GASTROENTEROLOGY 2006;130:2010–2022

Cleavage of Endoplasmic Reticulum Proteins in Hepatocellular Carcinoma: Detection of Generated Fragments in Patient Sera

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Background & Aims: In the past decade, there has been a rising incidence of hepatocellular carcinoma (HCC) and a progressive increase in HCC-related mortality in the United States and Western Europe. The poor survival of patients with HCC is largely related to the lack of reliable tools for early diagnosis. _Methods:_ We have applied proteomics tools to the comparative analysis of protein profiles between HCC and adjacent nontumor tissues as a means for discovering novel molecular markers. _Results:_ Forty-seven protein spots that showed reproducible variation were identified by mass spectrometry, corresponding to 23 distinct genes. A positive correlation between transcript and protein level variations was observed for only 7 out of the 23 genes. Proteolytic cleavage accounted for the discrepancies between messenger RNA and protein level changes for 7 genes including calreticulin, PDIA3, PDI, and GRP78. We detected a fragment of each of these 4 endoplasmic reticulum proteins in the culture supernatant of the PLC-PRF5 hepatoma cell line, suggesting that their cleavage leads to release of selected cleaved products in the extracellular compartment. We also detected calreticulin and PDIA3 cleavage products in sera of patients with HCC. A statistically highly significant difference in calreticulin and PDIA3 fragment serum levels between patients with HCC and healthy individuals was observed. Amounts of calreticulin and PDIA3 fragments were also significantly different between patients with HCC and at-risk patients (patients with chronic hepatitis or cirrhosis). _Conclusions:_ Specific isoforms in general and cleavage products in particular should therefore be further evaluated as new markers for HCC.

Hepatocellular carcinoma (HCC), the major histologic 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.[1] The etiology of HCC is mainly associated with hepatitis B virus (HBV) or hepatitis C virus (HCV) chronic infection.[2] 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.[3,4] 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%.[3] 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 α-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).[5] However, the usefulness of AFP as a marker has been overshadowed by its inability to diagnose early-stage tumors efficiently[6,7]; 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 serologic markers such as α-1-antitrypsin,[8] des-γ-carboxyprothrombin,[9,10] glycipan-3,[11] or isoenzymes of γ-glutamyltransferase.[12,13]

The discovery of new molecular targets for HCC diagnostics and therapeutics has the potential to change significantly the clinical outcome of this disease. Proteomics promises the discovery of biomarkers for early HCC detection and diagnosis.[14] Proteomics-based profiling uniquely allows delineation of global changes in expression patterns resulting from transcriptional and posttranscriptional control, posttranslational modifications, and shifts in proteins between cellular compartments. Therefore, we have applied proteomics tools to the comparative analysis of protein profiles between HCC

Abbreviations used in this paper: AFP, α-fetoprotein; ER, endoplasmic reticulum; ESI/Q-TOF MS/MS, electrospray ionization quadrupole time of flight tandem mass spectrometry; HCC, hepatocellular carcinoma; MW, molecular weight; PDI, protein disulfide isomerase.

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0016-5085/06/$32.00
doi:10.1053/j.gastro.2006.02.058

and adjacent nontumor liver tissues as a means for discovering novel molecular markers.

Materials and Methods

Tumor Tissues, Sera, and Cell Line

Tumor and nontumor counterpart tissues were obtained from 7 patients with HCC. Following excision, the tissues were immediately frozen at −80°C. The tumoral or nontumoral localizations of samples used for protein extraction were verified histologically. Histologic analysis of nontumor 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 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 nontumor tissues were solubilized in lysis buffer containing 9.5 mol/L urea (Bio-Rad Laboratories, Hercules, CA), 2% Nonidet P-40 (BDH Chemicals, Poole, Dorest, UK), 2% carrier ampholytes, pH 4–8 (Gallard-Schlesinger Industries, Plainview, NY), 2% β-mercaptoethanol, and 10 mmol/L phenylmethanesulfonyl fluoride. Proteins (200 μg) 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 mmol/L 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, NY) was used. Proteins were visualized by a photochemical silver-based staining technique.

Protein Spot Detection and Quantitation

After the 2-dimensional (2-D) separation, each gel was scanned using a Kodak CCD camera (Eastman Kodak Company, Rochester, NY). A 1024 × 1024 pixel format was used, yielding pixel widths of 163 μm in which each pixel had 256 possible gray-scale values (optical density). Spot detection was accomplished by Bio Image Visage System software (Bioimage, Ann Arbor, MI). The background-subtracted integrated intensity of each spot was obtained in optical density units multiplied by mm^2. A set of 999 selected spots from each gel was matched to the spots on a master gel.[15] A total of 250 spots was 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.[16]

In-Gel Enzymatic Digestion and Mass Spectrometry

Additional 2-D gels were silver stained by successive incubations in 0.02% sodium thiosulfate for 2 minutes, 0.1% silver nitrate for 40 minutes, and 0.014% formaldehyde plus 2% sodium carbonate. The protein spots of interest were excised from the 2-D gels and destained for 10 minutes in 15 mmol/L potassium ferricyanide and 50 mmol/L sodium thiosulfate. Following 3 washes with water, the gel pieces were dehydrated in 100% acetonitrile for 5 minutes and dried for 20 minutes in a vacuum centrifuge. Digestion was performed by addition of 100 ng trypsin (Promega Corporation, Madison, WI) in 200 mmol/L ammonium bicarbonate. Following overnight enzymatic digestion at 37°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 ionization quadrupole time of flight tandem mass spectrometry (ESI/Q-TOF MS/MS) in the Q-Tof micro mass spectrometer (Waters Corporation, Milford, MA). 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 nonredundant SwissProt protein sequence database using the ProteinLynx global server (available at www.waters.com). The molecular weight (MW) and isoelectric point (pI) of identified proteins were calculated using Compute pI/Mw software tool from ExPASy (http://us.expasy.org/tools/pi_tool.html).

2-D, 1-D, and Dot Immunoblotting

Proteins separated by 2D-polyacrylamide gel electrophoresis (PAGE) were transferred to polyvinylidene difluoride (PVDF) membranes. Immunoblotting was then performed with antibodies against calreticulin, SPA-600 (StressGen Biotechnologies Corporation, Victoria, Canada), and T-19 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with horseradish peroxidase–conjugated anti-rabbit (Amersham Biosciences, Piscataway, NJ) 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 mmol/L Tris-HCl, pH 7.5, buffer containing 5 mmol/L EDTA and 100 mmol/L KCl. Proteins (50 μg) 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 μg) and cell culture supernatant (5–25 μL) were blotted onto nitrocellulose membranes, using a dot-blot apparatus (Schleicher & Schuell, Keene, NH). The following antibodies were used: anticalreticulin antibodies, PA3-900 (Af-

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