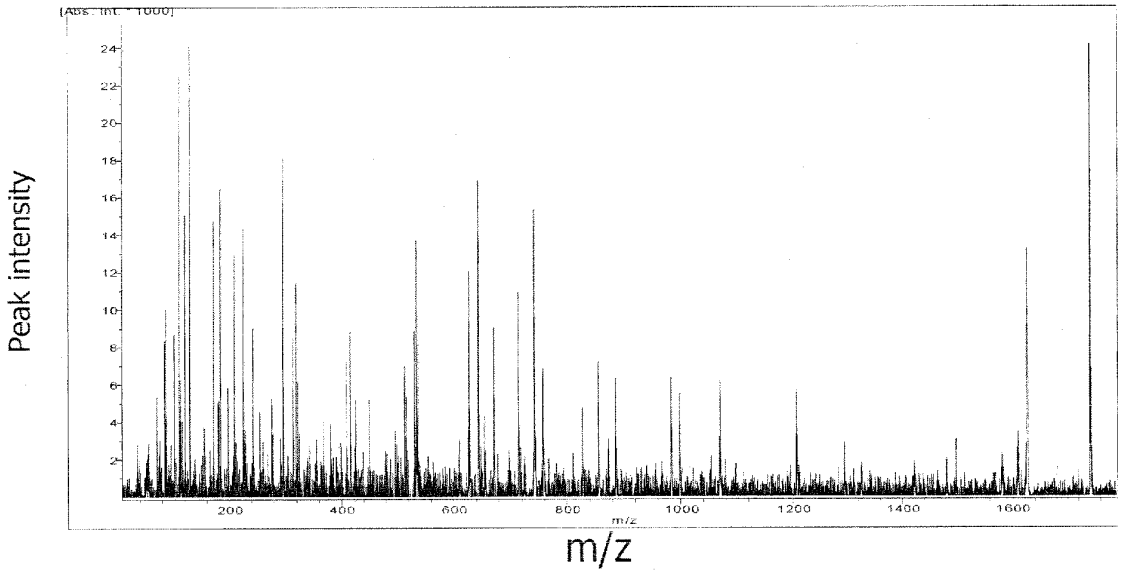

(A)
ASC patient
Healthy individual
Peak intensity
m/z
(B)
1738m/z
1896m/z
ASC patient
Healthy individual
Peak intensity
m/z
(C)
(a) 1738m/z
(b) 1896m/z
Peak intensity
ASC patients (n=20)
Healthy individuals (n=10)

Fig. 13

(A) 1738m/z



(B) 1896m/z

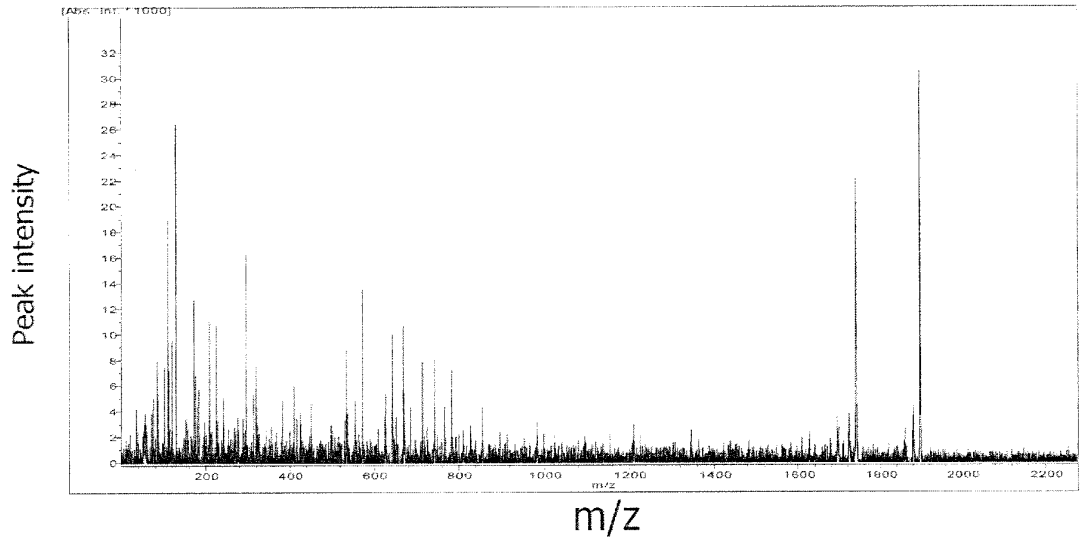
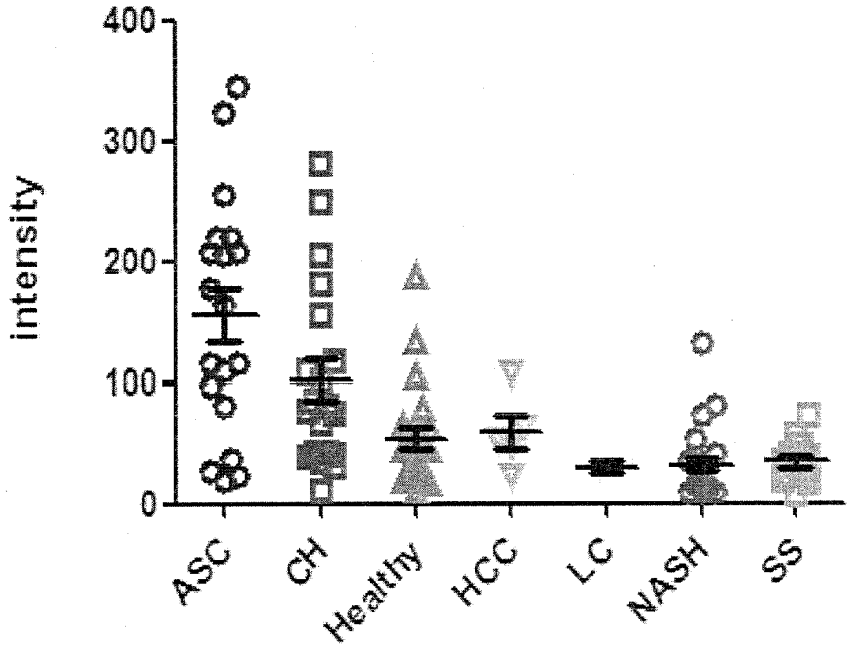


Fig. 14

(A)


intensity
400
300
200
100
0
ASC
CH
Healthy
HCC
LC
NASH
SS

(B)

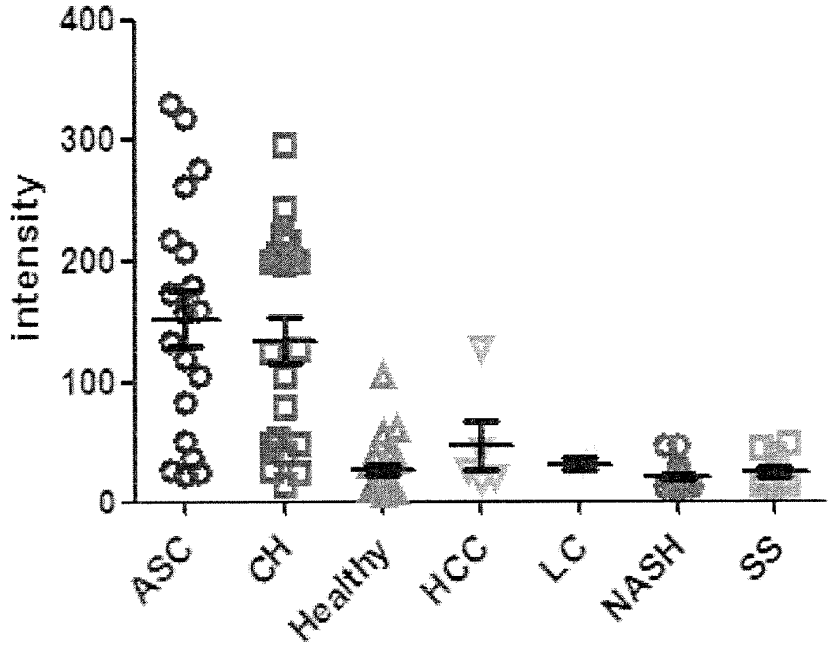

intensity
400
300
200
100
0
ASC
CH
Healthy
HCC
LC
NASH
SS

Fig. 15

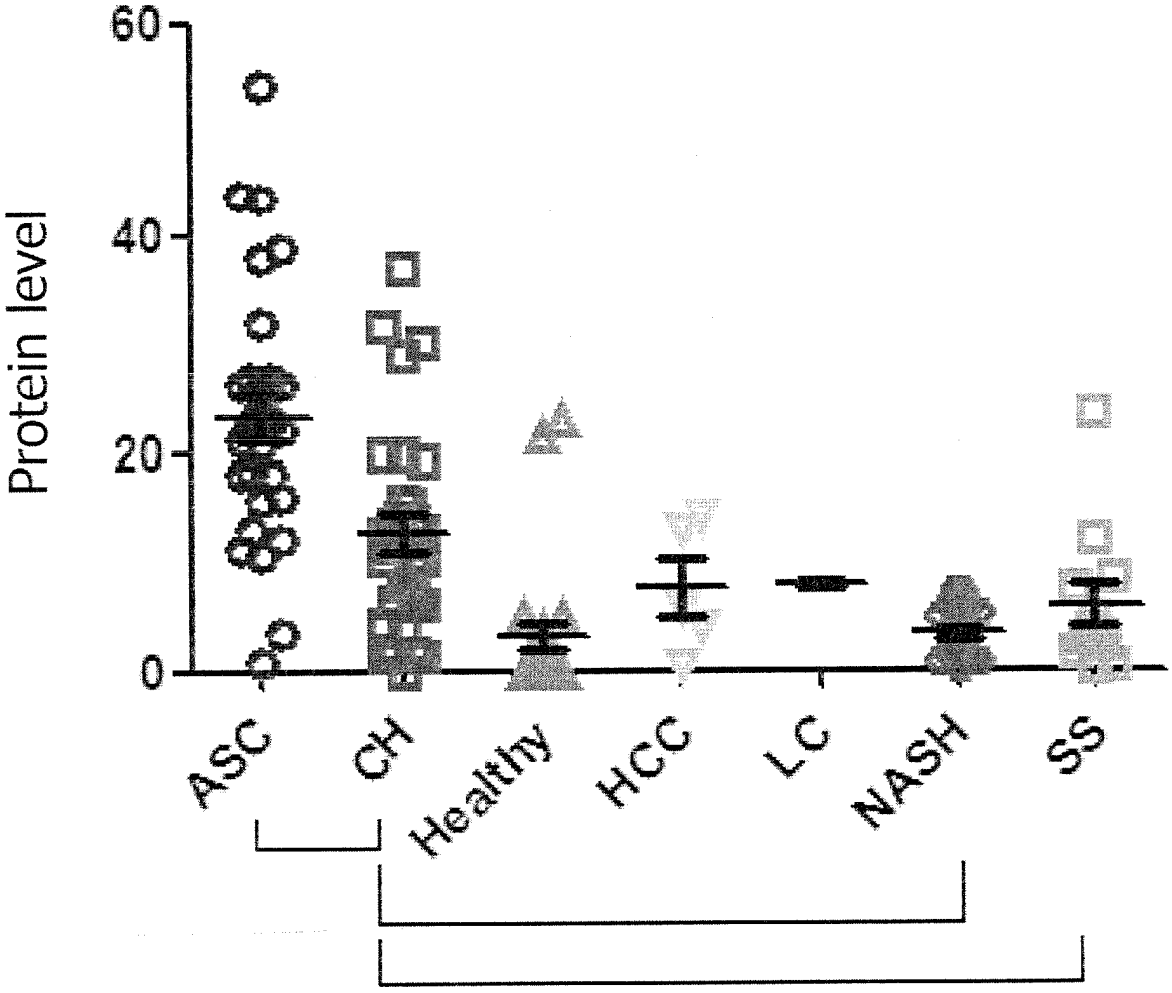


Fig. 16

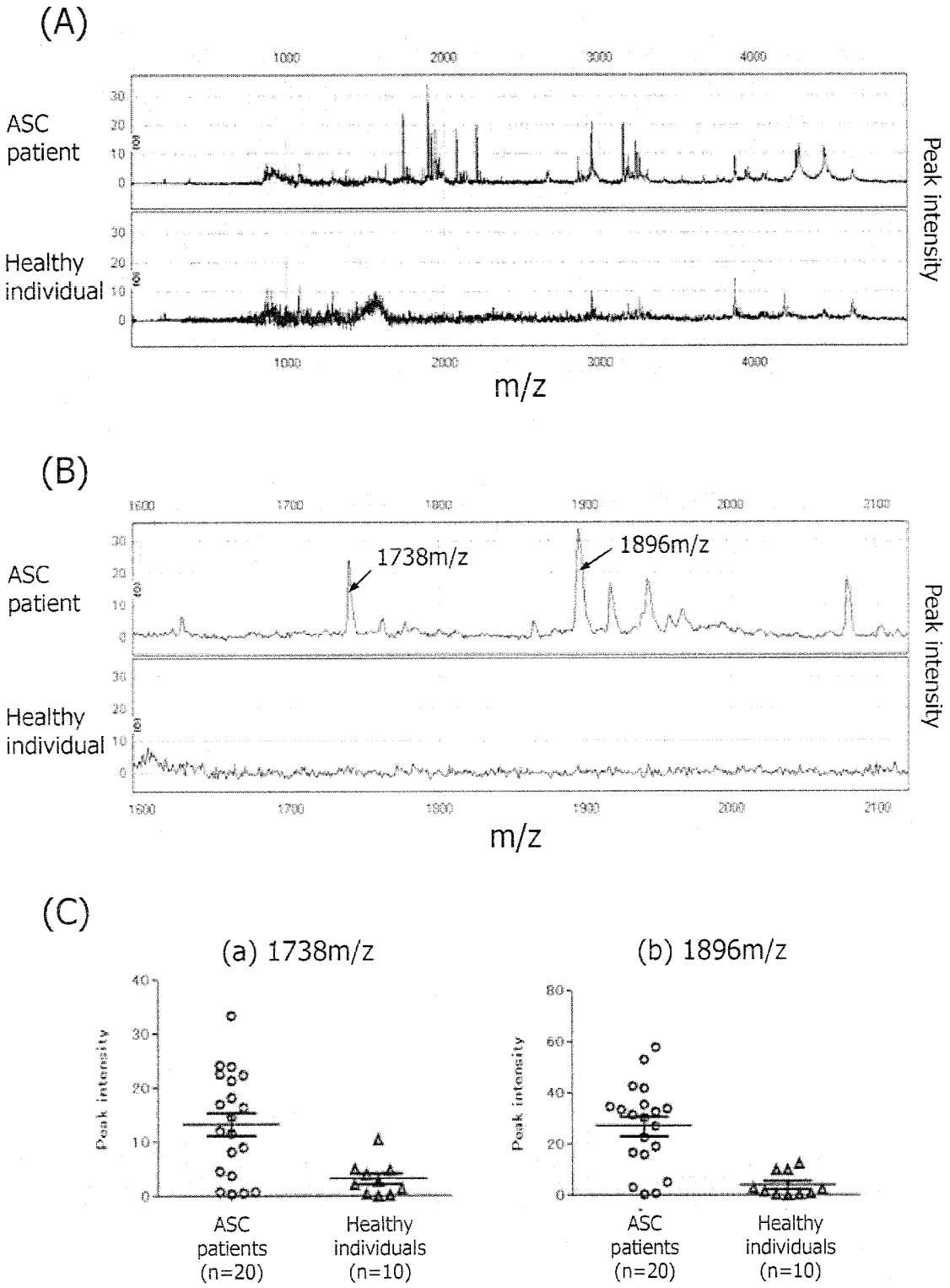

(A)
ASC patient
Healthy individual
Peak intensity
m/z
(B)
ASC patient
Healthy individual
1738m/z
1896m/z
Peak intensity
m/z
(C)
(a) 1738m/z
(b) 1896m/z
Peak intensity
ASC patients (n=20)
Healthy individuals (n=10)

Fig. 17

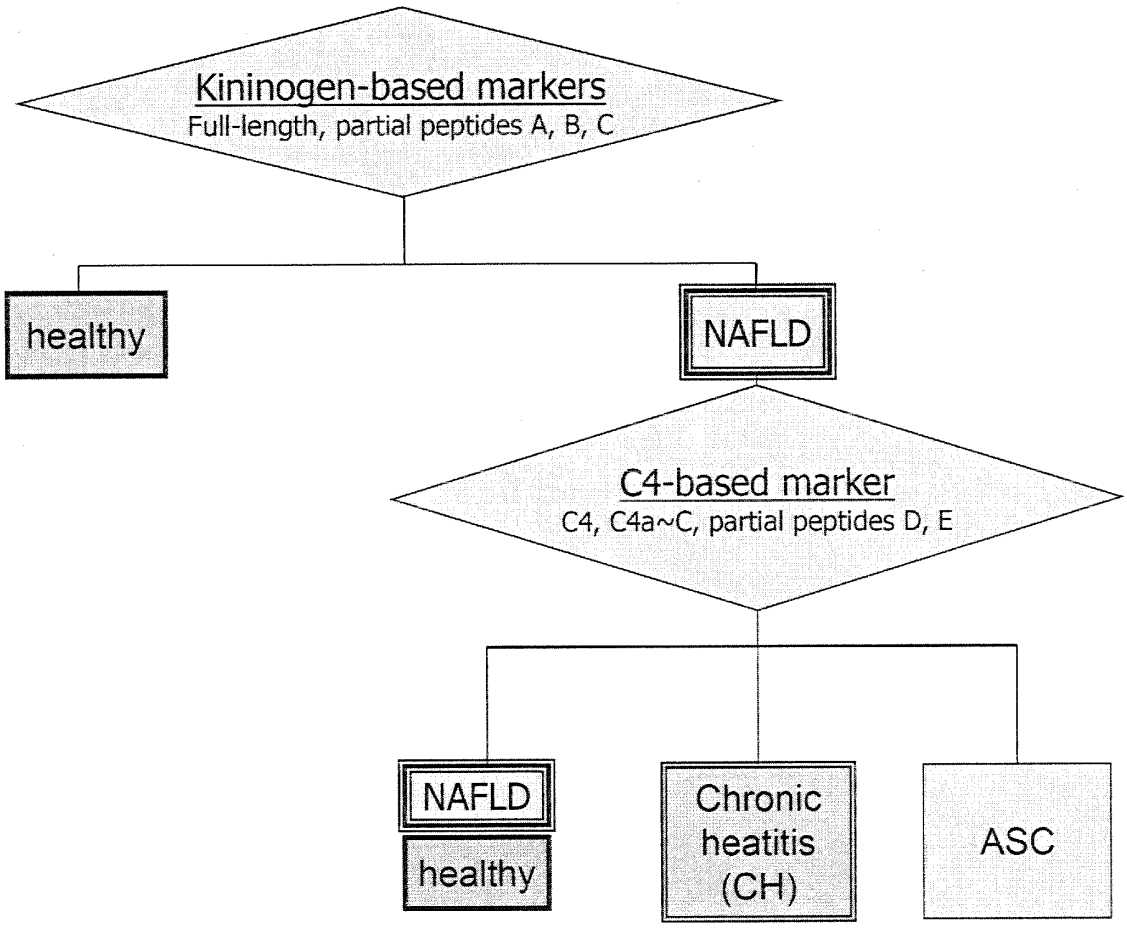
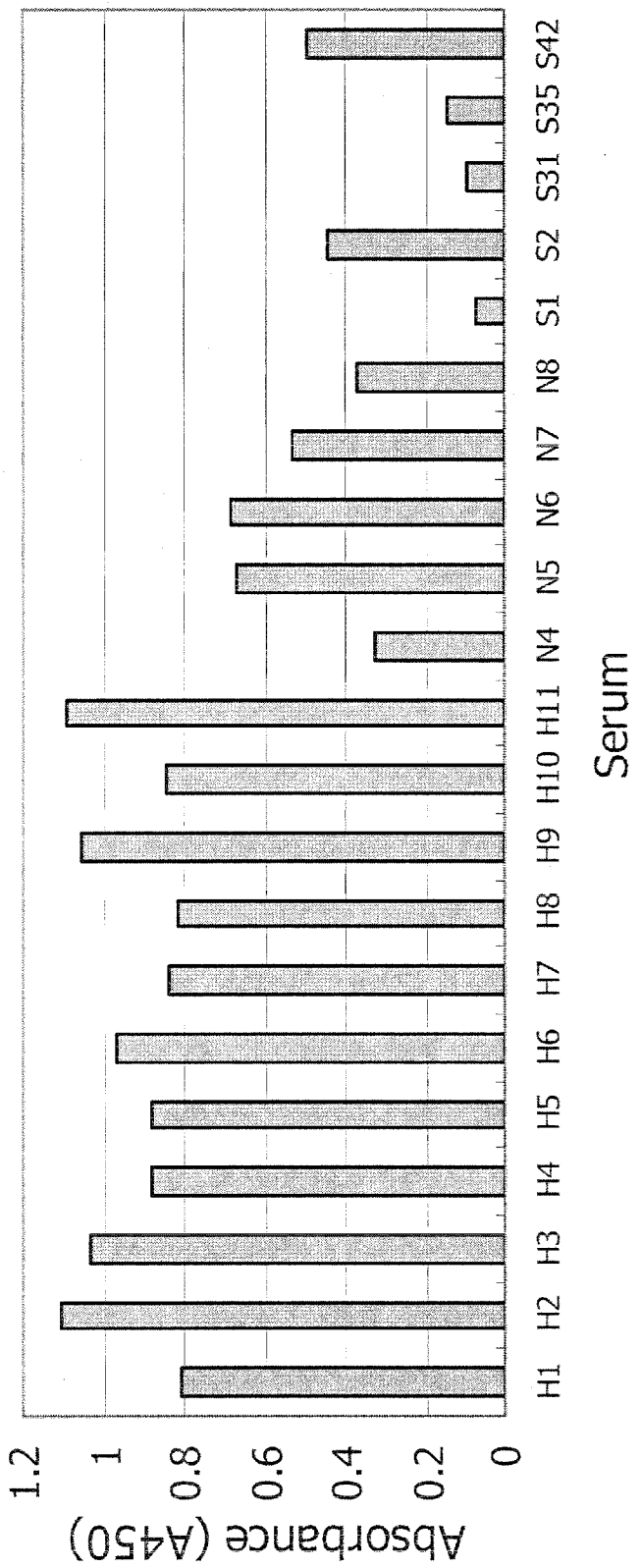

Kininogen-based markers
Full-length, partial peptides A, B, C
healthy
NAFLD
C4-based marker
C4, C4a~C, partial peptides D, E
NAFLD
healthy
Chronic heatitis (CH)
ASC

Fig. 18


Absorbance (A450)
1.2
1
0.8
0.6
0.4
0.2
0
H1
H2
H3
H4
H5
H6
H7
H8
H9
H10
H11
N4
N5
N6
N7
N8
S1
S2
S31
S35
S42
Serum

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2008/068985

A. CLASSIFICATION OF SUBJECT MATTER
G01N33/53(2006.01)i, *C07K16/18*(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N33/53, C07K16/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2008
Kokai Jitsuyo Shinan Koho 1971-2008 Toroku Jitsuyo Shinan Koho 1994-2008

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAplus(STN), JSTPlus(JDreamII), JMEDPlus(JDreamII), JST7580(JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOUHNIK J. et al., "Biochemical and physiological studies on two T-kininogen species using monoclonal antibodies.", Biochimica et Biophysica Acta, 1992.07.13, Vol.1122, No.1, P.70-76	3-5
X	JP 2006-308533 A (MCBI Inc.), 09 November, 2006 (09.11.06), (Family: none)	8-10
A	Mamoru FUJII, 'Kakushu Kan Shikkan ni Okeru Kessho Kallikrein-kinin-kei no Hendo to sono Rinshoteki Igi -Tokuni Alcohol-sei Kan Shogai o Chushin to shite-', Japanese Journal of Gastroenterological Surgery, 05 March, 1985 (05.03.85), Vol.82, No.3, pages 450 to 458	1-6,12,13, 15-18

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
26 November, 2008 (26.11.08)	09 December, 2008 (09.12.08)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2008/068985

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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

Form PCT/ISA/210 (continuation of second sheet) (April 2007)

REFERENCES CITED IN THE DESCRIPTION

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

Patent documents cited in the description

- JP 4110660 A **[0003] [0006]**
- JP 2006300689 A **[0005] [0006]**
- JP 2006308533 A **[0005] [0006]**

Non-patent literature cited in the description

- **C Cordova et al.** Hageman factor, high molecular weight kininogen, and prekallikrein in chronic liver disease. *J Clin Pathol,* 1986, vol. 39, 1003-1005 **[0006]**
- **Dumestre-Perard et al.** *Clin Exp Immunol,* 2002, vol. 127, 131-136 **[0006]**
- **Robert W. Colman et al.** Contact System: A Vascular Biology Modulator With Anticoagulant, Profibrinolytic, Antiadhesive, and Proinflammatory Attributes. *Blood,* 1997, vol. 90 (10), 3819-3833 **[0035]**

专利名称(译)	用于诊断肝病的生物标志物		
公开(公告)号	EP2216651A4	公开(公告)日	2010-12-01
申请号	EP2008840152	申请日	2008-10-20
[标]申请(专利权)人(译)	宫崎县立INDAL支持FOUND		
申请(专利权)人(译)	宫崎县立产业支撑基础 鹿儿岛大学 中外SEIYAKU株式会社		
当前申请(专利权)人(译)	宫崎县立产业支撑基础 鹿儿岛大学 中外SEIYAKU株式会社		
[标]发明人	TSUBOUCHI HIROHITO UTO HIROFUMI OKANOUE TAKESHI ISHIDA YO ICHI SATO YUKO SUDO MASAYUKI		
发明人	TSUBOUCHI, HIROHITO UTO, HIROFUMI OKANOUE, TAKESHI ISHIDA, YO-ICHI SATO, YUKO SUDO, MASAYUKI		
IPC分类号	G01N33/53 C07K16/18		
CPC分类号	G01N33/6893 G01N2333/8139 G01N2800/085		
代理机构(译)	法思博事务所		
优先权	2007270799 2007-10-18 JP 2008145337 2008-06-03 JP		
其他公开文献	EP2216651A1		
外部链接	Espacenet		

摘要(译)

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