US 20100248256A1

(19) **United States**

(12) **Patent Application Publication**

Minagawa et al.

(10) **Pub. No.: US 2010/0248256 A1**

(43) **Pub. Date: Sep. 30, 2010**

(54) **HEPATOCELLULAR CARCINOMA PROTEIN MARKER, AND METHOD FOR DETECTION OF HEPATOCELLULAR CARCINOMA USING THE SAME**

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(21) Appl. No.: **12/452,216**

(22) PCT Filed: **Jun. 16, 2008**

(86) PCT No.: **PCT/JP2008/061375**

§ 371 (c)(1),
(2), (4) Date: **Dec. 22, 2009**

(30) **Foreign Application Priority Data**

Jun. 20, 2007 (JP) 2007-162857

Publication Classification

(51) **Int. Cl.**
G01N 33/53 (2006.01)
C07K 14/435 (2006.01)

(52) **U.S. Cl.** **435/7.1**; 530/350; 436/501

(57) **ABSTRACT**

Provided are: a method of assessing hepatocellular carcinoma by using a protein with a different phosphorylated state in hepatocellular carcinoma cells compared with non-hepatocellular carcinoma cells; and a hepatocellular carcinoma protein marker for detecting hepatocellular carcinoma formed of the protein. The hepatocellular carcinoma protein marker for detecting hepatocellular carcinoma includes tumor rejection antigen gp96 formed of the amino acid represented by SEQ ID NO: 1, and is measured for its phosphorylated state to detect the presence or absence of hepatocellular carcinoma.

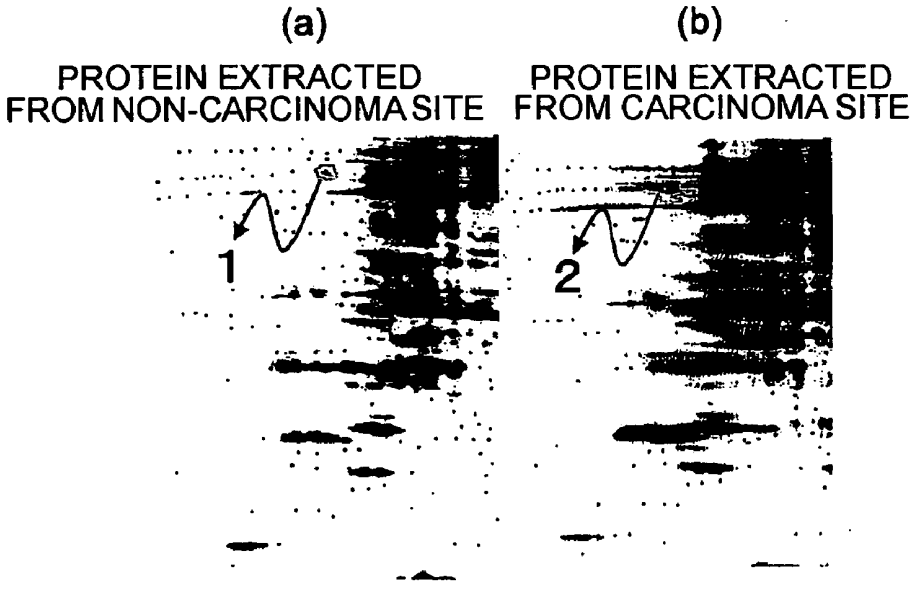
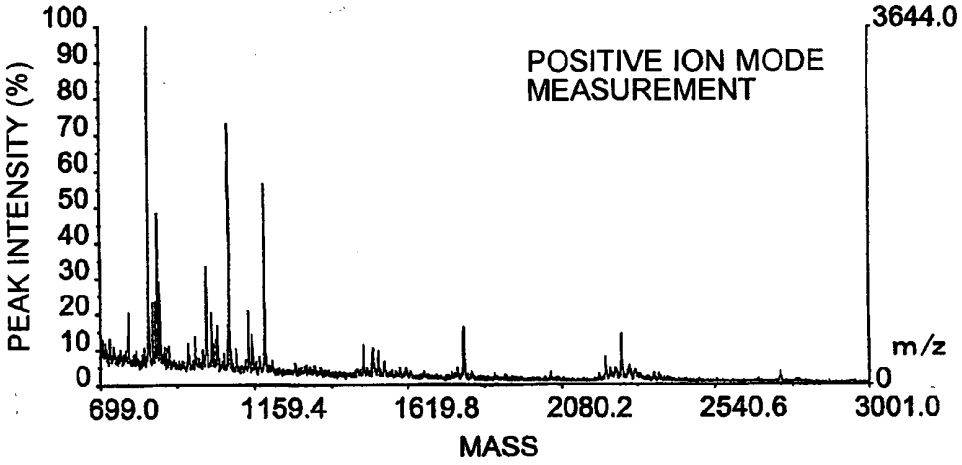

(a)
(b)
PROTEIN EXTRACTED FROM NON-CARCINOMA SITE
PROTEIN EXTRACTED FROM CARCINOMA SITE
1
2
STAINING WITH SOLUTION FOR STAINING PHOSPHORYLATED PROTEIN

FIG. 1

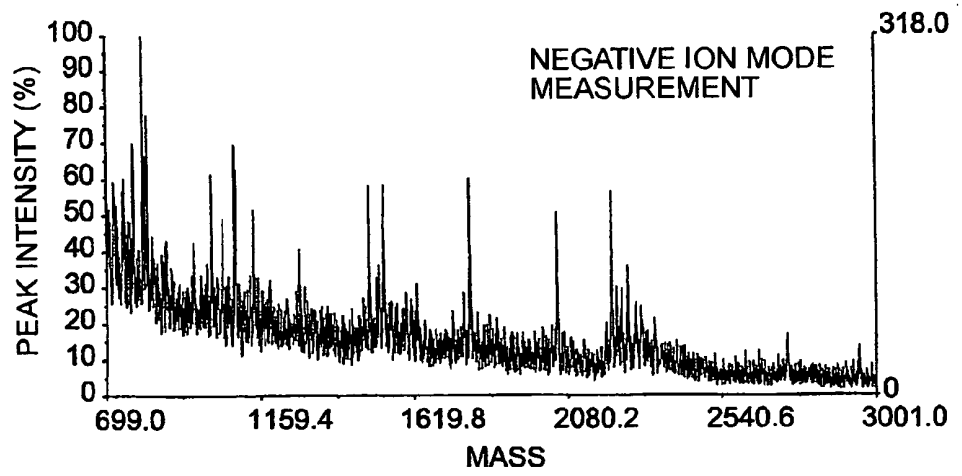
1 MRALWVLGLC CVLLTFGSVR ADDEVDVDGT VEEDLGKSRE GSR**TDDEVVQ**
51 **REEEAIQLDG LNASQIR**ELR EKSEK**FAFQA EVNR**MMKLII NSLYKNKEIF
101 LR**ELISNASD ALDK**IRLISL TDENALSGNE ELTVKIKCDK EKNLLHVTDT
151 GVGMTREELV K**NLGTIAKSG TSEFLNK**MTE AQEDGQSTSE LIGQFGVGFY
201 SAFLVADKVI VTSKHNNDTQ HIWESDSNEF SVIADPRGNT LGRGTTITLV
251 LK**EEASDYLE LDTIK**NLVKK YSQFINFPIY VWSSK**TETVE EPMEEEEAAK**
301 **EEKEESDDEA AVEEEEEEK**K PKTKKVEKTV WDWELMNDIK PIWQRPSK**EV**
351 **EEDEYK**AFYK SFSKESDDPM AYIHFTAEGE VTFK**SILFVP TSAPR**GLFDE
401 YGSKKSDYIK LYVRR**VFITD DFHDMMPKYL NFVKGVVDSD DLPLNVSR**ET
451 LQQHKLLKVI RK**KLVR**KTLD MIKKIADDKY NDTFWKEFGT NIKLGVIEDH
501 SNRTRLAKLL R**FQSSHHPTD ITSLDQYVER** MKEKQDK**IYF MAGSSR**KEAE
551 SSPFVERLLK KGYEVIYLTE PVDEYCIQAL PEFDGKRFQN VAKEGVKFDE
601 SEKTKESREA VEK**EFEPLLN WMK**DKALKDK IEKAVVSQRL TESPCALVAS
651 QYGWSGNMER IMKAQAYQTG K**DISTNYYAS QK**KTFEINPR HPLIRDMLRR
701 IKEDEDDKTV LDLAVVLFET ATLRSGYLLP DTKAYGDRIE RMLRLSLNID
751 PDAKVEEEPE EEPEETAEDT TEDTEQDEDE EMDVGTDEEE ETAKESTAEK
801 DEL

FIG. 2

(a)


100
90
80
70
60
50
40
30
20
10
0
PEAK INTENSITY (%)
POSITIVE ION MODE
MEASUREMENT
3644.0
m/z
0
699.0
1159.4
1619.8
2080.2
2540.6
3001.0
MASS

(b)


100
90
80
70
60
50
40
30
20
10
0
PEAK INTENSITY (%)
NEGATIVE ION MODE
MEASUREMENT
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0
699.0
1159.4
1619.8
2080.2
2540.6
3001.0
MASS

FIG. 3

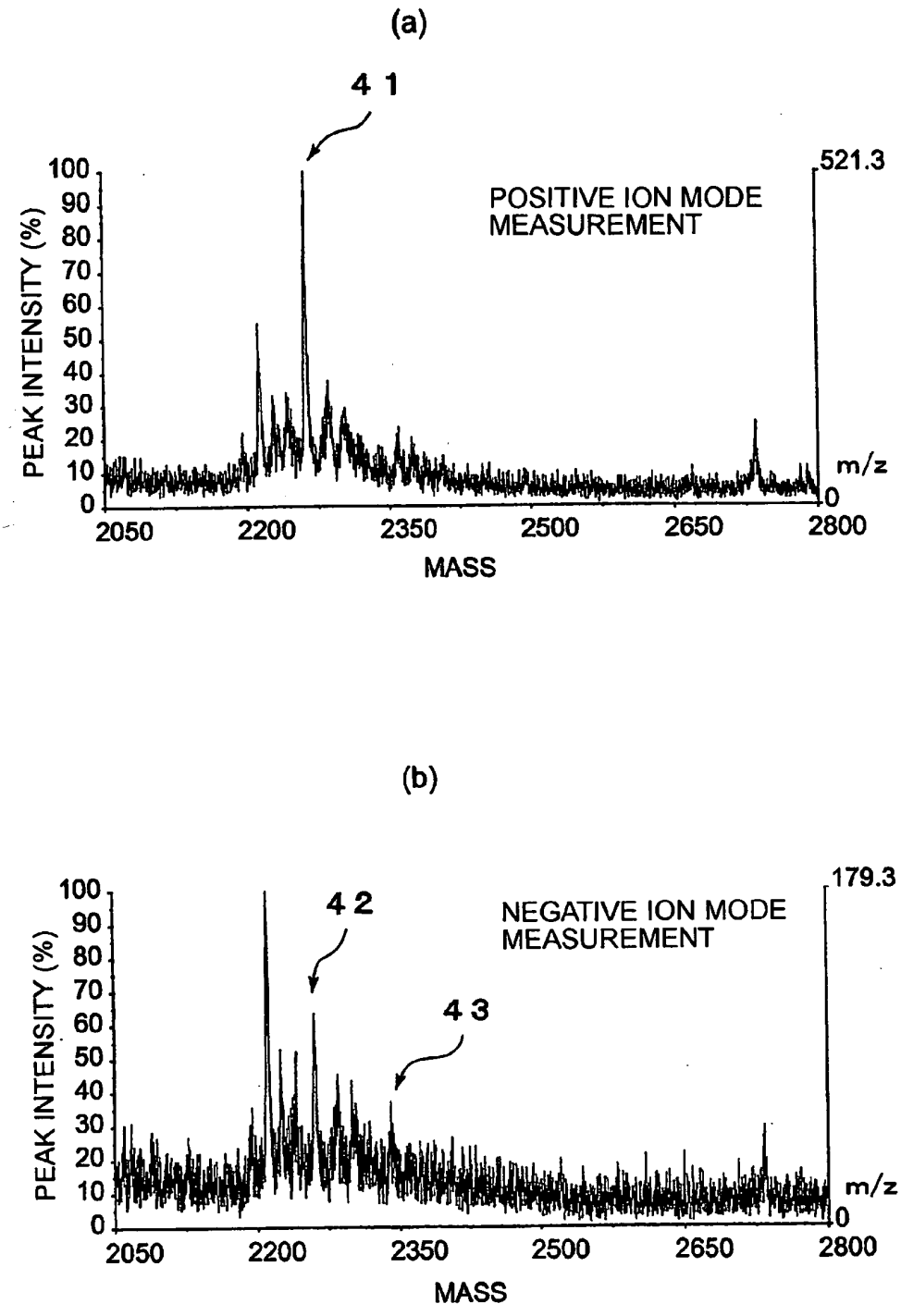
(a)
41
521.3
POSITIVE ION MODE
MEASUREMENT
PEAK INTENSITY (%)
100
90
80
70
60
50
40
30
20
10
0
m/z
0
2050
2200
2350
2500
2650
2800
MASS
(b)
179.3
42
43
NEGATIVE ION MODE
MEASUREMENT
PEAK INTENSITY (%)
100
90
80
70
60
50
40
30
20
10
0
m/z
0
2050
2200
2350
2500
2650
2800
MASS

FIG. 4

HEPATOCELLULAR CARCINOMA PROTEIN MARKER, AND METHOD FOR DETECTION OF HEPATOCELLULAR CARCINOMA USING THE SAME

TECHNICAL FIELD

[0001] This invention relates to a phosphorylating-modified, i.e. phosphorylated, protein marker that can be used in detection of hepatocellular carcinoma and a method of detecting a hepatocellular carcinoma including a step of using the protein marker.

BACKGROUND ART

[0002] The hepatocellular carcinoma is one of epithelial malignant tumors developed as a primary carcinoma in a liver and formed of tumor cells similar to hepatocytes. In general, a large number of tumors are formed in a liver and are likely to grow and progress in a hepatic vessel, and a tumor thrombosis is frequently formed in a portal vein. The hepatocellular carcinoma often breaks out in the Asian region including Japan and the African region, and cirrhosis concurs in many cases.

[0003] As a marker for detecting hepatocellular carcinoma, use is made have been conventionally of α-fetoprotein (AFP) and PIVKA-II (see Non-patent Document 1), KM-2 (see Non-patent Document 2), CAl25 (see Non-patent Document 3), and the like.

[0004] Meanwhile, it is known that a post-translational modified, in particular, phosphorylated protein reflects conditions of various organs and tissues. For example, a phosphorylated troponin 1 protein for recognizing a condition of a muscle tissue that has suffered damage (see Patent Document 1); and phosphorylated urokinase to be used for detecting various types of carcinoma (see Patent Document 2).

[Non-patent Document 1] Liebman H A, Furie B C, Tong M J, Blanchard R A, Lo K J, Lee S D, Coleman M S, and Furie B., New Engl. J. Med. 310, pp. 1427-1431. (1984)

[Non-patent Document 2] Kumagai Y, Chiba J, Sata T, Ohtaki S, and Mitamura K. Cancer Res. 52, pp 4987-4994. (1992)

[0005] [Non-patent Document 3] Elias J, Kew M C. Int. J. Cancer. 46, pp 805-807. (1990)

[Patent Document 1] JP-A-2006-502203, Title of the Invention "ISOLATED POST-TRANSLATIONALLY MODIFIED PROTEINS FOR MONITORING AND DIAGNOSING MUSCLE DAMAGE"

[Patent Document 2] JP-B-3129430, Title of the Invention "METHOD FOR DETECTING TUMOR DISEASE"

DISCLOSURE OF THE INVENTION

Problem to be Solved by the Invention

[0006] However, markers disclosed in Non-patent Documents 1 to 3 have not been sufficient in assessment rate of positive results.

[0007] For example, the screening rates of AFP and PIVKA-II in hepatocellular carcinoma assessment are 60% to 70%. Therefore, there is a demand for a marker with additional reliability.

[0008] An object of this invention is to provide a protein other than those described in the above-mentioned Patent Documents 1 and 2, including a method of assessing hepatocellular carcinoma by using a protein which is present in a hepatocellular carcinoma cell in a different phosphorylated state from that in a non-hepatic carcinoma cell.

[0009] Further, another object of this invention is to provide a hepatocellular carcinoma protein marker for detecting the hepatocellular carcinoma, including a protein having a different phosphorylated state.

Means to Solve the Problem

[0010] According to an aspect of this invention, there is provided a hepatocellular carcinoma protein marker, including tumor rejection antigen gp96 formed of an amino acid represented by SEQ ID NO: 1, in which the protein marker is phosphorylated.

[0011] According to another aspect of this invention, there is provided a method of detecting hepatocellular carcinoma, which includes using the hepatocellular carcinoma protein marker as above-described.

[0012] According to still another aspect of this invention, there is provided a method of detecting hepatocellular carcinoma as above described in which the using the protein marker is verifying phosphorylation of the protain marker.

[0013] According to yet another aspect of this invention, there is provided a method of detecting hepatocellular carcinoma, in which the using verification is performed based on measurement of any one of a serine residue, a threonine residue, and a tyrosine residue of the protein marker for its phosphorylation.

[0014] According to a further aspect of this invention, there is provided a method of detecting hepatocellular carcinoma, in which using the protein marker is extracted from a surgically sampled biological sample.

[0015] According to a still further aspect of this invention, there is provided a method of detecting hepatocellular carcinoma, in which the biological sample includes any one kind of a biopsy sample, blood, plasma, serum, and urine.

[0016] According to a yet further aspect of this invention, there is provided a method of detecting hepatocellular carcinoma, which includes: measuring phosphorylation of tumor rejection antigen gp96 formed of an amino acid represented by SEQ ID NO: 1; and judging the measurements as positive based on a comparison with a normal value.

[0017] According to another aspect of this invention, there is provided a method of detecting hepatocellular carcinoma, which comprises measuring phosphorylation of tumor rejection antigen gp96 as the hepatocellular carcinoma protein marker as above-described in a biological sample, wherein the measuring is performed by a method selected from the group consisting of an enzyme immunoassay, a fluorescence-labeled antibody method, a western blot method, a radioimmunoassay, an immunoprecipitation method, electrophoresis, liquid chromatography, and mass spectrometry.

[0018] According to still another aspect of this invention, there is provided a method of detecting hepatocellular carcinoma, in which the phosphorylation is measured with respect to any one of a serine residue, a threonine residue, and a tyrosine residue of the hepatocellular carcinoma protein marker.

[0019] According to yet another aspect of this invention, there is provided a method of detecting hepatocellular carcinoma as above-described, in which the measuring comprises: extracting a protein from a surgically sampled biological sample; subjecting the protein to a multicycle of electrophoresis with different dimensions; fixing and staining the protein on a solid carrier; and comparing an increase or decrease in the tumor rejection antigen gp96 in a protein spot.

[0020] According to a further aspect of this invention, there is provided a method of detecting hepatocellular carcinoma,

in which the biological sample includes any one kind of a biopsy sample, blood, plasma, serum, and urine.

[0021] According to a still further aspect of this invention, there is provided a method of detecting hepatocellular carcinoma as above-described, in which judging a presence or absence of hepatocellular carcinoma based on a comparison of the results obtained from measuring phosphorylation of a digestion product of the tumor rejection antigen gp96 obtained from a surgically sampled biological sample in positive ion mode measurement and in negative ion mode measurement by using a mass spectrometer.

[0022] According to a yet further aspect of this invention, there is provided a method of detecting hepatocellular carcinoma, in which the biological sample includes any one kind of a biopsy sample, blood, plasma, serum, and urine.

[0023] According to another aspect of this invention, there is provided a method of detecting hepatocellular carcinoma, in which an antibody that recognizes a phosphorylated site of the tumor rejection antigen gp96 is used.

[0024] According to still another aspect of this invention, there is provided a method of detecting hepatocellular carcinoma, in which the antibody recognizes any one of a phosphorylated serine residue, a phosphorylated threonine residue, and a phosphorylated tyrosine residue.

EFFECT OF THE INVENTION

[0025] The measurement of phospholation the tumor rejection antigen gp96 formed of the amino acid represented by SEQ ID NO: 1 can distinct hepatocellular carcinoma.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] FIG. **1** shows a comparison between gels each stained with a solution for staining a phosphorylated protein after a protein sample extracted from a non-carcinoma site (a) and a protein sample extracted from a carcinoma site (b), which are obtained from 18 patients, have been developed by two-dimensional electrophoresis.

[0027] FIG. **2** is a view showing the whole amino acid sequence of tumor rejection antigen gp 96.

[0028] FIG. **3** includes charts each showing a MALDI-TOF/MS spectrum of a product obtained by digesting tumor rejection antigen gp96 with trypsin. (a) shows the results in positive ion mode measurement and (b) shows the results in negative ion mode measurement.

[0029] FIG. **4** includes charts each showing a MALDI-TOF/MS spectrum of a product obtained by digesting tumor rejection antigen gp96 with trypsin, and showing a comparison between the results in positive ion mode measurement (a) and the results in negative ion mode measurement (b) in a mass range of 2,050 Da to 2,800 Da.

DESCRIPTION OF REFERENCE NUMBERS

[0030] **1** spot of tumor rejection antigen gp96 of sample derived from non-carcinoma site

[0031] **2** spot of tumor rejection antigen gp96 of sample derived from carcinoma site

[0032] **41** peak derived from FQSSHHPT-DITSLDQYVER peptide of tumor rejection antigen gp96 measured in positive ion mode

[0033] **42** peak derived from FQSSHHPT-DITSLDQYVER peptide of tumor rejection antigen gp96 measured in negative ion mode

[0034] **43** peak derived from FQSSHHPT-DITSLDQYVER peptide of phosphorylated tumor rejection antigen gp96 measured in negative ion mode

BEST MODE FOR CARRYING OUT THIS INVENTION

[0035] Hereinafter, this invention is described in more detail.

[0036] The inventors of this invention have researched a protein whose phosphorylation is accelerated in hepatocellular carcinoma tissues compared with non-carcinoma cells. As a result, the inventors have found that the phosphorylation amount of a specified protein may be measured to distinguish hepatocellular carcinoma cells from non-carcinoma cells. Thus, this invention has been completed.

[0037] That is, this invention relates to a method of assessing hepatocellular carcinoma by measuring the variation in phosphorylation of tumor rejection antigen gp96 formed of the amino acid represented by SEQ ID NO: 1 in a biological sample obtained from a subject.

[0038] Specifically, this invention provides a method of measuring tumor rejection antigen gp96 formed of the amino acid represented by SEQ ID NO: 1 for its phosphorylation by a method selected from the group consisting of an enzyme immunoassay, a fluorescence-labeled antibody method, a western blot method, a radioimmunoassay, an immunoprecipitation method, electrophoresis, liquid chromatography, and mass spectrometry.

[0039] In this case, the biological sample, such as a biopsy sample, blood, plasma, serum, and urine, can be used in this invention.

[0040] Examples of the method of measuring such variation in protein phosphorylation as described above in such a biological sample include: a method using in combination two-dimensional electrophoresis for separating a protein from the biological sample, which is a combination of isoelectric point electrophoresis and SDS polyacrylamide gel electrophoresis, and a staining method for allowing a phosphorylated state of the separated protein to be visualized; a method involving separating the biological sample by multi-dimensional chromatography, which is a combination of various types of chromatography such as ion-exchange chromatography, reverse-phase chromatography, and gel filtration chromatography, and determining the phosphorylation of the separated protein by mass spectrometry; and a method using a specified antibody that recognizes a phosphorylated site of a protein.

[0041] Further, the specified antibody according to this invention is an antibody which recognizes a phosphorylated serine residue, a phosphorylated threonine residue, and a phosphorylated tyrosine residue. The protein phosphorylation in a sample may be detected by using those antibodies alone or in combination, and employing a known method such as an enzyme immunoassay (ELISA), a western blotting method, a radioimmunoassay, and an immunoprecipitation method.

EXAMPLES

[0042] This invention is described in detail by way of examples, but this invention is not limited by those examples.

Example 1

[0043] In Example 1 of this invention, a two-dimensional electrophoresis analysis of proteins extracted from carcinoma

cells and proteins extracted from non-carcinoma cells, which are derived from hepatocellular carcinoma patients, is described.

[0044] Carcinoma site tissues and non-carcinoma site tissues, which were obtained from tissues surgically excised from 18 hepatocellular carcinoma patients and were diagnosed pathologically, each were crushed in a cell lysis solution (30 mM Tris-Cl (pH 8.5), 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5 mM EDTA, PMSF, Aprotinin, and Pepstatin) by using a glass homogenizer, followed by incubation at 37° C. for 1 hour. After the sample was centrifuged (13,000 rpm, 20 minutes), the supernatant was collected. The protein concentration in the supernatant was measured using a protein assay kit (manufactured by Bio-Rad Laboratories, Inc.) by a Bradford method. 25 μg of the protein extracted from a carcinoma site and 25 μg of the protein extracted from a non-carcinoma site, which had been sampled from each of the patients, were mixed to prepare a protein sample extracted from a carcinoma site (total: 450 μg) and a protein sample extracted from a non-carcinoma site (total: 450 μg). The protein samples extracted from a carcinoma site and the protein samples extracted from a non-carcinoma site were subjected to an isoelectric point electrophoresis at 71,500 Volt/hour by using an immobilized pH gradient gel (Immobiline DryStrip pH 3-10, 24 cm: GE Healthcare) (one-dimensional electrophoresis). The gel after being subjected to the one-dimensional electrophoresis was reduced-alkylated, a two-dimensional SDS electrophoresis was performed by using a 12.5% polyacrylamide gel (24 cm×20 cm). The gel subjected to the above-mentioned separation was stained with a solution for staining a phosphorylated protein (Pro-Q Diamond phosphoprotein gel stain: Invitrogen Corporation). The stained gel was detected for its protein spots with an image analyzer (Typhoon 9400: GE Healthcare UK Ltd).

[0045] A protein spot at which the staining with the solution for staining a phosphorylated protein varied more greatly in the carcinoma site compared with the non-carcinoma site was specified. The specified protein spot was cut out from the gel, and converted into a peptide by in-gel tryptic digestion. The in-gel digested peptide was measured with an ion spray mass spectrometer (ESI-MS, LCQ-Deca: Thermoelectron Corporation), and the protein at the spot was identified by a peptide mass fingerprint method (PMF method) using a Mascot software (MATRIX SCIENCE Ltd.).

[0046] The results revealed that the staining of the protein spot of the tumor rejection antigen gp96 with the solution for staining a phosphorylated protein varied more greatly in the carcinoma site compared with the non-carcinoma site.

[0047] FIG. **1** shows a comparison between gels each stained with the solution for staining a phosphorylated protein after the protein sample extracted from a carcinoma site and the protein sample extracted from a non-carcinoma site has been developed by two-dimensional electrophoresis. The reference numeral **1** in FIG. **1** denotes a spot of the tumor rejection antigen gp96 of a sample derived from the non-carcinoma site, and the reference numeral **2** denotes a spot of the tumor rejection antigen gp96 of a sample derived from the carcinoma site. With regard to the respective spots denoted as the reference numerals **1** and **2**, the sample derived from the non-carcinoma site (1) and the sample derived from the carcinoma site (2) differ from each other in the staining with the solution for staining a phosphorylated protein. The spot denoted as the reference numeral **2** in FIG. **1** was cut out and subjected to in-gel tryptic digestion. After that, the protein was identified by the PMF method. As a result, as underlined in FIG. **2**, a peptide having mass that corresponds to a partial peptide of the tumor rejection antigen gp96 was observed as a peptide obtained by subjecting the protein spot gel to tryptic digestion.

Example 2

[0048] In Example 2 of this invention, there is described the verification of the tumor rejection antigen gp96 for its phosphorylation by MALDI-TOF/MS measurement.

[0049] A product obtained by digesting the tumor rejection antigen gp96 with trypsin was dissolved in 10 μl of an aqueous solution containing 0.1% TFA and 50% methanol to obtain a sample solution. 1 μl of the sample solution was dropped onto a target plate for a mass spectrometer, and dried at room temperature. To the dried sample spot, dropped were 0.7 μl of a matrix solution (a solution obtained by dissolving α-cyano-4-hydroxycinnamic acid (α-CHCA) in a solution containing 0.1% trifluoroacetic acid (TFA), 70% methanol, and 4 mM mono ammonium phosphate so that the concentration would be a saturated concentration), followed by drying at room temperature. The target plate was measured with a time-of-flight mass spectrometer (MALDI-TOF/MS, Voyager DE STR: Applied Biosystems, Inc.) in a linear mode, and the ion polarity to be measured was measured in two ways, i.e., a positive ion mode and a negative ion mode. The mass in the spectrum was calibrated by an external standard method using a standard peptide.

[0050] FIG. **3** includes charts showing a comparison between the results in positive ion mode measurement and the results in negative ion mode measurement in a mass range of 699 Da to 3,001 Da. The negative ion mode can measure a mass spectrum of a phosphorylated peptide difficult to be measured in the positive ion mode.

[0051] FIG. **4** includes charts showing a comparison between mass spectra in a mass range of 2,050 Da to 2,800 Da. In FIG. **4**, the reference numeral **41** denotes a peak derived from the FQSSHHPTDITSLDQYVER peptide of the tumor rejection antigen gp96 measured in the positive ion mode. Further, the reference numeral **42** denotes a peak derived from the FQSSHHPTDITSLDQYVER peptide of the tumor rejection antigen gp96 measured in the negative ion mode. Still further, **43** denotes a peak derived from the FQSSHHPTDITSLDQYVER peptide of the phosphorylated tumor rejection antigen gp96 measured in the negative ion mode. As clear from FIG. **4**, there is a peak observed only in the negative ion mode measurement (**43** in FIG. **4**) by a mass difference of +80 Da from a peak commonly observed in both of the positive ion mode measurement and the negative ion mode measurement (**41** in FIGS. **4** and **42** in FIG. **4**). The mass of the peak commonly observed in both of the positive ion mode measurement and the negative ion mode measurement (**41** in FIGS. **4** and **42** in FIG. **4**) coincides with the mass of the peptide of 512th to 530th residues (FQSSHHPTDITSLDQYVER) in the tumor rejection antigen gp96, and it is conceivable that any one of a serine residue (S), a threonine residue (T), and a tyrosine residue (Y) in FQSSHHPTDITSLDQYVER has been subjected to phosphorylation.

[0052] Accordingly, the measurement of the tumor rejection antigen gp96 for its phosphorylation is useful for diagnosis of hepatocellular carcinoma.

INDUSTRIAL APPLICABILITY

[0053] This invention can provide the hepatocellular carcinoma protein marker and the method of detecting hepatocellular carcinoma cells using the hepatocellular carcinoma protein marker.

[0054] Note that the application of this invention insists advantage thereof based on the priority of Japanese Patent Application No. 2007-162857 filed on 20 Jun. 2007, and the disclosure of the filed application is taken into the whole of this application.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 1

<210> SEQ ID NO 1
<211> LENGTH: 803
<212> TYPE: PRT
<213> ORGANISM: homo sapiens

<400> SEQUENCE: 1

```
Met Arg Ala Leu Trp Val Leu Gly Leu Cys Cys Val Leu Leu Thr
1               5                  10                  15

Phe Gly Ser Val Arg Ala Asp Asp Glu Val Asp Val Asp Gly Thr
                20                  25                  30

Val Glu Glu Asp Leu Gly Lys Ser Arg Glu Gly Ser Arg Thr Asp
                35                  40                  45

Asp Glu Val Val Gln Arg Glu Glu Glu Ala Ile Gln Leu Asp Gly
                50                  55                  60

Leu Asn Ala Ser Gln Ile Arg Glu Leu Arg Glu Lys Ser Glu Lys
                65                  70                  75

Phe Ala Phe Gln Ala Glu Val Asn Arg Met Met Lys Leu Ile Ile
                80                  85                  90

Asn Ser Leu Tyr Lys Asn Lys Glu Ile Phe Leu Arg Glu Leu Ile
                95                 100                 105

Ser Asn Ala Ser Asp Ala Leu Asp Lys Ile Arg Leu Ile Ser Leu
                110                 115                 120

Thr Asp Glu Asn Ala Leu Ser Gly Asn Glu Glu Leu Thr Val Lys
                125                 130                 135

Ile Lys Cys Asp Lys Glu Lys Asn Leu Leu His Val Thr Asp Thr
                140                 145                 150

Gly Val Gly Met Thr Arg Glu Glu Leu Val Lys Asn Leu Gly Thr
                155                 160                 165

Ile Ala Lys Ser Gly Thr Ser Glu Phe Leu Asn Lys Met Thr Glu
                170                 175                 180

Ala Gln Glu Asp Gly Gln Ser Thr Ser Glu Leu Ile Gly Gln Phe
                185                 190                 195

Gly Val Gly Phe Tyr Ser Ala Phe Leu Val Ala Asp Lys Val Ile
                200                 205                 210

Val Thr Ser Lys His Asn Asn Asp Thr Gln His Ile Trp Glu Ser
                215                 220                 225

Asp Ser Asn Glu Phe Ser Val Ile Ala Asp Pro Arg Gly Asn Thr
                230                 235                 240

Leu Gly Arg Gly Thr Thr Ile Thr Leu Val Leu Lys Glu Glu Ala
                245                 250                 255

Ser Asp Tyr Leu Glu Leu Asp Thr Ile Lys Asn Leu Val Lys Lys
                260                 265                 270

Tyr Ser Gln Phe Ile Asn Phe Pro Ile Tyr Val Trp Ser Ser Lys
                275                 280                 285

Thr Glu Thr Val Glu Glu Pro Met Glu Glu Glu Glu Ala Ala Lys
                290                 295                 300

Glu Glu Lys Glu Glu Ser Asp Asp Glu Ala Ala Val Glu Glu Glu
                305                 310                 315

Glu Glu Glu Lys Lys Pro Lys Thr Lys Lys Val Glu Lys Thr Val
```

-continued

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                320                 325                 330

Trp Asp Trp Glu Leu Met Asn Asp Ile Lys Pro Ile Trp Gln Arg
                335                 340                 345

Pro Ser Lys Glu Val Glu Glu Asp Glu Tyr Lys Ala Phe Tyr Lys
                350                 355                 360

Ser Phe Ser Lys Glu Ser Asp Asp Pro Met Ala Tyr Ile His Phe
                365                 370                 375

Tyr Ala Glu Gly Glu Val Thr Phe Lys Ser Ile Leu Phe Val Pro
                380                 385                 390

Thr Ser Ala Pro Arg Gly Leu Phe Asp Glu Tyr Gly Ser Lys Lys
                395                 400                 405

Ser Asp Tyr Ile Lys Leu Tyr Val Arg Arg Val Phe Ile Thr Asp
                410                 415                 420

Asp Phe His Asp Met Met Pro Lys Tyr Leu Asn Phe Val Lys Gly
                425                 430                 435

Val Val Asp Ser Asp Asp Leu Pro Leu Asn Val Ser Arg Glu Thr
                440                 445                 450

Leu Gln Gln His Lys Leu Leu Lys Val Ile Arg Lys Lys Leu Val
                455                 460                 465

Arg Lys Thr Leu Asp Met Ile Lys Lys Ile Ala Asp Asp Lys Tyr
                470                 475                 480

Asn Asp Thr Phe Trp Lys Glu Phe Gly Thr Asn Ile Lys Leu Gly
                485                 490                 495

Val Ile Glu Asp His Ser Asn Arg Thr Arg Leu Ala Lys Leu Leu
                500                 505                 510

Arg Phe Gln Ser Ser His His Pro Thr Asp Ile Thr Ser Leu Asp
                515                 520                 525

Gln Tyr Val Glu Arg Met Lys Glu Lys Gln Asp Lys Ile Thr Phe
                530                 535                 540

Met Ala Gly Ser Ser Arg Lys Glu Ala Glu Ser Ser Pro Phe Val
                545                 550                 555

Glu Arg Leu Leu Lys Lys Gly Tyr Glu Val Ile Tyr Leu Thr Glu
                560                 565                 570

Pro Val Asp Glu Tyr Cys Ile Gln Ala Leu Pro Glu Phe Asp Gly
                575                 580                 585

Lys Arg Phe Gln Asn Val Ala Lys Glu Gly Val Lys Phe Asp Glu
                590                 595                 600

Ser Glu Lys Thr Lys Glu Ser Arg Glu Ala Val Glu Lys Glu Phe
                605                 610                 615

Glu Pro Leu Leu Asn Trp Met Lys Asp Lys Ala Leu Lys Asp Lys
                620                 625                 630

Ile Glu Lys Ala Val Val Ser Gln Arg Leu Thr Glu Ser Pro Cys
                635                 640                 645

Ala Leu Val Ala Ser Gln Tyr Gly Trp Ser Gly Asn Met Glu Arg
                650                 655                 660

Ile Met Lys Ala Gln Ala Tyr Gln Thr Gly Lys Asp Ile Ser Thr
                665                 670                 675

Asn Tyr Tyr Ala Ser Gln Lys Lys Thr Phe Glu Ile Asn Pro Arg
                680                 685                 690

His Pro Leu Ile Arg Asp Met Leu Arg Arg Ile Lys Glu Asp Glu
                695                 700                 705
```

-continued

```
Asp Asp Lys Thr Val Leu Asp Leu Ala Val Val Leu Phe Glu Thr
                710                 715                 720

Ala Thr Leu Arg Ser Gly Tyr Leu Leu Pro Asp Thr Lys Ala Tyr
                725                 730                 735

Gly Asp Arg Ile Glu Arg Met Leu Arg Leu Ser Leu Asn Ile Asp
                740                 745                 750

Pro Asp Ala Lys Val Glu Glu Glu Pro Glu Glu Glu Pro Glu Glu
                755                 760                 765

Thr Ala Glu Asp Thr Thr Glu Asp Thr Glu Gln Asp Glu Asp Glu
                770                 775                 780

Glu Met Asp Val Gly Thr Asp Glu Glu Glu Glu Thr Ala Lys Glu
                785                 790                 795

Ser Thr Ala Glu Lys Asp Glu Leu
                800
```

1. A hepatocellular carcinoma protein marker, comprising tumor rejection antigen gp96 formed of an amino acid represented by SEQ ID NO: 1, wherein the protein marker is phosphorylated.

2. A method of detecting hepatocellular carcinoma, comprising using the hepatocellular carcinoma protein marker according to claim **1**.

3. A method of detecting hepatocellular carcinoma according to claim **2**, wherein said using the protein marker includes extracting from a surgically sampled biological sample.

4. A method of detecting hepatocellular carcinoma according to claim **3**, wherein said using the protein marker further comprises preparing the biological sample comprising any one kind of a biopsy sample, blood, plasma, serum, and urine.

5. A method of detecting hepatocellular carcinoma according to claim **2**, wherein said using the protein marker includes verifying phosphorylation of the protain marker.

6. A method of detecting hepatocellular carcinoma according to claim **5**, wherein said using the protein marker further includes extracting from a surgically sampled biological sample.

7. A method of detecting hepatocellular carcinoma according to claim **6**, wherein said using the protein marker further comprises preparing the biological sample comprises any one kind of a biopsy sample, blood, plasma, serum, and urine.

8. A method of detecting hepatocellular carcinoma according to claim **5**, wherein the verifying is performed based on measurement of any one of a serine residue, a threonine residue, and a tyrosine residue of the protein marker for its phosphorylation.

9. A method of detecting hepatocellular carcinoma according to claim **8**, wherein said using the protein marker comprises extracting the protein marker from a surgically sampled biological sample.

10. A method of detecting hepatocellular carcinoma according to claim **9**, wherein said using the protein marker further comprises preparing the biological sample comprising any one kind of a biopsy sample, blood, plasma, serum, and urine.

11. A method of detecting hepatocellular carcinoma, comprising:

measuring phosphorylation of tumor rejection antigen gp96 formed of an amino acid represented by SEQ ID NO: 1; and

judging the measurements as positive based on a comparison with a normal value.

12. A method of detecting hepatocellular carcinoma, comprising measuring phosphorylation of tumor rejection antigen gp96 as the hepatocellular carcinoma protein marker according to claim **1** in a biological sample, wherein the measuring is performed by a method selected from the group consisting of an enzyme immunoassay, a fluorescence-labeled antibody method, a western blot method, a radioimmunoassay, an immunoprecipitation method, electrophoresis, liquid chromatography, and mass spectrometry.

13. A method of detecting hepatocellular carcinoma according to claim **12**, wherein said measuring comprises:

extracting a protein from a surgically sampled biological sample;

subjecting the protein to a multicycle of electrophoresis with different dimensions;

fixing and staining the protein on a solid carrier; and

comparing an increase or decrease in the tumor rejection antigen gp96 in a protein spot.

14. A method of detecting hepatocellular carcinoma according to claim **13**, wherein said measuring further comprises preparing the biological sample comprising any one kind of a biopsy sample, blood, plasma, serum, and urine.

15. A method of detecting hepatocellular carcinoma according to claim **12**, wherein said measuring comprises judging a presence or absence of hepatocellular carcinoma based on a comparison of the results obtained from measuring phosphorylation of a digestion product of the tumor rejection antigen gp96 obtained from a surgically sampled biological sample in positive ion mode measurement and in negative ion mode measurement by using a mass spectrometer.

16. A method of detecting hepatocellular carcinoma according to claim **15**, wherein said measuring further comprises preparing the biological sample comprising any one kind of a biopsy sample, blood, plasma, serum, and urine.

17. A method of detecting hepatocellular carcinoma according to claim **12**, wherein said measuring phosphorylation of the hepatocellular carcinoma protein marker is per-

formed with respect to any one of a serine residue, a threonine residue, and a tyrosine residue of the protein marker.

18. A method of detecting hepatocellular carcinoma according to claim **17**, wherein said measuring comprises:

extracting a protein from a surgically sampled biological sample;

subjecting the protein to a multicycle of electrophoresis with different dimensions;

fixing and staining the protein on a solid carrier; and

comparing an increase or decrease in the tumor rejection antigen gp96 in a protein spot.

19. (canceled)

20. A method of detecting hepatocellular carcinoma according to claim **17**, wherein said measuring comprises judging a presence or absence of hepatocellular carcinoma based on a comparison of the results obtained from measuring phosphorylation of a digestion product of the tumor rejection antigen gp96 obtained from a surgically sampled biological sample in positive ion mode measurement and in negative ion mode measurement by using a mass spectrometer.

21. (canceled)

22. A method of detecting hepatocellular carcinoma according to claim **17**, wherein said measuring further comprises using an antibody that recognizes a phosphorylated site of the tumor rejection antigen gp96.

23. (canceled)

* * * * *

专利名称(译)	肝细胞癌蛋白质标记物和使用其的肝细胞癌检测方法		
公开(公告)号	US20100248256A1	公开(公告)日	2010-09-30
申请号	US12/452216	申请日	2008-06-16
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IPC分类号	G01N33/53 C07K14/435		
CPC分类号	G01N33/57438 C07K14/4748		
优先权	2007162857 2007-06-20 JP		
其他公开文献	US8207301		
外部链接	Espacenet USPTO		

摘要(译)

提供了一种与非肝细胞癌细胞相比，通过在肝细胞癌细胞中使用具有不同磷酸化状态的蛋白质来评估肝细胞癌的方法。用于检测由该蛋白质形成的肝细胞癌的肝细胞癌蛋白质标记物。用于检测肝细胞癌的肝细胞癌蛋白质标记物包括由SEQ ID NO：1代表的氨基酸形成的肿瘤排斥抗原gp96，并测量其磷酸化状态以检测肝细胞癌的存在或不存在。

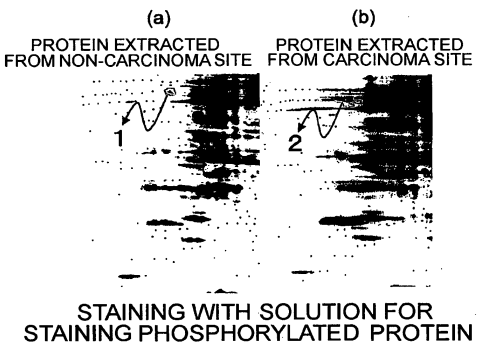
(a)
(b)
PROTEIN EXTRACTED FROM NON-CARCINOMA SITE
PROTEIN EXTRACTED FROM CARCINOMA SITE
1
2
STAINING WITH SOLUTION FOR STAINING PHOSPHORYLATED PROTEIN

FIG. 1