

US008067152B2

(12) **United States Patent**  
**Beretta**

(10) **Patent No.:** **US 8,067,152 B2**  
(45) **Date of Patent:** **Nov. 29, 2011**

(54) **LIVER CANCER BIOMARKERS**

(75) Inventor: **Laura Beretta**, Mercer Island, WA (US)

(73) Assignee: **The Fred Hutchinson Cancer Research Center**, Seattle, WA (US)

(\* ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 716 days.

(21) Appl. No.: **11/708,202**

(22) Filed: **Feb. 20, 2007**

(65) **Prior Publication Data**

US 2007/0202496 A1 Aug. 30, 2007

**Related U.S. Application Data**

(60) Provisional application No. 60/777,087, filed on Feb. 27, 2006.

(51) **Int. Cl.**

**C12Q 1/00** (2006.01)

**G01N 33/53** (2006.01)

**G01N 33/574** (2006.01)

(52) **U.S. Cl.** ..... **435/4; 435/7.1; 435/7.21; 435/7.23**

(58) **Field of Classification Search** ..... None  
See application file for complete search history.

(56) **References Cited**

**U.S. PATENT DOCUMENTS**

7,387,881	B2 *	6/2008	Hanash et al.	435/7.23
2002/0052308	A1 *	5/2002	Rosen et al.	514/1
2002/0115094	A1	8/2002	Farnham et al.	
2003/0108553	A1 *	6/2003	Hanash et al.	424/155.1

**OTHER PUBLICATIONS**

- Denning et al. (Blood, 1997, 90:372-381).\*
- Boyd (The Basic Science of Oncology, 1992, McGraw-Hill, Inc., p. 379).\*
- Lebert et al. (Cancer. Sep. 1, 2003; 98(5):970-7).\*
- Glaessgen et al. (APMIS 2008 116: 888-95).\*
- Tockman et al (Cancer Res., 1992, 52:2711s-2718s).\*
- Kim et al. (Electrophoresis, 2002, 23:4142-4156).\*
- ERp57 (N-20): sc-18619 (Santa Cruz Biotechnology, Inc., 2006).\*
- Erp57 (Grp58) Monoclonal Antibody (Map.Erp57) (Assay Designs/Stressgen® Jun. 11, 2008).\*
- Tang, Z-Y. (Surgical Treatment: Evidence-Based and Problem Oriented, Multimodality treatment for hepatocellular carcinoma, Holzheimer and Mannick, eds. 2001, NCBI Bookshelf).\*
- Le Naor, F, et al., A Distinct Repertoire of Autoantibodies in Hepatocellular Carcinoma Identified by Proteomic Analysis, Molecular & Cellular Proteomics (2002) 197-203, 1:3.
- Hong, S-H, et al., An Autoantibody-Mediated Immune Response to Calreticulin Isoforms in Pancreatic Cancer, Cancer Research (Aug. 1, 2004) 5504-5510, 64.

\* cited by examiner

*Primary Examiner* — Peter J Reddig

(74) *Attorney, Agent, or Firm* — Myers Bigel Sibley & Sajovec, P.A.

(57) **ABSTRACT**

A method of detecting whether a subject is afflicted with or at increased risk of developing liver cancer is carried out by detecting cleavage of a marker in a sample such as a blood sample from the subject. Suitable markers include but are not limited to, calreticulin, calreticulin precursor, protein disulfide isomerase family A member 3 (PDIA3), and cleavage products thereof. The cleavage, or extent of cleavage, can be as compared to that found in a control sample.

**24 Claims, 7 Drawing Sheets**

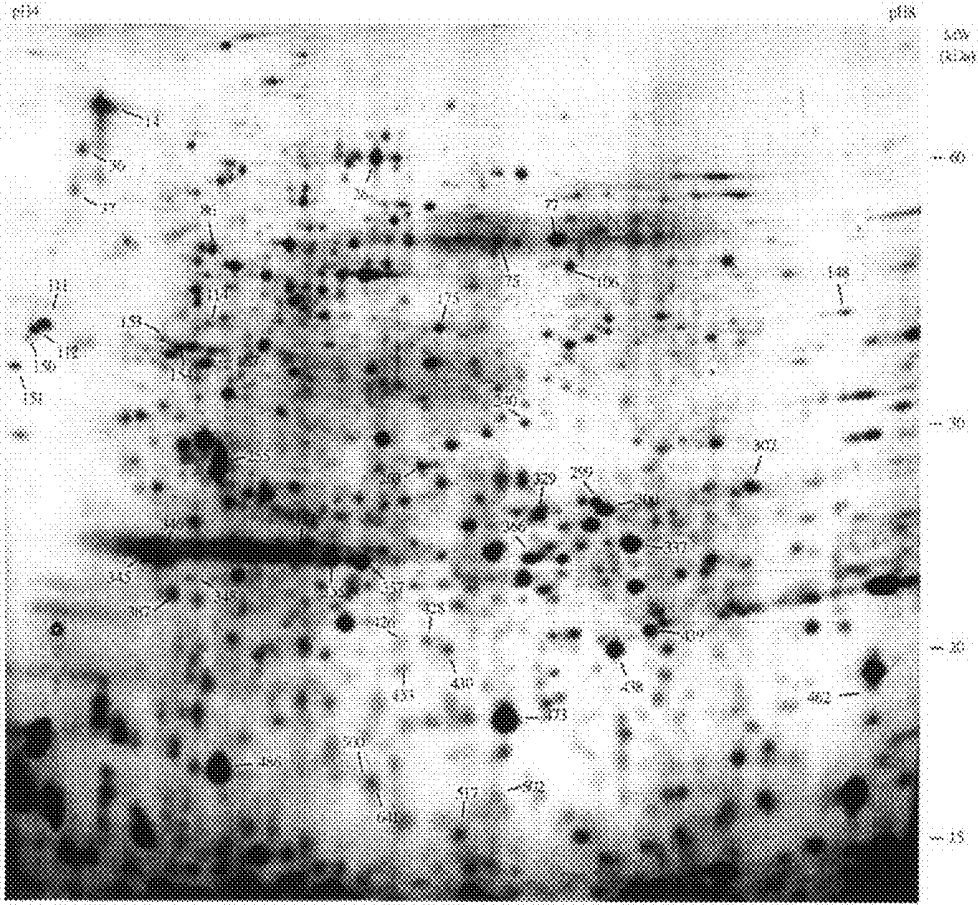
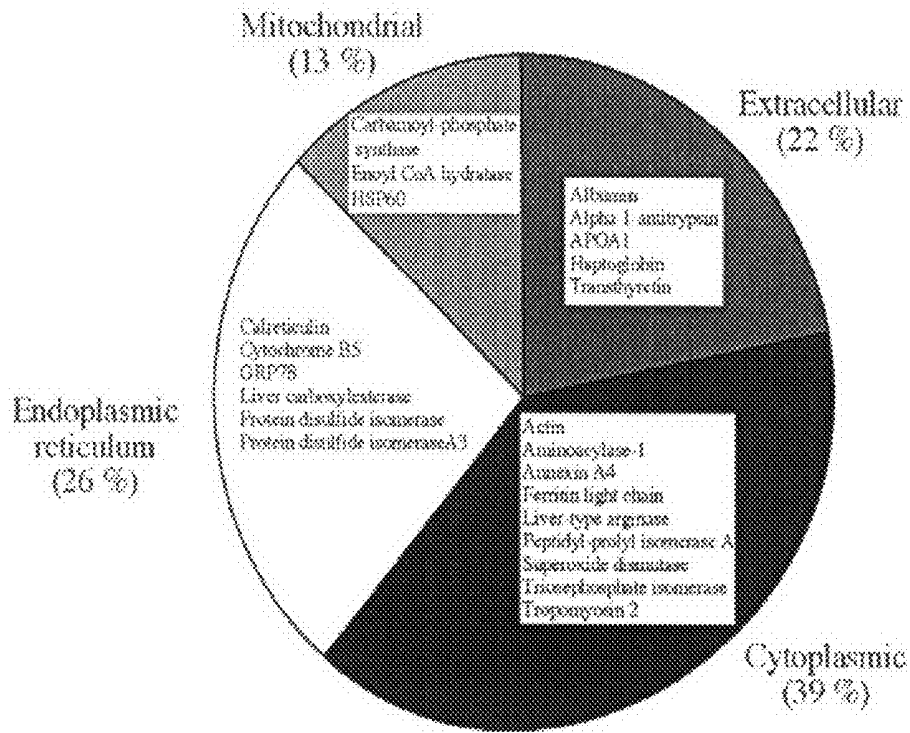
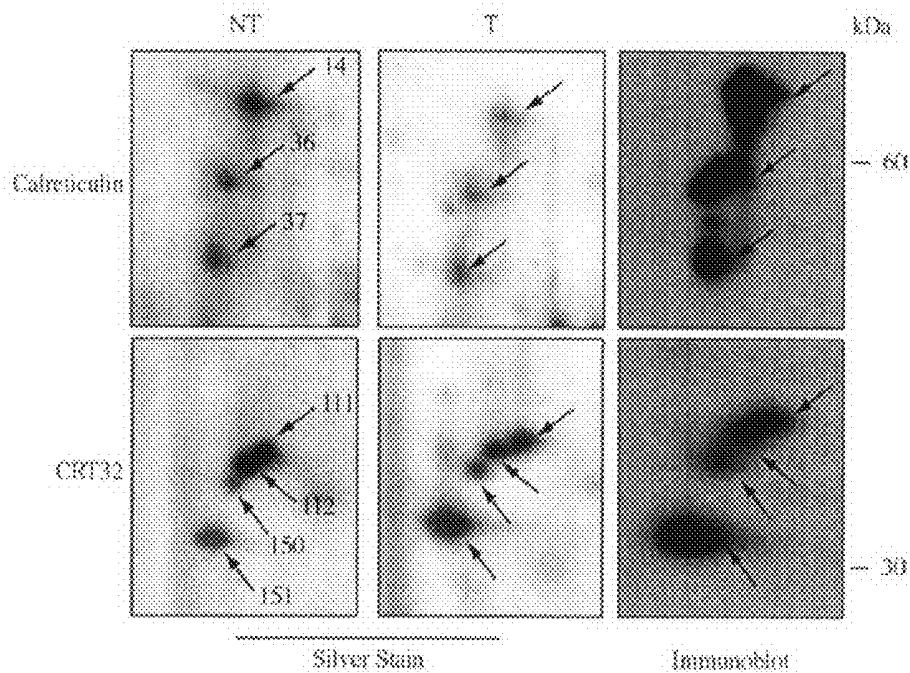


Figure 1.  
2-D pattern of human liver proteins.



**Figure 2.**  
Subcellular localization of identified proteins.



**Figure 3.**  
2-D pattern of calreticulin isoforms in tumor tissue.

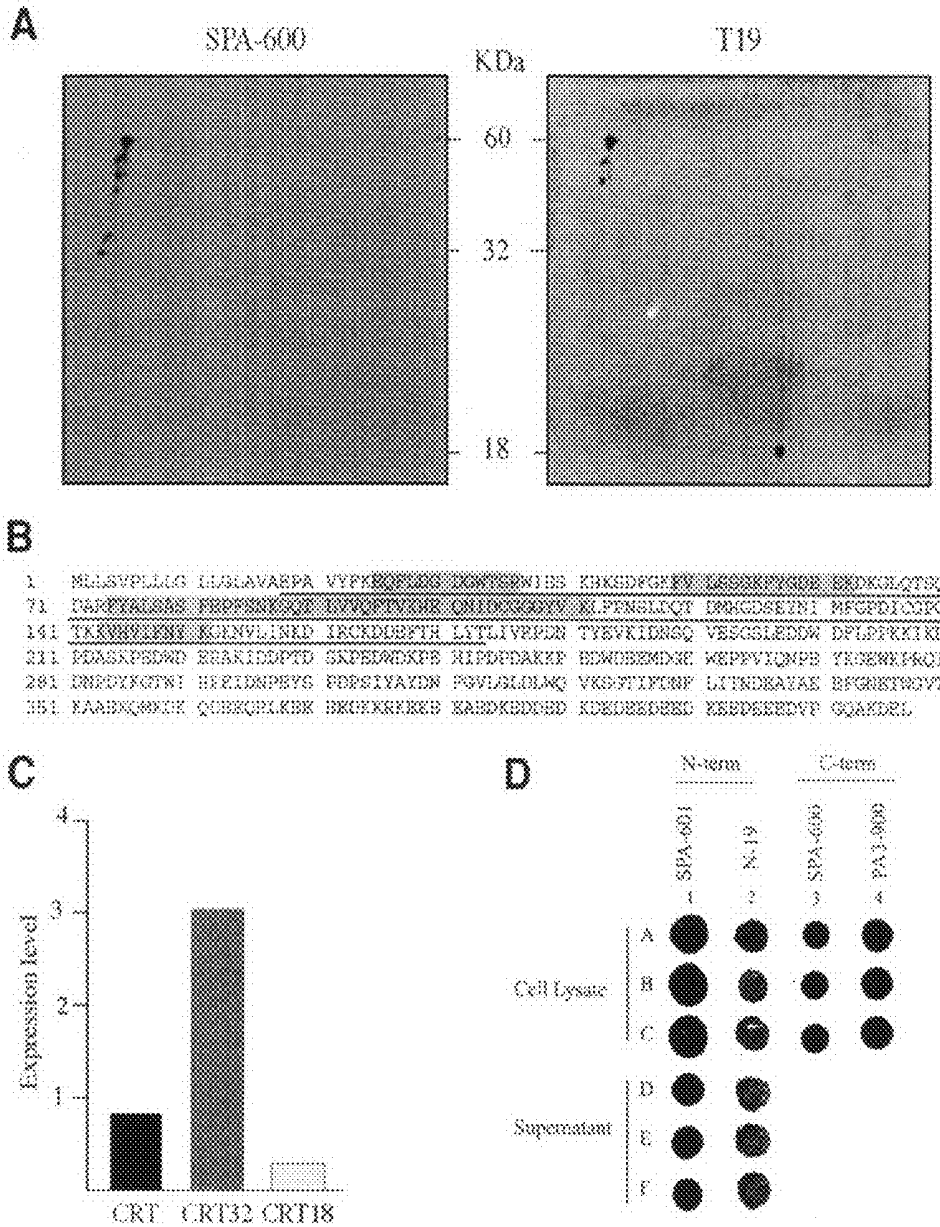
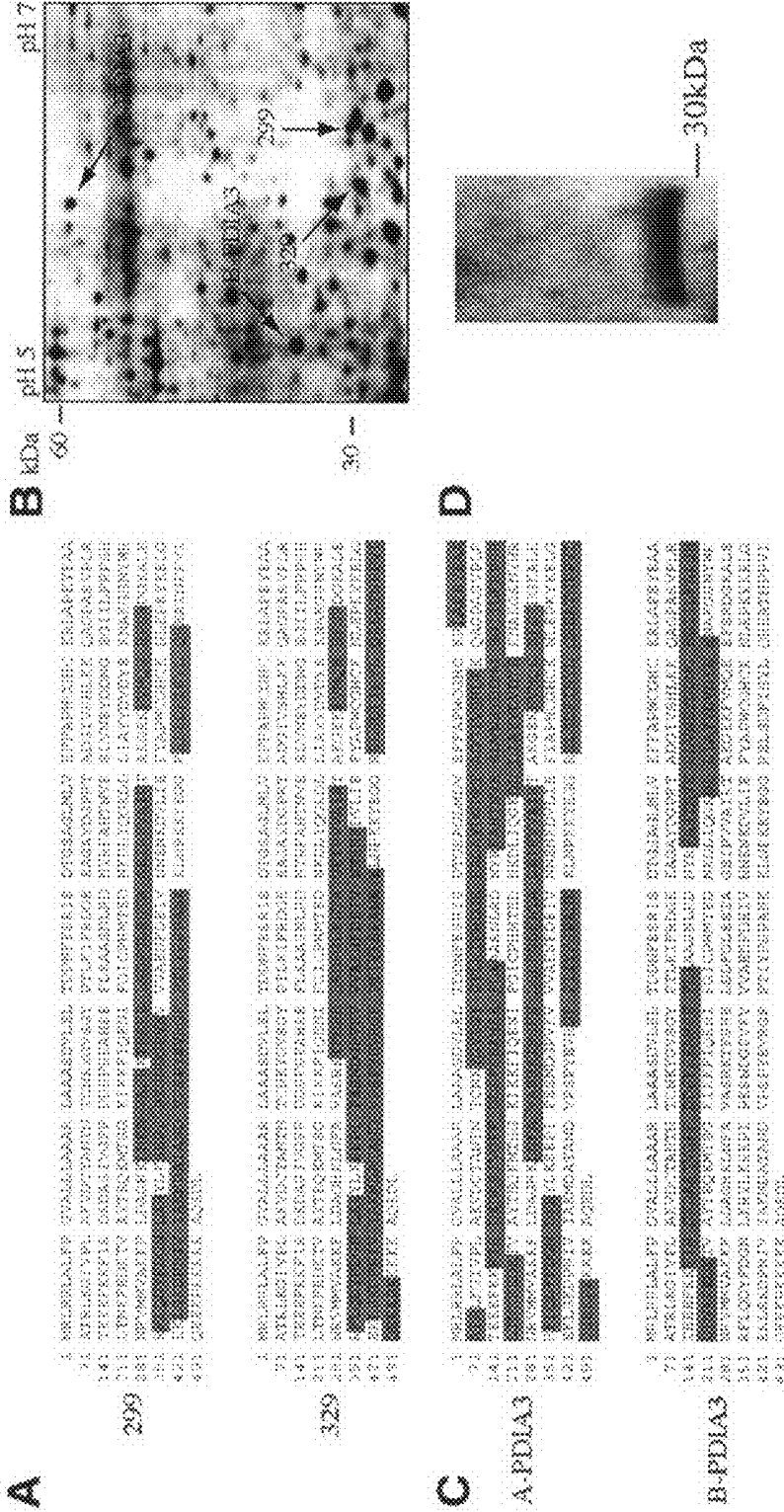


Figure 4.  
Analysis of calreticulin isoforms.



**A**

1 MKLSLVAAML LLSAARAEE EDKEDVGTV VGIDLGTTYS CVGVFKNGRV EIIANDQGNR ITPSYVAFTP  
 71 EGERLIGDAA KNQLTSPEN TVFDAKRLIG RTWNDESVQQ DIKFLPFKVV EKKTKPYIQV DIGGGQTKTF  
 141 APEEISAMVL TKMKETAAY LGKKVTHAVV TVPAYFNDAQ RQATKDAGTI AGLNVMRLIN EPTAAAIAYG  
 211 LDKREGEKNI LVFDLGGGTF DVSLLTIDNG VFEVVAATNGD THLGGEDFDQ RVMEHFIKLY KKKTGKDVRK  
 281 DNRAVQKLR EYKAKRALS SQHQARIEIE SFYEGEDFSE TLTRAKFEEL NMDLFRSTMK PVQXVLEDS  
 351 LKKSDDIDEIV LVGGSTRIPK IQQLVKEFFN GKEPSRGINP DEAVAYGAAV QAGVLSGDQD TGDVLLDVC  
 421 PLTLGIBTVG GVMTKLIPRN TVVPTKKSQI FSTASDNQPT VTIKVYEGER PLTKDNHLLG TFDLTGIPPA  
 491 PRGVPQIEVT FEIDVNGILR VTAEDKGTGN KNKITITNDQ NRLTPEEIER MVNDAEKFAE EDKLLKERID  
 561 TRNELESYAY SLKNQIGDKE KLGKGLSSED KETMEKAVEE KIEWLESHQD ADIEDFKAKK KELEEIVQPI  
 631 ISKLYGSAGP PPTGEEDTAE KDEL

**B**

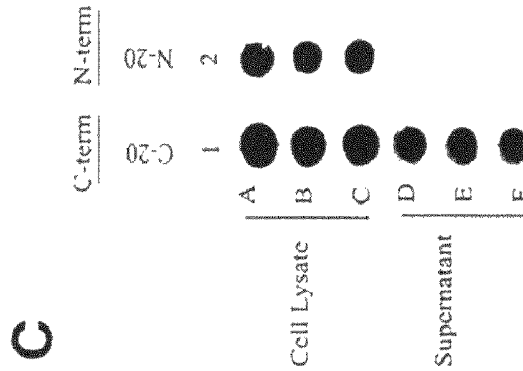
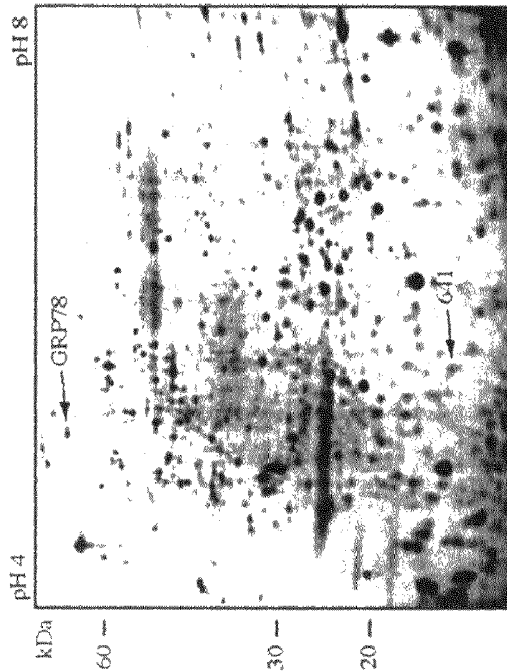


Figure 6.  
Analysis of GRP78 isoforms.

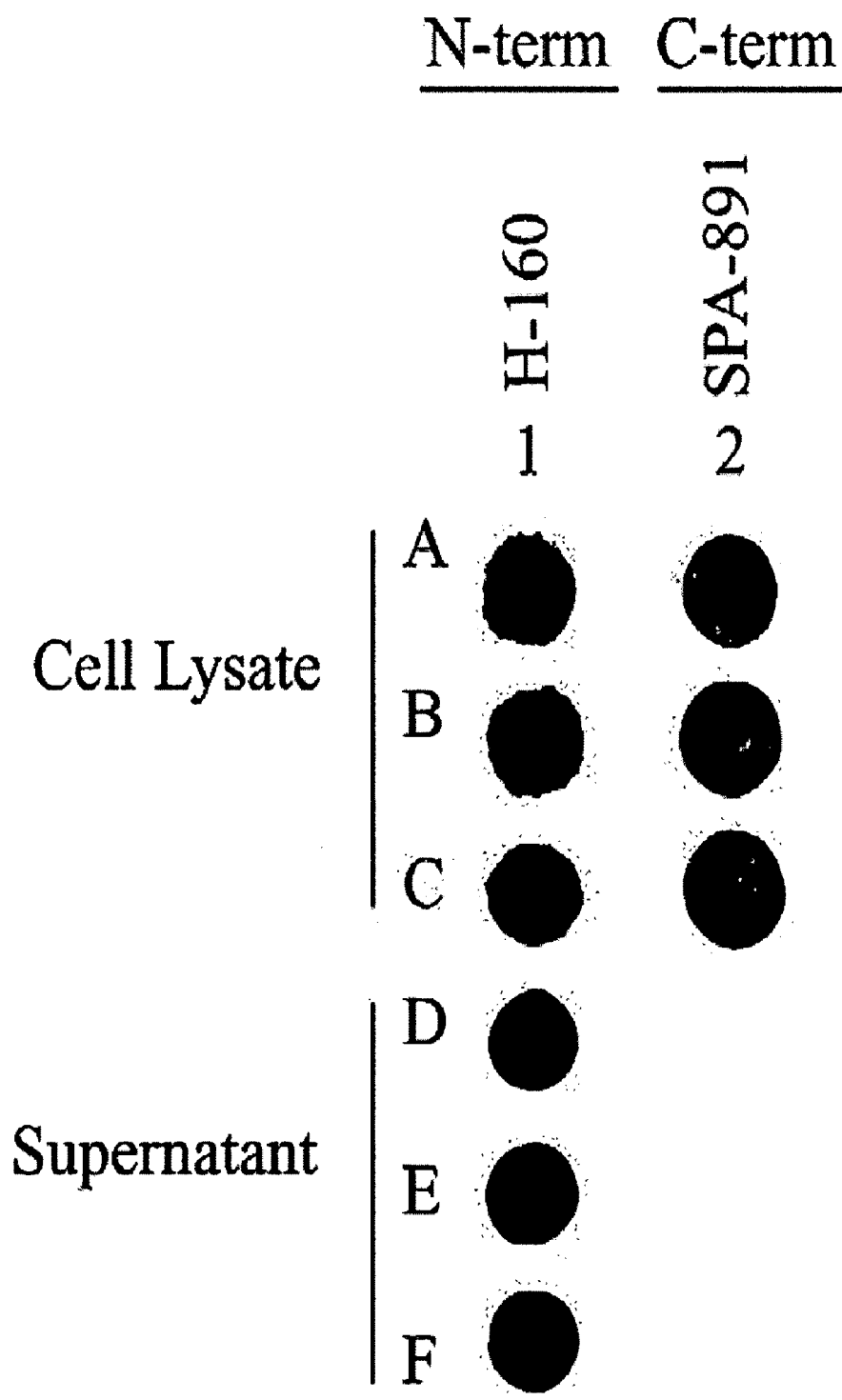
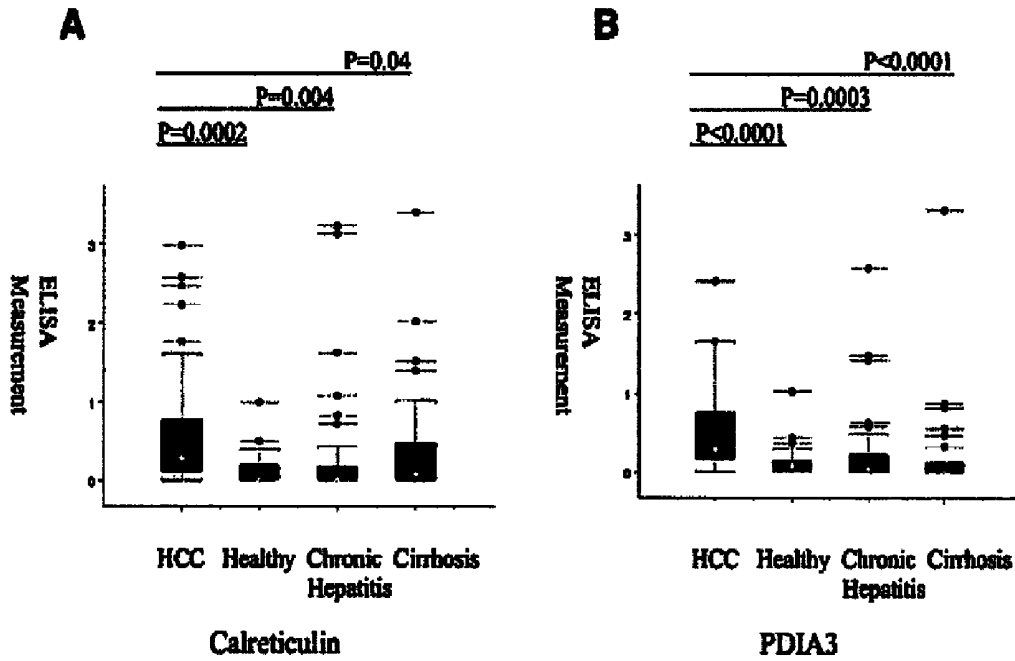
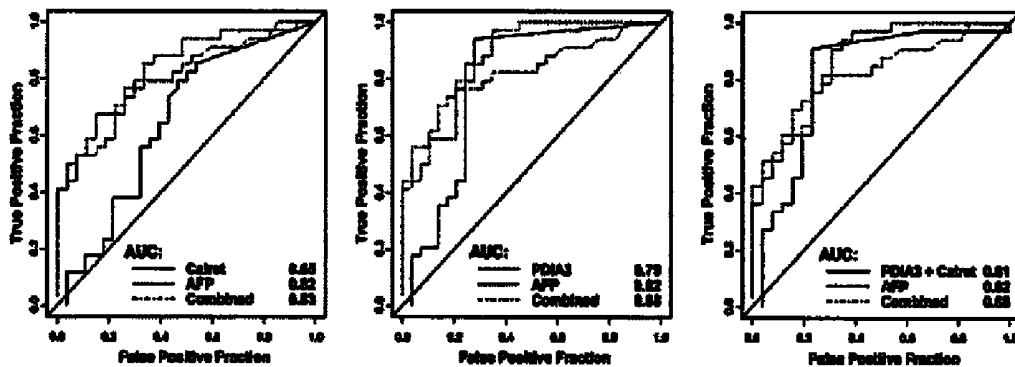


Figure 7.  
Analysis of PDI isoform.



**Figure 8.**  
Measurement by ELISA of calreticulin and PDIA3 fragments in sera.



**Figure 9.**  
ROC curves comparing calreticulin, PDIA3 and AFP individually or in combination in patients with HCC versus those with cirrhosis.

**LIVER CANCER BIOMARKERS****CROSS-REFERENCE TO RELATED APPLICATION**

This application claims the benefit of provisional application Ser. No. 60/777,087, filed Feb. 27, 2006, the disclosure of which is hereby incorporated herein by reference in its entirety.

**GOVERNMENT FUNDING**

This invention was made with government support under grant numbers CA084986 and DK066840 awarded by the National Institutes of Health. The government has certain rights to this invention.

**FIELD OF THE INVENTION**

The present invention concerns methods of screening for or detecting liver cancer in subjects, including subjects with or without previously detected risk factors for liver cancer.

**BACKGROUND OF THE INVENTION**

Hepatocellular carcinoma (HCC), the major histological form of primary liver cancer, affects approximately half a million persons each year, making it the fifth most common malignancy and the third most common cause of cancer death worldwide.<sup>1</sup> The etiology of HCC is mainly associated with hepatitis B virus (HBV) or hepatitis C virus (HCV) chronic infection.<sup>2</sup> In the past decade, there has been a rising incidence of HCC and a progressive increase in HCC-related mortality in the United States and Western Europe.<sup>3, 4</sup> The similarity between incidence and mortality rates is indicative of the rapid death after diagnosis in most cases of HCC, with a 5-year survival rate of less than 5%.<sup>3</sup> The poor survival of patients with HCC is largely related to the lack of reliable tools for early diagnosis. At-risk patients with chronic viral hepatitis and cirrhosis are routinely screened for HCC with annual serum alpha-fetoprotein (AFP) and ultrasonography (for healthy hepatitis B virus carriers) or with twice-yearly serum AFP and ultrasonography (for patients with cirrhosis of any etiology).<sup>5</sup> However, the usefulness of AFP as a marker has been overshadowed by its inability to diagnose early-stage tumors efficiently;<sup>6, 7</sup> AFP levels may increase transiently, intermittently, or permanently in patients with viral hepatitis without HCC. The lack of efficiency of AFP as a serum marker for HCC surveillance or diagnosis has led to assessment of other serological markers such as alpha-1-antitrypsin,<sup>8</sup> des-gamma-carboxyprothrombin,<sup>9, 10</sup> glypican-3,<sup>11</sup> or isoenzymes of gamma-glutamyltransferase.<sup>12, 13</sup>

**SUMMARY OF THE INVENTION**

A method of screening for or detecting liver cancer in a subject, comprises (a) collecting a sample from the subject; and then (b) detecting an altered level of a liver cancer marker in the sample, an altered level of said marker indicating the subject is afflicted with or at risk of developing liver cancer.

The present invention provides a method of screening for or detecting liver cancer in a subject. The method may be carried out on a sample such as a blood or tissue sample collected from the subject.

In general, the method is carried out by detecting cleavage of a marker in that sample, with cleavage of the marker indicating the subject is afflicted with, or at risk of develop-

ing, liver cancer. Suitable markers include but are not limited to, calreticulin, calreticulin precursor, protein disulfide isomerase family A member 3 (PDIA3), and cleavage products thereof. The cleavage, or extent of cleavage, can be as compared to that found in a control sample. Such a control sample may be taken from the same subject at a different (e.g., earlier) point in time or from a different subject or population of subjects considered to be free of, or at no more than normal risk of, liver cancer.

A second aspect of the invention is the use of a means of detecting cleavage of a marker as described herein for carrying out a method of detecting affliction with, or risk of developing, liver cancer as described herein.

The foregoing and other objects and aspects of the present invention are explained in greater detail in the drawings herein and the specification set forth below.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**FIG. 1.** 2-D pattern of human liver proteins. Two hundred micrograms of proteins were separated in the first dimension by isoelectric focusing. Separation in the second dimension was performed using an acrylamide gradient (11 to 14%) followed by silver staining. The 47 spots identified by computer-assisted analysis as being dysregulated in HCC are labeled according to Table 1.

**FIG. 2.** Subcellular localization of identified proteins. The subcellular localization of the 23 unique proteins identified by ESI-QTOF-MS/MS is shown in the pie chart. Each individual protein was linked to a specific subcellular localization according to GO terms.

**FIG. 3.** 2-D pattern of calreticulin isoforms in tumor tissue. Close-up sections of 2-D gels from non-tumor (NT) and tumor (T) tissues are shown for the full-length calreticulin and CRT32. 2-D immunoblot analysis was also performed using a specific antibody directed against the C-terminal end of calreticulin (SPA-600).

**FIG. 4.** Analysis of calreticulin isoforms. A, Immunoblot analysis of 2-D gels from tumor tissues performed with antibodies directed against the C-terminal end (SPA-600) or the N-terminal end (T19) of calreticulin. B, The underlined sequence of the full sequence shown (SEQ ID NO: 1) represents the expected sequence of CRT18. The dark boxes in the sequence (SEQ ID NO: 1) represent the peptides identified by mass spectrometry. C, After digitalization of silver stained 2-D gels, the integrated intensity of spots corresponding to the full length calreticulin, to CRT32, and to CRT18 in tumor tissues were measured. D, PLC-PRF5 cell lysate (A,B,C) and supernatant (D,E,F) were blotted onto a nitrocellulose membrane. Immunodetection was then performed using antibodies directed against the N-terminal end (SPA-601, N-19) or the C-terminal end (SPA-600, PA3-900) of calreticulin.

**FIG. 5.** Analysis of PDIA3 isoforms. A, The dark boxes in the two sequences (both SEQ ID NO: 2) represent the peptides identified by mass spectrometry for spots 299 and 329. B, 2-D silver-stained gel from liver tissue with the position of the full-length PDIA3 (A-PDIA3) and PDIA3 cleavage isoforms (B-PDIA3, 299, 329). C, The dark boxes in the two sequences (both also SEQ ID NO: 2) represent the peptides identified by mass spectrometry for spots A-PDIA3 and B-PDIA3. D, Immunoblot analysis of PDIA3 expression in PLC-PRF5 supernatant. Fifty microliters of cell culture supernatant from PLC-PRF5 were subjected to 1D-PAGE and immunodetection was performed with a polyclonal antibody directed against PDIA3 (SPA-585).

**FIG. 6.** Analysis of GRP78 isoforms. A, The dark boxes in the sequence (SEQ ID NO: 3) represent the peptides identi-

fied by mass spectrometry for spot 641. B, 2-D silver-stained gel from liver tissue with the position of the full-length GRP78 and GRP78 cleavage isoform 641. C, PLC-PRF5 cell lysate (A,B,C) and supernatant (D,E,F) were blotted onto a nitrocellulose membrane. Immunodetection was then performed using antibodies directed against the C-terminal end (C-20) or the N-terminal end (N-20) of GRP78.

FIG. 7. Analysis of PDI isoform. PLC-PRF5 cell lysate (A,B,C) and supernatant (D,E,F) were blotted onto a nitrocellulose membrane. Immunodetection was then performed using antibodies directed against the N-terminal end (H-160) or the C-terminal end (SPA-891) of PDI.

FIG. 8. Measurement by ELISA of calreticulin and PDIA3 fragments in sera. Levels of calreticulin (A) and PDIA3 (B) fragments were measured by ELISA in sera obtained from patients with HCC (n=34), patients with chronic hepatitis (n=33), patients with cirrhosis (n=28) and healthy individuals (n=27).

FIG. 9. ROC curves comparing calreticulin, PDIA3 and AFP individually or in combination in patients with HCC versus those with cirrhosis.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### 1. Definitions

“Altered level” as used with respect to marker proteins herein refers to an increased level (e.g., a one or two fold increase, or more) or a decreased level (e.g., a one or two-fold decrease, or more) in the quantity of marker protein detectable in or via a biological sample from a subject, as compared to a level of marker protein in a corresponding subject not afflicted with a liver disease such as liver cancer.

“Biological sample” as used herein refers to any material taken from the body of a subject which carries the target compound or compounds of the tests described herein, including both tissue samples and biological fluids such as blood samples, saliva samples, urine samples, etc.

“Blood sample” as used herein refers to whole blood or any fraction thereof that contains detectable levels of marker proteins therein (if marker proteins are present in the whole blood sample from which said fraction is obtained), and in particular embodiments refers to a blood sera or blood plasma sample.

“Detecting” or “screening” as used herein means providing an indication that a subject is afflicted with or at risk of developing a disease, particularly a liver disease such as liver cancer, and includes other terms such as screening for a disease, providing a risk assessment for disease, prognosing disease, diagnosing disease, etc. It will be appreciated that no such technique is perfect and that such diagnosis, prognosis or the like may be confirmed by other procedures such as physical examination, imaging, histological examination of tissue samples, etc.

“Isoform” as used herein refers to a different form of a protein, regardless of whether it originates from a different gene or splice variant or by modification of a single gene product. Thus “isoform” as used herein refers to a form of a protein that migrates differently from another form of that protein on a 2 dimensional gel.

“Marker protein” as used herein refers to any protein which can be detected, directly or indirectly (e.g., via an analog, metabolite, fragment or breakdown product) in a biological sample from a subject, an increase or decrease of the amount of which, compared to amounts found in similar subjects without disease, is indicative of the presence or risk of liver

cancer in a subject. Marker proteins described herein include any protein listed in Table 1-2 herein. The analog, metabolite, fragment or breakdown product of the marker protein may or may not possess the functional activity of the marker protein.

“Additional liver cancer marker” as used herein can be an elevation (or decrease) in a patient sample of alpha fetoprotein, des-carboxy-prothrombin (DCP), carcinoembryonic antigen (CEA), a marker as described in Farnham and Braveel, US Patent Application No. US 2002/0115094, an additional marker as described in Tables 1-2 herein, or any other liver cancer marker.

“Liver cancer” as used herein includes any type of liver cancer, such as those arising from hepatocytes (hepatomas or hepatocellular carcinomas), as well as fibrolamellar hepatomas and cholangiocarcinomas (or bile duct cancer).

“Subjects” as described herein are generally human subjects and includes “patients”. The subjects may be male or female and may be of any race or ethnicity, including but not limited to Caucasian, African American, African, Asian, Indian, etc. The subjects may be of any age, including newborn, neonate, infant, child, adolescent, adult, and geriatric. Subjects may also include animal subjects, particularly mammalian subjects such as dog, cat, horse, mouse, rat, etc., screened for veterinary medicine or pharmaceutical drug development purposes.

Subjects include but are not limited to those who have, possess, have been exposed to, or have been previously diagnosed as afflicted with one or more risk factors for liver cancer, including but not limited to: subjects afflicted with or suspected of being afflicted with cirrhosis (e.g., caused by excessive alcohol consumption), subjects afflicted with, infected with, or suspected of being exposed to or infected with hepatitis B or hepatitis C virus, subjects afflicted with or suspected of being afflicted with haemochromatosis, and subjects who have consumed, or are suspected of having consumed, a toxin such as aflatoxin (e.g., found in moldy foods such as grain, peanuts, etc.).

The disclosures of all United States patent references cited herein are to be incorporated herein by reference in their entirety.

While the following description focuses primarily on liver cancer, it will be appreciated that the present invention may also be utilized in connection with other liver diseases as noted above.

##### 2. Assay Procedures

The step of collecting a sample such as a blood sample from a subject can be carried out by phlebotomy or any other suitable technique, with the blood sample processed further to provide a serum sample or other suitable blood fraction. The step of determining the presence of an altered level of a marker protein in the sample, and/or depressed level of a marker protein in the sample, can also be carried out in accordance with known techniques, including but not limited to mass spectrometry, chromatography, electrophoresis, sedimentation, isoelectric focusing, and antibody assay. See, e.g., U.S. Pat. No. 6,589,748; U.S. Pat. No. 6,027,896. Marker proteins may also be identified by two-dimensional electrophoresis (2-D electrophoresis). 2D-electrophoresis is a technique comprising denaturing electrophoresis, followed by isoelectric focusing; this generates a two-dimensional gel (2D gel) containing a plurality of separated proteins. For an example of a preferred means of carrying out 2D-electrophoresis to identify marker proteins, see, e.g. WO 98/23950; U.S. Pat. No. 6,064,654 and U.S. Pat. No. 6,278,794. Briefly, spots identified in a 2D gel are characterized by their isoelec-

tric point (pI) and apparent molecular weight (MW) as determined by 2D gel electrophoresis. Altered levels of marker proteins in a first sample or sample set with respect to a second sample or sample set can be determined when 2D gel electrophoresis gives a different signal when applied to the first sample and second samples or sample sets. Altered levels of marker proteins may be present in first sample or sample sets at increased, elevated, depressed or reduced levels as compared to the second sample or sample sets. By "increased level" is meant (a) any level of a particular isoform of marker protein when that isoform is not present in a normal subject without liver cancer, as well as (b) an elevated level (e.g., a two or three-fold increase) of marker protein or a particular isoform of a marker protein when that protein or a particular isoform is present in a normal subject without liver cancer. By "depressed level" is meant (a) an absence of a particular isoform of a marker protein when that isoform is present in a normal subject without liver cancer, as well as (b) a reduced level (e.g., a two or three-fold reduction) of a marker protein or a particular isoform of a marker protein when that protein or a particular isoform is present in a normal subject without liver cancer. In general, the steps of (a) assaying a sample for an elevated level of a marker protein and/or depressed level of a marker protein, and (b) correlating an elevated level of a marker protein and/or a depressed level of a marker protein in said sample with liver cancer, can be carried out in accordance with known techniques, or variations thereof that will be apparent to persons skilled in the art. See, e.g., U.S. Pat. No. 4,940,658 to Allen et al.

Signals obtained upon analyzing a biological sample or sample set from subjects having liver cancer relative to signals obtained upon analyzing a biological sample or sample set from normal subjects without liver cancer will depend upon the particular analytical protocol and detection technique that is used. Accordingly, the invention contemplates that each laboratory will establish a reference range for each marker protein identifier (e.g. pI and/or MW) in normal subjects without liver cancer according to the analytical protocol and detection technique in use, as is conventional in the diagnostic art.

Antibody assays (immunoassays) may, in general, be homogeneous assays or heterogeneous assays. In a homogeneous assay the immunological reaction usually involves the specific antibody, a labeled analyte, and the sample of interest. The signal arising from the label is modified, directly or indirectly, upon the binding of the antibody to the labeled analyte. Both the immunological reaction and detection of the extent thereof are carried out in a homogeneous solution. Immunochemical labels which may be employed include free radicals, radioisotopes, fluorescent dyes, enzymes, bacteriophages, coenzymes, and so forth.

In a heterogeneous assay approach, the reagents are usually the specimen, the antibody of the invention and a system or means for producing a detectable signal. Similar specimens as described above may be used. The antibody is generally immobilized on a support, such as a bead, plate or slide, and contacted with the specimen suspected of containing the antigen in a liquid phase. The support is then separated from the liquid phase and either the support phase or the liquid phase is examined for a detectable signal employing means for producing such signal. The signal is related to the presence of the analyte in the specimen. Means for producing a detectable signal include the use of radioactive labels, fluorescent labels, enzyme labels, and so forth. For example, if the antigen to be detected contains a second binding site, an antibody which binds to that site can be conjugated to a detectable group and added to the liquid phase reaction solution before the separa-

tion step. The presence of the detectable group on the solid support indicates the presence of the antigen in the test sample. Examples of suitable immunoassays are the radioimmunoassay, immunofluorescence methods, enzyme-linked immunoassays, and the like.

Those skilled in the art will be familiar with numerous specific immunoassay formats and variations thereof which may be useful for carrying out the method disclosed herein. See generally E. Maggio, *Enzyme-Immunoassay*, (1980) (CRC Press, Inc., Boca Raton, Fla.); see also U.S. Pat. No. 4,727,022 to Skold et al. titled "Methods for Modulating Ligand-Receptor Interactions and their Application." U.S. Pat. No. 4,659,678 to Forrest et al., U.S. Pat. No. 4,316,110 to David et al., U.S. Pat. No. 4,275,149 to Litman et al., U.S. Pat. No. 4,233,402 to Maggio et al., and U.S. Pat. No. 4,230,767 to Boguslaski et al.

Antibodies for immunoassays can be polyclonal or monoclonal antibodies, Fab fragments, humanized antibodies and chimeric antibodies (including fragments thereof) and can be produced in accordance with known techniques, based on one or more marker protein. For example, monoclonal antibodies may be produced in a hybridoma cell line according to the techniques of Kohler and Milstein, *Nature* 265, 495-97 (1975). Monoclonal Fab fragments may be produced in *Escherichia coli* from the known sequences by recombinant techniques known to those skilled in the art. See, e.g., W. Huse, *Science* 246, 1275-81 (1989) (recombinant Fab techniques). Polyclonal antibodies can be produced in animals such as goats, rabbits and horses by administration of one or more marker protein, optionally in combination with an adjuvant, as an immunogen, optionally administering booster doses thereof, and collecting the polyclonal antibodies from the animal.

### 3. Panel Tests

The marker proteins described herein can be used individually or in panels with one another or other additional markers for liver cancer such as described above, and including the marker alpha fetoprotein (upregulated in the blood of subjects afflicted with liver cancer). Where used in a panel test, the levels of the various markers are optionally but preferably tested from the same biological sample obtained from the subject (e.g., by detecting the quantities or amounts of various proteins in the same blood sample obtained from a patient). When combined in a panel test, the panel test may comprise determining an altered level for each of 2, 3 or 4 different proteins, up to 6, 8 or 10 or more different proteins (e.g., a panel of all proteins set forth in Tables 1-2 below). The combination of multiple marker proteins in a panel test serves to reduce the number of false positives and false negatives should an aberrant value for one particular member of the panel be found.

The present invention is explained in greater detail in the following non-limiting examples.

## EXPERIMENTAL

### Material and Methods

Tumor Tissues, Sera, and Cell Line. Tumor and non-tumor counterpart tissues were obtained from 7 patients with HCC. Following excision, the tissues were immediately frozen at  $-80^{\circ}$  C. The tumoral or non-tumoral localizations of samples used for protein extraction were verified histologically. Histological analysis of non-tumor tissues showed that HCCs had developed on cirrhotic tissues. The tumors were classified

histologically using the Edmondson grading system. All HCCs were defined as well or moderately differentiated. Sera were obtained from 27 healthy individuals, from 33 patients with chronic hepatitis, from 28 patients with cirrhosis, and from 34 patients with HCC, following informed consent. All patients were HCV-positive. The human hepatoma cell line PLC-PRF5 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE). Tumor and non-tumor tissues were solubilized in lysis buffer containing 9.5 M urea (Bio-Rad Laboratories, Hercules, Calif.), 2% Nonidet P-40, 2% carrier ampholytes, pH 4-8 (Gallard-Schlesinger Industries, Plainview, N.Y.), 2%  $\beta$ -mercaptoethanol, and 10 mM phenylmethanesulfonyl fluoride. Proteins (200  $\mu$ g) were applied onto isofocusing gels. Isoelectric focusing was conducted using pH 4-8 carrier ampholytes. The first-dimension gel was loaded onto the second-dimension gel, after equilibration in 125 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 1% dithiothreitol, and bromophenol blue. For the second-dimension separation, a gradient of 11 to 14% acrylamide (Serva; Crescent Chemical Company, Hauppauge, N.Y.) was used. Proteins were visualized by a photochemical silver-based staining technique.

Protein spot detection and quantitation. After the two-dimensional separation, each gel was scanned using a Kodak CCD camera. A 1,024 $\times$ 1,024 pixel format was used, yielding pixel widths of 163  $\mu$ m, where each pixel had 256 possible gray-scale values (optical density). Spot detection was accomplished by Bio Image Visage System software (Bioimage, Ann Arbor, Mich.). The background-subtracted integrated intensity of each spot was obtained in Optical Density Units multiplied by  $\text{mm}^2$ . A set of 999 selected spots from each gel was matched to the spots on a master gel.<sup>15</sup> A total of 250 spots were chosen as ubiquitously expressed reference spots to allow adjustment for variation in protein loading and gel staining. Each of the 999 spots was then normalized in relation to the 10 closest neighboring reference spots.<sup>16</sup>

In-gel Enzymatic Digestion and Mass Spectrometry. Additional 2-D gels were silver-stained by successive incubations in 0.02% sodium thiosulfate for 2 min, 0.1% silver nitrate for 40 min, and 0.014% formaldehyde plus 2% sodium carbonate. The protein spots of interest were excised from the 2-D gels and destained for 10 min in 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. Following three washes with water, the gel pieces were dehydrated in 100% acetonitrile for 5 min and dried for 20 min in a vacuum centrifuge. Digestion was performed by addition of 100 ng of trypsin (Promega Corporation, Madison, Wis.) in 200 mM ammonium bicarbonate. Following overnight enzymatic digestion at 37 $^\circ$  C., the peptides were extracted twice with 10% acetonitrile/10% formic acid. After centrifugation in a vacuum centrifuge, 5% acetonitrile/0.1% formic acid was added to the peptides. Peptide mixtures were analyzed by nanoflow capillary liquid chromatography coupled with electrospray quadrupole time of flight tandem mass spectrometry (ESI/Q-TOF MS/MS) in the Q-ToF Micro<sup>TM</sup> mass spectrometer (Waters Corporation, Milford, Mass.). ESI/Q-TOF MS/MS tandem spectra were recorded in the automated MS to MS/MS switching mode, with an m/z-dependent set of collision offset values. Singly- to triply-charged ions were selected and fragmented, with argon used as the collision gas. The acquired spectra were processed and searched against a non-redundant SwissProt protein sequence database using the ProteinLynx global server (available from Waters Corporation, 34 Maple Street, Milford, Mass. 01757 (telephone 508 478-2000)). The molecular weight (MW) and pI of identified

proteins were calculated using Compute pI/Mw software tool from ExPASy (the Expert Protein Analysis System of the Swiss Institute of Bioinformatics (Biozentrum—University of Basel, Klingelbergstrasse 50-70, 4056 Basel, Switzerland)).

2-D, 1-D and Dot Immunoblotting. Proteins separated by 2D-PAGE were transferred to PVDF membranes. Immunoblotting was then performed with antibodies against calreticulin, SPA-600 (StressGen Biotechnologies Corporation, Victoria, Canada) and T-19 (Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase-conjugated anti-rabbit (Amersham Biosciences, Piscataway, N.J.) or anti-goat (Sigma-Aldrich, St. Louis, Mo.) IgG antibodies. Immunoreactivity was revealed by enhanced chemiluminescence using an ECL kit (Amersham Biosciences). For 1-D immunoblots, PLC-PRF5 cells were lysed in 20 mM Tris-HCl, pH 7.5, buffer containing 5 mM EDTA and 100 mM KCl. Proteins (50  $\mu$ g) were loaded onto a 12% polyacrylamide gel, separated, and transferred onto nitrocellulose membrane (Amersham Biosciences). Immunoblotting was performed using a polyclonal anti-PDIA3 antibody (Stressgen Biotechnologies Corporation). Immunodetection was realized by ECL (Amersham Biosciences). For dot-immunoblot assays, PLC-PRF5 protein extracts (1-10  $\mu$ g) and cell culture supernatant (5-25  $\mu$ l) were blotted onto nitrocellulose membranes, using a dot-blot apparatus (Schleicher & Schuell, Keene, N.H.). The following antibodies were used: anti-calreticulin antibodies, PA3-900 (Affinity BioReagents, Golden, Colo.), N-19 (Santa Cruz Biotechnology), SPA-600 and SPA-601 (Stressgen Biotechnologies Corporation), anti-PDI antibodies, SPA-891 (Stressgen Biotechnologies Corporation), and H-160 (Santa Cruz Biotechnology), and anti-GRP78 antibodies N-20 and C-20 (Santa Cruz Biotechnology). Immunoreactivity was revealed by enhanced chemiluminescence using an ECL kit (Amersham Biosciences).

ELISA. Ninety six-well microplates (Nalge Nunc International, Rochester, N.Y.) were coated overnight with 0.5  $\mu$ g/ml anti-calreticulin N-19 or 1  $\mu$ g/ml anti-ERp57 N-20 antibodies (Santa Cruz Biotechnology), diluted in 0.05M carbonate-bicarbonate. After blocking with 1% BSA for 2 hours at room temperature, 50  $\mu$ l of serum (diluted 1:10 with 0.67% BSA) or 50  $\mu$ l of 0.67% BSA used as a negative control, were added. Following four washes with PBS/0.05% Tween20, 0.45  $\mu$ g/ml anti-calreticulin SPA-601 or 1  $\mu$ g/ml anti-Erp57 SPA-725 antibodies (Stressgen Biotechnologies Corporation) diluted in 0.67% BSA were added. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (DakoCytomation California, Carpinteria, Calif.) (1:500 dilution in 0.67% BSA) followed by addition of 50  $\mu$ l TMB one solution (Promega Corporation) were used for detection. The reaction was stopped by addition of 1N sulfuric acid (Acros Organics, Morris Plains, N.J.) and the absorbance was measured at 450 nm using SpectraMax<sup>®</sup> Plus<sup>384</sup> spectrophotometer (Molecular Devices, Sunnyvale, Calif., USA).

## 55 Results

Protein Quantification. Liver tumor tissues and non-tumor counterpart regions isolated from 7 patients with HCC were analyzed by high-resolution 2-D PAGE. Nine hundred and ninety-nine protein spots were detected in 2-D gels that localized in the pI range of 4 to 8 and relative molecular mass of 10 to 150 kDa. Using computer-assisted analysis, each individual protein spot was quantified according to integrated intensity, allowing the comparison of protein spot intensities between tumor and non-tumor counterpart regions from the same patient. We selected protein spots that showed variation in at least 4 out of the 7 patients. Using this criterion, 47 protein spots were selected, corresponding to 16 up-regulated

and 31 down-regulated spots (Table 1). The position of these protein spots on 2-D PAGE is shown in FIG. 1.

TABLE 1

Protein spots modified in HCC					
Up-regulated spots	Samples displaying change (n = 7)	Mean change	Down-regulated spots	Samples displaying change (n = 7)	Mean change
26	4	2.8	14	5	5.2
75	5	2.5	36	4	2.2
77	5	2.0	37	5	~10.1
114	4	~26.8	86	5	~28.0
151	4	5.2	106	4	5.8
153	5	4.9	111	4	4.9
154	5	4.8	112	5	2.9
157	5	2.4	148	4	~67.0
240	4	3.5	150	4	4.9
307	5	6.3	175	5	4.4
353	4	4.0	255	5	~13.9
355	4	6.5	263	6	1.8
357	4	3.0	299	5	~26.3
500	5	7.2	300	4	~27.4
517	4	3.8	329	4	3.0
641	5	7.2	337	6	1.5
			345	5	~41.0
			346	5	~80.0
			348	5	~14.8
			366	4	~27.0
			387	4	2.7
			426	5	~33.0
			428	5	~50.0

TABLE 1-continued

Protein spots modified in HCC					
Up-regulated spots	Samples displaying change (n = 7)	Mean change	Down-regulated spots	Samples displaying change (n = 7)	Mean change
			430	5	~33.0
			438	4	~18.2
			439	5	~52.0
			453	4	~51.1
			462	5	~75.0
			473	6	1.7
			486	5	7.8
			502	5	~50.0

The ~ indicates fold change calculation for which the smaller value is replaced by an estimate of the minimum value for detectable signal.

Protein Spot Identification. Of the 47 selected protein spots, 3 (spots 111, 112 and 150) had been previously identified by our group.<sup>17</sup> The remaining 44 protein spots were cut from additional 2-D PAGE gels and processed for mass spectrometry identification. Forty-one of these protein spots were identified unambiguously by ESI/Q-TOF MS/MS, while 2 protein identifications were obtained for spot 366, and no identification was obtained for spots 345 and 346. Protein spot identifications are presented in Table 2. Overall, the 45 protein spots identified corresponded to 23 distinct genes, belonging to 4 major sub-cellular groups: cytoplasmic proteins (39%), mitochondrial proteins (13%), extracellular proteins (22%), and components of the endoplasmic reticulum (ER) (26%) (FIG. 2).

TABLE 2

Protein spot identification						
Spot	Name	MS Identification (Peptide Matched)	Calculated MW/pI	Estimated MW/pI	Uni-gene	Swiss-prot
Up-regulated						
26	HSP60	17	61/5.8	60/5.3	Hs.79037	P10809
75	Albumin	5	69/5.9	48/5.6	Hs.184411	P02768
77	Albumin	5	69/5.9	48/5.8	Hs.184411	P02768
114	Tropomyosin 2	4	33/4.6	35/4.4	Hs.300772	P07951
151	CRT32 (calreticulin fragment)	6	—	32/3.3	Hs.353170	P27797
153	Alpha-1-antitrypsin	1	48/7.7	35/4.0	Hs.297681	P01009
154	Alpha-1-antitrypsin	1	48/7.7	33/4.2	Hs.297681	P01009
157	Alpha-1-antitrypsin	2	48/7.7	31/4.3	Hs.297681	P01009
240	Annexin A4	2	36/5.8	30/5.7	Hs.422986	P09525
307	Triosephosphate isomerase	1	26/6.5	27/7.0	Hs.83848	P00938
353	APOA1	9	31/5.7	26/5.0	Hs.93194	P02647
355	APOA1	—	31/5.7	25/5.1	Hs.93194	P02647
357	APOA1	13	31/5.7	26/5.2	Hs.93194	P02647
500	Alpha-1-antitrypsin	7	48/7.7	16/5.2	Hs.297681	P01009
517	Transthyretin	3	16/5.7	15/5.5	Hs.427202	P02766
641	GRP78 (BIP)	3	72/5.1	16/5.2	Hs.75410	P11021
Down-regulated						
14	Calreticulin precursor	6	48/4.3	70/4.4	Hs.353170	P27797
36	Calreticulin	4	48/4.3	59/4.0	Hs.353170	P27797
37	Calreticulin	10	48/4.3	53/3.6	Hs.353170	P27797
86	Haptoglobin	3	45/6.5	45/5.2	Hs.75990	P00738
106	Aminoacylase-1	4	46/5.8	40/5.8	Hs.334707	Q03154
111	CRT32-C	Ref 15	48/4.3	36/3.5	Hs.353170	P27797
112	CRT32-B	Ref 15	48/4.3	36/3.5	Hs.353170	P27797
148	Liver-type arginase	9	35/7.2	35/7.0	Hs.332405	P05089
150	CRT32-A	Ref 15	48/4.3	35/3.4	Hs.353170	P27797
175	Actin	7	42/5.3	33/5.5	Hs.426930	P02570
255	HSP60	3	61/5.8	30/4.5	Hs.79037	P10809
263	Carbamoyl-phosphate synthase 1	2	165/6.7	30/5.5	Hs.50966	P31327

TABLE 2-continued

Protein spot identification						
Spot	Name	MS Identification		Uni-gene	Swiss-prot	
		(Peptide Matched)	Calculated MW/pI			
299	Protein disulfide isomerase A3	10	57/6.0	29/6.0	Hs.13751	P30101
300	Enoyl-CoA hydratase	6	31/8.5	29/6.0	Hs.76394	P30084
329	Protein disulfide isomerase A3	13	57/6.0	29/5.8	Hs.13751	P30101
337	Triosephosphate isomerase	6	26/6.5	26/6.0	Hs.83848	P00938
348	Protein disulfide isomerase	6	57/4.8	25/4.4	Hs.410578	P07237
366	Enoyl-CoA hydratase	7	31/8.5	26/5.8	Hs.76394	P30084
	Triosephosphate isomerase	2	26/6.5	26/5.8	Hs.83848	P00938
387	Liver-type arginase	1	35/7.2	23/4.3	Hs.332405	P05089
426	Ferritin light chain	3	20/5.7	22/5.3	Hs.39574	P02792
428	Ferritin light chain	1	20/5.7	22/5.4	Hs.39574	P02792
430	Ferritin light chain	3	20/5.7	21/5.5	Hs.39574	P02792
438	Alpha-1-antitrypsin	3	20/6.0	20/6.0	Hs.297681	P01009
	Liver carboxylesterase	2	62/6.5	22/6.2	Hs.76688	P23141
453	Carbamoyl-phosphate synthase 1	5	165/6.7	19/5.3	Hs.50966	P31327
462	Superoxide dismutase [Cu-Zn]	1	16/6.0	19/7.2	Hs.75428	P00441
473	Superoxide dismutase [Cu-Zn]	4	16/6.0	18/5.7	Hs.75428	P00441
486	Cytochrome b5	4	15/4.9	16/4.4	Hs.83834	P00167
502	Peptidyl-prolyl isomerase A	3	18/8.2	16/5.8	Hs.401787	P05092

Correlation with Transcript Levels. In parallel with the proteomic analysis, we compared the gene expression profiles of HCC and adjacent non-tumor tissues using oligonucleotide microarrays. Expression levels of the transcripts corresponding to the 23 individual proteins were examined (Table 3). Positive correlations between transcript and protein level variations were observed for only 7 out of the 23 proteins. Levels of Hsp60 and GRP78 mRNAs and proteins were higher in the tumor tissues, while levels of aminoacylase-1, carbamoyl-phosphate synthase 1, cytochrome b5, enoyl-CoA hydratase, and haptoglobin mRNAs and proteins were lower in the tumor tissues. It is likely that post-transcriptional and post-translational alterations account for the discrepancies between mRNA and protein levels.

TABLE 3

Correlation between transcript and protein level variations. A positive correlation was observed for the genes listed in bold. Mean NT and T are expressed in Affymetrix intensity values. As criteria for determining significant differences in mean gene mRNA expression levels, two-sample t tests were done on log-transformed data.				
Gene Name	Mean NT	Mean T	Fold	p value
Actin	19784	22515	1.14	0.530
Albumin	23641	11207	-2.11	0.000
Alpha-1-antitrypsin	69290	62071	-1.12	0.024
<b>Aminoacylase-1</b>	<b>10381</b>	<b>7675</b>	<b>-1.35</b>	<b>0.007</b>
Annexin A4	4583	4782	1.04	0.545
APOA1	60528	36653	-1.65	<b>0.018</b>
Calreticulin	5580	6061	1.09	0.254
<b>Carbamoyl-phosphate synthase 1</b>	<b>22269</b>	<b>13772</b>	<b>-1.62</b>	<b>0.008</b>
<b>Cytochrome b5</b>	<b>21562</b>	<b>13869</b>	<b>-1.55</b>	<b>0.004</b>
<b>Enoyl-CoA hydratase</b>	<b>15904</b>	<b>11123</b>	<b>-1.43</b>	<b>0.011</b>
Ferritin light chain	71534	78385	1.10	0.018
<b>GRP78</b>	<b>5581</b>	<b>7547</b>	<b>1.35</b>	<b>0.000</b>

TABLE 3-continued

Correlation between transcript and protein level variations. A positive correlation was observed for the genes listed in bold. Mean NT and T are expressed in Affymetrix intensity values. As criteria for determining significant differences in mean gene mRNA expression levels, two-sample t tests were done on log-transformed data.				
Gene Name	Mean NT	Mean T	Fold	p value
<b>Haptoglobin</b>	<b>41446</b>	<b>25451</b>	<b>-1.63</b>	<b>0.017</b>
<b>Hsp60</b>	<b>17124</b>	<b>23105</b>	<b>1.35</b>	<b>0.000</b>
Liver carboxylesterase	12887	15857	1.23	0.883
Liver-type arginase	10704	8231	-1.30	0.088
Peptidyl propyl isomerase	24377	32685	1.34	0.026
Protein disulfide isomerase	32404	38211	1.18	0.003
Protein disulfide isomerase A3	3370	4455	1.32	0.001
Superoxide dismutase [Cu-Zn]	18511	17217	-1.08	0.257
Transthyretin	50195	39128	-1.28	0.020
Triosephosphate isomerase	4164	5862	1.41	0.205
Tropomyosin 2	373	563	1.51	0.127

Identification of Protein Cleavages in HCC. Protein separation by 2D-PAGE discriminates between post-translationally modified or processed protein isoforms. Twelve out of the 45 protein spots identified by mass spectrometry and listed in Table 2 had an apparent molecular weight significantly smaller than the predicted value for the corresponding intact protein, suggesting that these protein spots may correspond to protein fragments resulting from proteolytic cleavages. These 12 protein spots correspond to 7 distinct genes, including 5 that encode proteins of the ER: calreticulin, PDIA3, PDI, GRP78 and liver carboxylesterase. The other 2 proteins, Hsp60 and carbamoyl-phosphate synthase 1, are mainly mitochondrial, although partial localization in the ER has been reported for both proteins.

Characterization of Calreticulin. The dysregulated proteins in HCC included the precursor and full-length calreti-

culin isoforms (spots 14, 36 and 37) which were reduced in HCC compared to non-tumor tissues. Concomitant with this down-regulation, an increase in another calreticulin form (Crt32, spot 151), with an estimated molecular mass of 32 kDa and pI of 4.1) was observed in the tumor tissues of the same patients (FIG. 3). Crt32 is a truncated form of calreticulin that we previously characterized in the hepatoma cell line PLC-PRF5, which corresponds to the C-terminal end of the protein (amino acids 157-400).<sup>17</sup> Identification of protein spots 14, 36, 37 and 151 as calreticulin isoforms was further confirmed by Western blot analysis, using an antibody directed against a C-terminal peptide of calreticulin (FIG. 3). Three additional spots (spots 111, 112 and 150) were also recognized by the antibody produced against the C-terminal region of calreticulin. These 3 calreticulin isoforms, which remain to be fully characterized, were reduced in HCC compared to non-tumor tissues. Taken together, these observations suggested that calreticulin is cleaved in HCC.

We sought to identify the N-terminal cleavage product of calreticulin in HCC. Two-dimensional Western blot experiments using the antibodies SPA-600 and T-19, directed against the C-terminal and N-terminal parts of calreticulin, respectively, were performed. A protein spot with estimated MW of 18 kDa and pI 6.5 reacted against the antibody T-19 but not against the antibody SPA-600, suggesting that it corresponds to an N-terminal fragment of calreticulin (FIG. 4A). Furthermore, the MW/pI estimated based on migration in 2-D gels were in agreement with the calculated MW/pI (19.5 kDa/5.7) of the N-terminal product that, together with Crt32, constitutes the complete calreticulin sequence. Identification of this protein spot as the N-terminal end of calreticulin was further confirmed by mass spectrometry. After tryptic digestion, 8 of the resulting peptides matched the calreticulin sequence in the N-terminal domain of the protein (FIG. 4B). We designated this N-terminal truncated form of calreticulin as Crt18.

In contrast with Crt32, the level of Crt18 was not significantly increased in HCC compared to non-tumor tissues. In addition, Crt18 intensity was significantly lower than Crt32 intensity in the HCC 2-D patterns (FIG. 4C). These observations suggested that Crt18 may be less stable than Crt32 or that Crt18 may be released in the extracellular compartment. To examine these possibilities, we used the PLC-PRF5 cell line, in which both Crt32 and Crt18 were detected by 2-D PAGE (data not shown). Using antibodies directed against the N-terminal end (antibodies SPA-601 and N-19) or the C-terminal end (antibodies SPA-600 and PA3-900) of calreticulin, we demonstrated that Crt18 but not Crt32 was detected in the culture supernatant of the PLC-PRF5 cells (FIG. 4D). Therefore, we conclude that calreticulin is cleaved in HCC, leading to the generation of Crt32 and Crt18 fragments and to the subsequent release of Crt18 into the extracellular compartments.

Characterization of Protein Disulfide Isomerase A3 (PDIA3). Spots 299 and 329, with apparent MW of 29 kDa and pI of 6 and 5.8, respectively, were down-regulated in tumor tissues as compared to non-tumor counterparts. After enzymatic digestion using trypsin and analysis of the resulting peptides by mass spectrometry, the peptides were consistent with those derived from protein disulfide isomerase A3 (PDIA3), a protein with a MW of 57 kDa and a pI of 6. The matched peptides clustered in the C-terminal region of the protein, suggesting that spots 299 and 329 correspond to C-terminal fragment products of PDIA3 (FIG. 5A).

As with calreticulin, we wished to characterize further the PDIA3 proteolytic processing occurring in HCC. In addition to spots 299 and 329, two other protein spots on the HCC 2-D

gels (A-PDIA3 and B-PDIA3), were identified as PDIA3 isoforms (FIG. 5B). A-PDIA3 was identified as the full-length PDIA3 protein, based on a total of 17 matched peptides that covered 41% of the full-length sequence. Moreover, the matched peptides did not cluster in a specific region of PDIA3 (FIG. 5C). B-PDIA3 is a PDIA3 fragment containing aa148-aa273 (FIG. 5C). Because of the size of this fragment, B-PDIA3 corresponds most likely to a fragment of PDIA3, complementary to spots 229 or 329. While no specific modulation in intensity of the full-length A-PDIA3 protein was observed between tumor and non-tumor tissues, oligonucleotide microarray analysis indicated that the expression of the PDIA3 transcript was slightly (1.3 fold) but significantly ( $p=0.001$ ) increased in tumor as compared to non-tumor tissues. Taken together, these observations suggested that PDIA3 is specifically cleaved in HCC, leading to the generation of two highly similar C-terminal fragments (spots 299 and 329) and one complementary N-terminal cleaved product (B-PDIA3).

As observed with calreticulin, the lack of correlation between the intensities of these three spots suggested that cleavage products of PDIA3 may be released in the extracellular compartment. This assumption was verified in the PLC-PRF5 cell line, in which an approximately 30 kDa band was detected in the culture supernatant by Western blot using an antibody against PDIA3 (FIG. 5D).

Cleavage of Other ER Proteins and their Detection in the Extracellular Compartment. Another ER protein, GRP78, exhibited cleavage in HCC. Spot 641, a protein spot with an apparent molecular weight of 16 kDa, up-regulated in HCC, was identified by mass spectrometry as GRP78, a protein of 72 kDa. The peptides obtained by mass spectrometry from spot 641 matched the N-terminal end of the GRP78 sequence, suggesting that spot 641 corresponds to an N-terminal fragment of GRP78 (FIG. 6A). The full-length GRP78 protein was localized on the HCC 2-D patterns (FIG. 6B). No variation in intensity of the full-length protein was observed in tumor compared to non-tumor tissues. However, oligonucleotide microarray analysis indicated that the expression of the GRP78 transcript was slightly (1.35 fold) but significantly ( $p<0.001$ ) increased in tumor as compared to non-tumor tissues. These observations suggested that GRP78 is cleaved in HCC. Using antibodies directed against the N-terminal end (N-20) or the C-terminal end (C-20) of GRP78, we detected a C-terminal fragment of GRP78 in the culture supernatant of PLC-PRF5 cells (FIG. 6C).

Spot 348, with an apparent MW of 25 kDa was identified by ESI/Q-TOF MS/MS as protein disulfide isomerase (PDI), a protein of 57 kDa. All generated peptides matched with the N-terminal end of the protein, to a region spanning amino acids 79 to 230 (data not shown). Using antibodies directed against the N-terminal end (H-160) or the C-terminal end (SPA-891) of the protein, we detected an N-terminal fragment of PDI in the culture supernatant of PLC-PRF5 cells (FIG. 7).

Detection of Calreticulin and PDIA3 in Sera of HCC Patients. We investigated the possibility that calreticulin and PDIA3 cleavage fragments could be detected in serum of patients with HCC. We developed sandwich ELISAs using antibodies directed against the N-terminal end of calreticulin and antibodies directed against the N-terminal end of PDIA3. A statistically highly significant difference in Crt18 serum levels between patients with HCC ( $n=34$ ) and healthy individuals ( $n=27$ ) was observed ( $p=0.0002$ ) (FIG. 8A). Similar data were obtained for the PDIA3 fragment. The difference in PDIA3 fragment serum levels between patients with HCC and healthy individuals was highly significant ( $p<0.0001$ )

(FIG. 8B). We then investigated the possibility that Crt18 and PDIA3 fragments could be present in significantly different amounts in sera from patients with HCC and from patients at-risk of developing HCC, namely patients with chronic hepatitis and patients with cirrhosis. Levels of Crt18 and PDIA3 fragment were measured by ELISA in serum obtained from 33 patients with chronic hepatitis and 28 patients with cirrhosis. While Crt18 and PDIA3 fragment were detected at high levels in some patients in the high risk groups, amounts of both Crt18 and PDIA3 fragment were significantly different between patients with HCC and patients with chronic hepatitis ( $p=0.004$  and  $p=0.0003$  for Crt18 and PDIA3, respectively) or patients with cirrhosis ( $p=0.04$  and  $p<0.0001$  for Crt18 and PDIA3, respectively) (FIG. 8).

ROC curves were plotted to define the optimal cutoff values and to identify the sensitivity and specificity for serum calreticulin, PDIA3) and AFP in differentiating patients with HCC vs. those with cirrhosis (FIG. 9). Using 0.3 as pre-specified acceptable False Positive Fraction, sensitivity and specificity for calreticulin with an optimal cutoff point of 0.44 relative unit, were 58% and 71%, respectively; sensitivity and specificity for PDIA3 with an optimal cutoff point of 0.06 relative unit, were 94% and 72%, respectively; sensitivity and specificity of AFP with an optimal cutoff point of 11.7 ng/ml were 76% and 69%, respectively. At 20 ng/ml (recommended cutoff for AFP), sensitivity and specificity of AFP were 70% and 86%, respectively. Used alone, PDIA3 showed the greatest sensitivity (94%) in differentiating patients with HCC with those with cirrhosis. We then evaluated the sensitivity and specificity of calreticulin and PDIA3 combined and in combination with AFP. The best results were obtained by combining PDIA3 with AFP with 97% sensitivity (Table 4). Indeed, PDIA3 levels were elevated in all HCC patients with an AFP <20 ng/ml and in all HCC patients with an AFP <11.7 ng/ml (cutoff established by the ROC curve in this study).

TABLE 4

Calreticulin, PDIA3 and AFP, alone or in combination, for differentiation of patients with HCC from those with cirrhosis.				
Protein	Sensitivity	Specificity	Positive Pred. Value	Negative Pred. Value
AFP(>11.7 ng/ml)	76	69	75	74
AFP(>20 ng/ml)	71	86	86	71
Calreticulin (>0.44 relative unit)	58	71	62	49
PDIA3 (>0.059 relative unit)	94	72	80	91
Calreticulin + PDIA3	91	77	83	87
Calreticulin + AFP	79	70	77	73
PDIA3 + AFP	97	69	77	95
Calreticulin + PDIA3 + AFP	94	69	78	88

## DISCUSSION

Most proteomic analyses of HCC tissues, including this study, have been performed using 2-D PAGE followed by MS. This technique has led to the identification of a large number of proteins modified in HCC compared to adjacent non-tumor tissues.<sup>14</sup> In agreement with these studies, we describe here a decrease in carbamoyl-phosphate synthase 1, cytochrome b5, liver-carboxylesterase, liver-type arginase, PDI, superoxide dismutase [Cu—Zn] and enoyl-CoA hydratase expression in tumor tissues compared to non-tumor counterparts.<sup>18, 19</sup> We also observed an increase in Hsp60,

GRP78, triosephosphate isomerase, and ferritin light chain expression in HCC, as also previously reported.<sup>18-21</sup> Most proteins we identified are cytoplasmic. Other important groups include mitochondrial proteins, extracellular proteins and components of the ER. In the human proteome, the proportion of proteins assigned to the mitochondria and ER components is 4% and 3%, respectively.<sup>22</sup> Our findings therefore suggest that mitochondria- and ER-associated cellular functions may be specifically dysregulated in HCC. Protein separation by 2-D PAGE discriminates between post-translationally modified or processed protein isoforms. However, most studies reported to date have not presented any information regarding the specific protein isoforms, with the exception of the identification of specific protein alterations of aldehyde dehydrogenase<sup>23</sup> and aldose reductase-like protein<sup>24</sup> in HCC.

In our analysis, 27% of the protein spots we identified as modified in HCC corresponded to proteolytic cleavages as demonstrated by the smaller apparent Mw and the cluster of the peptides identified by MS within a specific region of the protein sequence. Interestingly, most of them corresponded to proteins expressed in the ER, including calreticulin, PDIA3, PDI, GRP78, and liver carboxylesterase. The ER is the site of synthesis and maturation of proteins destined for secretion, for the plasma membrane, and for the secretory and endocytic organelles. For glycoproteins, the ER possesses a dedicated maturation system, which assists folding and ensures the quality of final products before ER release. ER chaperones and folding enzymes such as GRP78, calreticulin, PDIA3, and PDI are not only responsible for assisting the folding and assembly process but also serve as retention anchors for immature proteins.<sup>25</sup> Calreticulin interacts with nascent and newly-synthesized glycoproteins in the ER and serves as a molecular chaperone during folding. Together with calnexin and PDIA3, calreticulin is responsible for quality control and folding in newly-synthesized glycoproteins.<sup>26, 27</sup> The amino acid sequence of PDIA3 is highly similar to that of PDI and these two proteins show strong analogies in function. GRP78 is a resident protein of the ER and is one of the most abundant ER chaperones. Many secretion incompetent proteins are found in stable association with GRP78 and are retained in the ER. Some proteins which are destined for secretion transiently associate with GRP78. The abundance of GRP78 and other ER chaperones influences the secretion efficiency of many proteins. Decrease in hepatic ER chaperone protein levels (GRP78, PDIA3, calreticulin) or in PDI activity leads to increased efficiency and amount of hepatic serum protein secretion.<sup>28-31</sup>

In addition, major histocompatibility complex (MHC) Class I maturation involves interactions with classical chaperones (e.g., GRP78), substrate-feature-specific chaperones (e.g., calreticulin), and catalyzing enzymes (e.g., PDIs), as well as with MHC class I dedicated accessory proteins (e.g., tapasin).<sup>32</sup> MHC class I down-regulation is a widespread mechanism used by tumor cells to escape destruction by anti-tumor cytotoxic T lymphocytes.<sup>33, 34</sup> Down-regulation of MHC class I antigen expression of tumors of different origins is frequently associated with the absence of or deficiencies in multiple components of the antigen presentation machinery.<sup>35-41</sup> Alterations in the expression of calreticulin, GRP78, PDI and PDIA3 in HCC may have important implications for MHC class I assembly, peptide loading, and presentation on the tumor cell surface and may be involved in the failure of the immune system to control HCC tumor progression.

Remarkably, following proteolytic cleavage, we demonstrated that fragments of calreticulin, GRP78, PDIA3 and

PDI are released in the extracellular compartment. Furthermore, fragments of calreticulin and PDIA3 were detected at significantly higher levels in serum of patients with HCC compared to serum from healthy individuals, from patients with chronic hepatitis or patients with cirrhosis. We therefore identified HCC biomarkers with enough initial characterization to make them highly attractive as candidates for full validation studies.

We previously reported that in the serum of patients with HCC, circulating antibodies against calreticulin can be detected.<sup>17</sup> We can hypothesize that the presence of these autoantibodies is a consequence of the cleavage of calreticulin in HCC. Recently, cleavage of glycan-3 and release of the NH<sub>2</sub>-terminal fragment was also reported as specifically detected in the sera of patients with HCC.<sup>42</sup> A catalytic fragment of vitronectin was also recently proposed as a new serum marker of HCC in patients with chronic liver disease.<sup>43</sup> Specific isoforms in general and cleavage products in particular should therefore be further evaluated as new markers arising from protein processing specific for HCC.

## REFERENCES

1. Bosch F X et al., *Gastroenterology* 2004; 127:S5-S16.
2. Llovet J M et al., *Lancet* 2003; 362:1907-1917.
3. El-Serag H B et al., *Hepatology* 2001; 33:62-65.
4. El-Serag H B. *Gastroenterology* 2004; 127:S27-34.
5. Bruix J, et al., *J Hepatol* 2001; 35:421-430.
6. Di Bisceglie A M et al., *Gastroenterology* 2004; 127:S104-107.
7. Daniele B et al., *Gastroenterology* 2004; 127:S108-112.
8. Hong W S et al., *J Korean Med Sci* 1991; 6:206-213.
9. Marrero J A et al., *Hepatology* 2003; 37:1114-1121.
10. Mita Y et al., *Cancer* 1998; 82:1643-1648.
11. Capurro M et al., *Gastroenterology* 2003; 125:89-97.
12. Yao D F et al., *Am J Clin Pathol* 1998; 110:743-749.
13. Cui R, et al., *Br J Cancer* 2003; 88:1878-1882.
14. Chignard N, Beretta L. *Gastroenterology* 2004; 127: S120-125.
15. Kuick R D et al., *Electrophoresis* 1991; 12:736-746.

16. Asakawa J et al., *Proc Natl Acad Sci USA* 1994; 91:9052-9056.
17. Le Naour F et al., *Mol Cell Proteomics* 2002; 1:197-203.
18. Kim W et al., *Clin Cancer Res* 2003; 9:5493-5500.
19. Lim S O et al., *Biochem Biophys Res Commun* 2002; 291:1031-1037.
20. Takashima M et al., *Proteomics* 2003; 3:2487-2493.
21. Park K S et al., *Hepatology* 2002; 35:1459-1466.
22. Anderson N L et al., *Mol Cell Proteomics* 2004; 3:311-326.
23. Park K S et al., *Int J Cancer* 2002; 97:261-265.
24. Zeindl-Eberhart E et al., *Hepatology* 2004; 39:540-549.
25. Kleizen B et al., *Curr Opin Cell Biol* 2004; 16:343-349.
26. Ellgaard L, Frickel E M. *Cell Biochem Biophys* 2003; 39:223-247.
27. Gelebart P et al., *Int J Biochem Cell Biol* 2005; 37:260-266.
28. Dhahbi J M et al., *Biochem Biophys Res Commun* 2001; 284:335-339.
29. Wang L et al., *J Biol Chem* 1997; 272:27644-27651.
30. Dorner A J et al., *Embo J* 1992; 11:1563-1571.
31. Dorner A J et al., *Mol Cell Biol* 1988; 8:4063-4070.
32. Paulsson K, Wang P. *Biochim Biophys Acta* 2003; 1641: 1-12.
33. Mariricola F M et al., *Adv Immunol* 2000; 74:181-273.
34. Chang C C et al., *Curr Opin Immunol* 2004; 16:644-650.
35. Seliger B et al., *Immunol Today* 2000; 21:455-464.
36. Seliger B et al., *Clin Cancer Res* 2003; 9:1721-1727.
37. Seliger B et al., *Cancer Res* 2001; 61:8647-8650.
38. Seliger B et al., *Cancer Res* 2001; 61:1095-1099.
39. Garcia-Lora A et al., *Int J Cancer* 2003; 106:521-527.
40. Ogino T et al., *Clin Cancer Res* 2003; 9:4043-4051.
41. Dissemmond J et al., *Cancer Lett* 2004; 203:225-231.
42. Hippo Y et al., *Cancer Res* 2004; 64:2418-2423.
43. Paradis V et al., *Hepatology* 2004; 41:40-47.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

## SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 3

<210> SEQ ID NO 1  
 <211> LENGTH: 417  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens  
 <220> FEATURE:  
 <221> NAME/KEY: MISC\_FEATURE  
 <222> LOCATION: (18)..(173)  
 <223> OTHER INFORMATION: Sequence of CRT18

<400> SEQUENCE: 1

Met Leu Leu Ser Val Pro Leu Leu Leu Gly Leu Leu Gly Leu Ala Val  
 1 5 10 15

Ala Glu Pro Ala Val Tyr Phe Lys Glu Gln Phe Leu Asp Gly Asp Gly  
 20 25 30

Trp Thr Ser Arg Trp Ile Glu Ser Lys His Lys Ser Asp Phe Gly Lys  
 35 40 45

Phe Val Leu Ser Ser Gly Lys Phe Tyr Gly Asp Glu Glu Lys Asp Lys  
 50 55 60

Gly Leu Gln Thr Ser Gln Asp Ala Arg Phe Tyr Ala Leu Ser Ala Ser

-continued

65		70		75		80									
Phe	Glu	Pro	Phe	Ser	Asn	Lys	Gly	Gln	Thr	Leu	Val	Val	Gln	Phe	Thr
				85					90					95	
Val	Lys	His	Glu	Gln	Asn	Ile	Asp	Cys	Gly	Gly	Gly	Tyr	Val	Lys	Leu
			100					105					110		
Phe	Pro	Asn	Ser	Leu	Asp	Gln	Thr	Asp	Met	His	Gly	Asp	Ser	Glu	Tyr
		115				120						125			
Asn	Ile	Met	Phe	Gly	Pro	Asp	Ile	Cys	Gly	Pro	Gly	Thr	Lys	Lys	Val
		130				135					140				
His	Val	Ile	Phe	Asn	Tyr	Lys	Gly	Lys	Asn	Val	Leu	Ile	Asn	Lys	Asp
145				150						155				160	
Ile	Arg	Cys	Lys	Asp	Asp	Glu	Phe	Thr	His	Leu	Tyr	Thr	Leu	Ile	Val
			165					170						175	
Arg	Pro	Asp	Asn	Thr	Tyr	Glu	Val	Lys	Ile	Asp	Asn	Ser	Gln	Val	Glu
			180					185					190		
Ser	Gly	Ser	Leu	Glu	Asp	Asp	Trp	Asp	Phe	Leu	Pro	Pro	Lys	Lys	Ile
		195					200					205			
Lys	Asp	Pro	Asp	Ala	Ser	Lys	Pro	Glu	Asp	Trp	Asp	Glu	Arg	Ala	Lys
	210					215					220				
Ile	Asp	Asp	Pro	Thr	Asp	Ser	Lys	Pro	Glu	Asp	Trp	Asp	Lys	Pro	Glu
225					230						235				240
His	Ile	Pro	Asp	Pro	Asp	Ala	Lys	Lys	Pro	Glu	Asp	Trp	Asp	Glu	Glu
			245						250					255	
Met	Asp	Gly	Glu	Trp	Glu	Pro	Pro	Val	Ile	Gln	Asn	Pro	Glu	Tyr	Lys
			260					265					270		
Gly	Glu	Trp	Lys	Pro	Arg	Gln	Ile	Asp	Asn	Pro	Asp	Tyr	Lys	Gly	Thr
		275				280						285			
Trp	Ile	His	Pro	Glu	Ile	Asp	Asn	Pro	Glu	Tyr	Ser	Pro	Asp	Pro	Ser
	290					295					300				
Ile	Tyr	Ala	Tyr	Asp	Asn	Phe	Gly	Val	Leu	Gly	Leu	Asp	Leu	Trp	Gln
305					310					315					320
Val	Lys	Ser	Gly	Thr	Ile	Phe	Asp	Asn	Phe	Leu	Ile	Thr	Asn	Asp	Glu
			325						330					335	
Ala	Tyr	Ala	Glu	Glu	Phe	Gly	Asn	Glu	Thr	Trp	Gly	Val	Thr	Lys	Ala
			340					345					350		
Ala	Glu	Lys	Gln	Met	Lys	Asp	Lys	Gln	Asp	Glu	Glu	Gln	Arg	Leu	Lys
		355					360					365			
Glu	Glu	Glu	Glu	Asp	Lys	Lys	Arg	Lys	Glu	Glu	Glu	Glu	Ala	Glu	Asp
	370					375						380			
Lys	Glu	Asp	Asp	Glu	Asp	Lys	Asp	Glu	Asp	Glu	Glu	Asp	Glu	Glu	Asp
385					390					395					400
Lys	Glu	Glu	Asp	Glu	Glu	Glu	Asp	Val	Pro	Gly	Gln	Ala	Lys	Asp	Glu
			405					410						415	

Leu

<210> SEQ ID NO 2  
 <211> LENGTH: 505  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens  
 <220> FEATURE:  
 <221> NAME/KEY: MISC\_FEATURE  
 <222> LOCATION: (148)..(273)  
 <223> OTHER INFORMATION: Sequence of B-PDIA3

&lt;400&gt; SEQUENCE: 2

Met Arg Leu Arg Arg Leu Ala Leu Phe Pro Gly Val Ala Leu Leu Leu

-continued

1	5	10	15
Ala Ala Ala Arg Leu Ala Ala Ala Ser Asp Val Leu Glu Leu Thr Asp	20	25	30
Asp Asn Phe Glu Ser Arg Ile Ser Asp Thr Gly Ser Ala Gly Leu Met	35	40	45
Leu Val Glu Phe Phe Ala Pro Trp Cys Gly His Cys Lys Arg Leu Ala	50	55	60
Pro Glu Tyr Glu Ala Ala Ala Thr Arg Leu Lys Gly Ile Val Pro Leu	65	70	75
Ala Lys Val Asp Cys Thr Ala Asn Thr Asn Thr Cys Asn Lys Tyr Gly	85	90	95
Val Ser Gly Tyr Pro Thr Leu Lys Ile Phe Arg Asp Gly Glu Glu Ala	100	105	110
Gly Ala Tyr Asp Gly Pro Arg Thr Ala Asp Gly Ile Val Ser His Leu	115	120	125
Lys Lys Gln Ala Gly Pro Ala Ser Val Pro Leu Arg Thr Glu Glu Glu	130	135	140
Phe Lys Lys Phe Ile Ser Asp Lys Asp Ala Ser Ile Val Gly Phe Phe	145	150	155
Asp Asp Ser Phe Ser Glu Ala His Ser Glu Phe Leu Lys Ala Ala Ser	165	170	175
Asn Leu Arg Asp Asn Tyr Arg Phe Ala His Thr Asn Val Glu Ser Leu	180	185	190
Val Asn Glu Tyr Asp Asp Asn Gly Glu Gly Ile Ile Leu Phe Arg Pro	195	200	205
Ser His Leu Thr Asn Lys Phe Glu Asp Lys Thr Val Ala Tyr Thr Glu	210	215	220
Gln Lys Met Thr Ser Gly Lys Ile Lys Lys Phe Ile Gln Glu Asn Ile	225	230	235
Phe Gly Ile Cys Pro His Met Thr Glu Asp Asn Lys Asp Leu Ile Gln	245	250	255
Gly Lys Asp Leu Leu Ile Ala Tyr Tyr Asp Val Asp Tyr Glu Lys Asn	260	265	270
Ala Lys Gly Ser Asn Tyr Trp Arg Asn Arg Val Met Met Val Ala Lys	275	280	285
Lys Phe Leu Asp Ala Gly His Lys Leu Asn Phe Ala Val Ala Ser Arg	290	295	300
Lys Thr Phe Ser His Glu Leu Ser Asp Phe Gly Leu Glu Ser Thr Ala	305	310	315
Gly Glu Ile Pro Val Val Ala Ile Arg Thr Ala Lys Gly Glu Lys Phe	325	330	335
Val Met Gln Glu Glu Phe Ser Arg Asp Gly Lys Ala Leu Glu Arg Phe	340	345	350
Leu Gln Asp Tyr Phe Asp Gly Asn Leu Lys Arg Tyr Leu Lys Ser Glu	355	360	365
Pro Ile Pro Glu Ser Asn Asp Gly Pro Val Lys Val Val Val Ala Glu	370	375	380
Asn Phe Asp Glu Ile Val Asn Asn Glu Asn Lys Asp Val Leu Ile Glu	385	390	395
Phe Tyr Ala Pro Trp Cys Gly His Cys Lys Asn Leu Glu Pro Lys Tyr	405	410	415
Lys Glu Leu Gly Glu Lys Leu Ser Lys Asp Pro Asn Ile Val Ile Ala	420	425	430

-continued

---

Lys Met Asp Ala Thr Ala Asn Asp Val Pro Ser Pro Tyr Glu Val Arg  
 435 440 445

Gly Phe Pro Thr Ile Tyr Phe Ser Pro Ala Asn Lys Lys Leu Asn Pro  
 450 455 460

Lys Lys Tyr Glu Gly Gly Arg Glu Leu Ser Asp Phe Ile Ser Tyr Leu  
 465 470 475 480

Gln Arg Glu Ala Thr Asn Pro Pro Val Ile Gln Glu Glu Lys Pro Lys  
 485 490 495

Lys Lys Lys Lys Ala Gln Glu Asp Leu  
 500 505

<210> SEQ ID NO 3  
 <211> LENGTH: 654  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Met Lys Leu Ser Leu Val Ala Ala Met Leu Leu Leu Leu Ser Ala Ala  
 1 5 10 15

Arg Ala Glu Glu Glu Asp Lys Lys Glu Asp Val Gly Thr Val Val Gly  
 20 25 30

Ile Asp Leu Gly Thr Thr Tyr Ser Cys Val Gly Val Phe Lys Asn Gly  
 35 40 45

Arg Val Glu Ile Ile Ala Asn Asp Gln Gly Asn Arg Ile Thr Pro Ser  
 50 55 60

Tyr Val Ala Phe Thr Pro Glu Gly Glu Arg Leu Ile Gly Asp Ala Ala  
 65 70 75 80

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

Arg Leu Ile Gly Arg Thr Trp Asn Asp Pro Ser Val Gln Gln Asp Ile  
 100 105 110

Lys Phe Leu Pro Phe Lys Val Val Glu Lys Lys Thr Lys Pro Tyr Ile  
 115 120 125

Gln Val Asp Ile Gly Gly Gly Gln Thr Lys Thr Phe Ala Pro Glu Glu  
 130 135 140

Ile Ser Ala Met Val Leu Thr Lys Met Lys Glu Thr Ala Glu Ala Tyr  
 145 150 155 160

Leu Gly Lys Lys Val Thr His Ala Val Val Thr Val Pro Ala Tyr Phe  
 165 170 175

Asn Asp Ala Gln Arg Gln Ala Thr Lys Asp Ala Gly Thr Ile Ala Gly  
 180 185 190

Leu Asn Val Met Arg Ile Ile Asn Glu Pro Thr Ala Ala Ala Ile Ala  
 195 200 205

Tyr Gly Leu Asp Lys Arg Glu Gly Glu Lys Asn Ile Leu Val Phe Asp  
 210 215 220

Leu Gly Gly Gly Thr Phe Asp Val Ser Leu Leu Thr Ile Asp Asn Gly  
 225 230 235 240

Val Phe Glu Val Val Ala Thr Asn Gly Asp Thr His Leu Gly Gly Glu  
 245 250 255

Asp Phe Asp Gln Arg Val Met Glu His Phe Ile Lys Leu Tyr Lys Lys  
 260 265 270

Lys Thr Gly Lys Asp Val Arg Lys Asp Asn Arg Ala Val Gln Lys Leu  
 275 280 285

Arg Arg Glu Val Glu Lys Ala Lys Arg Ala Leu Ser Ser Gln His Gln  
 290 295 300



That which is claimed is:

1. A method of screening for or diagnosing liver cancer in a subject, comprising:

(a) collecting a blood or blood fraction sample from said subject; and then

(b) detecting cleavage products of PDIA3 in said sample, cleavage of PDIA3 indicating said subject is afflicted with liver cancer.

2. The method of claim 1, wherein said blood sample comprises blood serum.

3. The method of claim 1 wherein said liver cancer is hepatocellular carcinoma.

4. The method of claim 1, wherein said cleavage is as compared to a control sample, with said control sample taken from the same or different subject.

5. The method of claim 1, wherein said subject is afflicted with chronic viral hepatitis, cirrhosis, or both.

6. The method of claim 1, wherein said detecting step is carried out by immunoassay.

7. The method of claim 1, further comprising the step of detecting an elevation of alpha fetoprotein in said sample, the presence of said elevation further indicating said subject is afflicted with liver cancer.

8. A method of screening for or diagnosing liver cancer in a subject, comprising:

(a) collecting a blood or blood fraction sample from said subject; and then

(b) detecting cleavage products of PDIA3 and an elevation of alpha fetoprotein in said sample; cleavage of PDIA3 and the presence of said elevation indicating said subject is afflicted with liver cancer.

9. The method of claim 8, wherein said blood sample comprises blood serum.

10. The method of claim 8, wherein said liver cancer is hepatocellular carcinoma.

11. The method of claim 8, wherein said cleavage is as compared to a control sample, with said control sample taken from the same or different subject.

12. The method of claim 8, wherein said subject is afflicted with chronic viral hepatitis, cirrhosis, or both.

13. The method of claim 8, wherein said detecting step is carried out by immunoassay.

14. A method of screening for or diagnosing liver cancer in a subject, comprising:

(a) collecting a blood or blood fraction sample from said subject; and then

(b) detecting a cleavage product of PDIA3 containing amino acid residues 148-273 in said sample; the cleaved PDIA3 product indicating said subject is afflicted with liver cancer.

15. The method of claim 14, wherein said blood sample comprises blood serum.

16. The method of claim 14, wherein said liver cancer is hepatocellular carcinoma.

17. The method of claim 14, wherein said cleavage is as compared to a control sample, with said control sample taken from the same or different subject.

18. The method of claim 14, wherein said subject is afflicted with chronic viral hepatitis, cirrhosis, or both.

19. The method of claim 14, wherein said detecting step is carried out by immunoassay, chromatography, or spectrometry.

20. The method of claim 14, further comprising the step of detecting an elevation of alpha fetoprotein in said sample, the presence of said elevation further indicating said subject is afflicted with liver cancer.

21. The method of claim 1, wherein said detecting step is carried out by chromatography.

22. The method of claim 1, wherein said detecting step is carried out by spectrometry.

23. The method of claim 8, wherein said detecting step is carried out by chromatography.

24. The method of claim 8, wherein said detecting step is carried out by spectrometry.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 8,067,152 B2  
APPLICATION NO. : 11/708202  
DATED : November 29, 2011  
INVENTOR(S) : Beretta

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

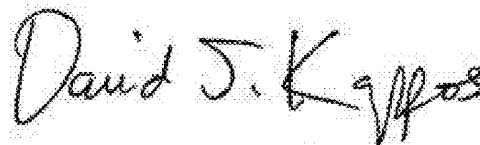
In the Patent:

Column 8, Line 51: Please correct "addition of IN"  
to read -- addition of 1N --

Column 11, Table 2, Headers Spot and Name: Please correct the Table entry  
immediately after 438 by inserting -- 439 -- as shown below.  
(Table headers are included for clarification)

Spot	Name	MS		Uni- gene	Swiss- prot
		Identification (Peptide Matched)	Calculated MW / pI		
438	Alpha-1-antitrypsin	3	20 / 6.0	20 / 6.0	Hs.297681 P01009
439	Liver carboxylesterase	2	62 / 6.5	22 / 6.2	Hs.76688 P23141

Signed and Sealed this  
Twenty-eighth Day of February, 2012



David J. Kappos  
Director of the United States Patent and Trademark Office

专利名称(译)	肝癌生物标志物		
公开(公告)号	<a href="#">US8067152</a>	公开(公告)日	2011-11-29
申请号	US11/708202	申请日	2007-02-20
[标]申请(专利权)人(译)	弗莱德哈钦森癌症研究中心		
申请(专利权)人(译)	Fred Hutchinson癌症研究中心		
当前申请(专利权)人(译)	美国国立卫生研究院 ( NIH ) , 美国DEPT.卫生与人类服务部 ( DHHS ) , 美国政府的		
[标]发明人	BERETTA LAURA		
发明人	BERETTA, LAURA		
IPC分类号	C12Q1/00 G01N33/53 G01N33/574		
CPC分类号	G01N33/57438		
优先权	60/777087 2006-02-27 US		
其他公开文献	US20070202496A1		
外部链接	<a href="#">Espacenet</a> <a href="#">USPTO</a>		

#### 摘要(译)

通过检测来自受试者的样品 ( 例如血液样品 ) 中的标记物的切割来进行检测受试者是否患有肝癌或患有增加的肝癌风险的方法。合适的标记包括但不限于钙网蛋白, 钙网蛋白前体, 蛋白质二硫键异构酶家族A成员3 ( PDIA3 ) 及其裂解产物。切割或切割程度可以与对照样品中发现的相比较。

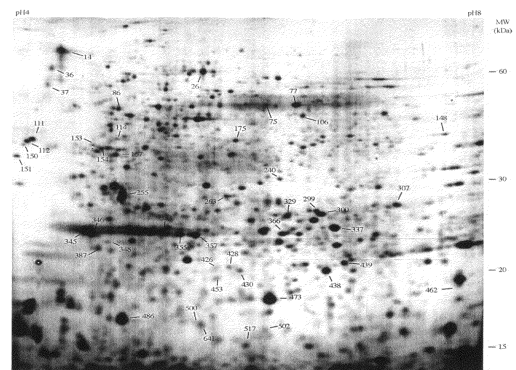


Figure 1.  
2-D pattern of human liver proteins.