A Distinct Repertoire of Autoantibodies in Hepatocellular Carcinoma Identified by Proteomic Analysis*

François Le Naour‡§, Franck Brichory¶, David E. Misek¶, Christian Bréchot¶, Samir M. Hanash¶, and Laura Beretta‡**

Chronic infections with hepatitis B (HBV) and hepatitis C (HCV) viruses are major risk factors for hepatocellular carcinoma (HCC). We have utilized a proteomic approach to determine whether a distinct repertoire of autoantibodies can be identified in HCC. Sera from 37 patients with HCC and 31 subjects chronically infected with HBV or HCV without HCC were investigated. Sera from 116 patients with other cancers, three patients with systemic lupus erythematosus, and 24 healthy subjects were utilized as controls. We report the identification of eight proteins, for each of which autoantibodies were detected in sera from more than 10% of patients with HCC but not in sera from healthy individuals (p < 0.05). Autoantibodies to four of these proteins were detected at a comparable frequency in sera from patients with chronic hepatitis. The other four proteins, which consisted of calreticulin isoforms, cytokeratin 8, nucleoside diphosphate kinase A, and F1-ATP synthase β-subunit, induced autoantibodies among patients with HCC, independently of their HBV/HCV status. Calreticulin, and a novel truncated form of calreticulin (Crt32) we have identified, most commonly elicited autoantibodies among patients with HCC (27%). We conclude that a distinct repertoire of autoantibodies is associated with HCC that may have utility in early diagnosis of HCC among high risk subjects with chronic hepatitis. Molecular & Cellular Proteomics 1:197–203, 2002.

There is increasing evidence for an immune response to cancer in humans, demonstrated in part by the identification of autoantibodies to tumor antigens (1–4). The identification of panels of tumor antigens that elicit a humoral response may have utility in cancer screening, diagnosis, or in establishing prognosis. Such antigens may also have utility in immunotherapy against the disease. Several approaches are currently available for the identification of tumor antigens. We have implemented a proteomic-based approach for the identification of tumor antigens that induce an antibody response, which we have applied to hepatocellular carcinoma (HCC), a major type of cancer worldwide (5).

HCC has a poor prognosis, with 5-year survival rates of less than 5%. Most cases of HCC are associated with cirrhosis (at least 90% in America and Europe) (6). Chronic infections with hepatitis B (HBV) and C (HCV) viruses are major risk factors for HCC, and development of a chronic carrier state is a most frequent event following acute viral infection (7–10). The most likely explanation for the rising incidence of HCC is the spread of hepatitis virus in the population. Antigens that have been shown to induce a humoral response in HCC include p53 (11, 12) and diverse other nuclear proteins (13–17). Autoantibodies to cyclin B1 (18) and to a novel cytoplasmic protein with RNA binding motifs (19) have also been reported. A SEREX (serological analysis of recombinant cDNA expression libraries) study of hepatocellular carcinoma has uncovered reactivity to diverse proteins involved in the transcription/translational machinery, as well as to chaperone proteins (20).

In contrast to approaches for identification of tumor antigens, based on the analysis of recombinant proteins, the proteomic approach we have utilized allows identification of autoantibodies to proteins in lysates prepared from tumors and tumor cell lines and thus may more readily uncover antigenicity associated with post-translational modification. We report the identification of eight proteins that elicited a humoral response in HCC patients but not in healthy individuals, based on our analysis of 37 patients with HCC and additional controls. Among these eight proteins, a truncated form of calreticulin, Crt32, was found to commonly induce autoantibodies in hepatocellular carcinomas. Crt32 autoantibodies were largely restricted to HCC.

EXPERIMENTAL PROCEDURES

Sera, Tumor Tissues, and Cell Lines—Sera were obtained at the time of diagnosis from 37 patients with HCC, following informed consent. This group consisted of 30 males and seven females with an age range of 20 to 66 (mean = 59 years). Clinical characteristics of the

* The abbreviations used are: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; SLE, systemic lupus erythematosus; 2-D, two-dimensional; NDPKA, nucleoside phosphatase kinase A; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight.
Tumor Antigens in Liver Cancer

37 patients are presented in Table I. HBV and HCV status was determined by combining serological assays for HBsAg and anti-HCV plus PCR for HBV DNA and HCV RNA. Sera from 116 patients with other cancers, consisting of 24 with breast cancer, 52 with lung cancer, 16 with brain tumor, 7 with melanoma, and 17 with esophageal cancer, from 31 subjects chronically infected with HBV or HCV without HCC, from three patients with active systemic lupus erythematosus (SLE), and from 24 healthy individuals, were utilized as controls. Tumor and non-tumor counterpart tissues were obtained from patients with HCC. Following excision, the tissues were immediately frozen at −80 °C. The human hepatoma cell lines PLC-PRF5 and Huh7 were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin (Invitrogen).

**Two-dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)**—The procedure followed was as described previously (21). Cultured cells and tumor and non-tumor tissues were solubilized in lysis buffer containing 9.5 M urea (Bio-Rad), 2% Nonidet P-40, 2% carrier ampholytes, pH 4–8 (Gallard/Schlessinger, Carle Place, NY), 2% β-mercaptoethanol, and 10 mM phenylmethanesulfonyl fluoride. Protein concentration was measured with the Bradford assay (Bio-Rad). Proteins (175 μg) were applied onto isofocusing gels. Isoelectric focusing was conducted using pH 4 to 8 carrier ampholytes at 700 V for 16 h, followed by 1000 V for an additional 2 h. 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 bromphenol blue. For the second-dimension separation, a gradient of 11 to 14% acrylamide (Serva; Crescent Chemical, Hauppauge, NY) was used. Proteins were transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA) or visualized by silver staining of the gels. The silver-stained gels were digitized at 1024 × 1024-pixel resolution using a Kodak CCD camera. When indicated, spots were detected and quantified with Visage software (Genomic Solutions, Ann Arbor, MI) as described (22).

**Western Blotting**—Following transfer, membranes were incubated for 2 h in blocking buffer containing 5% milk in 10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, pH 8, 50 mM NaCl. The membranes were incubated for 2 h with serum obtained either from patients or from controls (1:300 dilution). After three washes, the membranes were incubated with horseradish peroxidase-conjugated anti-human IgG antibody (1:1000 dilution) (Amersham Biosciences). Immunodetection was accomplished by enhanced chemiluminescence (ECL; Amersham Biosciences). Immunoblotting assays for the detection of calreticulin was performed by using antibodies against calreticulin, SPA-600 (StressGen, Victoria, Canada), and T-19 (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100,000 and 1:10,000, respectively. The membranes were then incubated for 1 h with horse-radish peroxidase-conjugated anti-rabbit (Amersham Biosciences) or anti-goat (SIGMA) IgG antibodies at a dilution of 1:1000.

**In-gel Enzymatic Digestion and Mass Spectrometry**—The 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 proteins of interest were excised from the 2-D gels and destained for 5 min in 15 mM potassium ferricyanide and 50 mM sodium thiosulfate as described (23). Following three washes with water, the gel pieces were dehydrated in 100% acetoneitrile for 5 min and dried for 30 min in a vacuum centrifuge. Digestion was performed by addition of 100 ng of trypsin (Promega, Madison, WI) in 200 mM ammonium bicarbonate. Following enzymatic digestion overnight at 37 °C, the peptides were extracted twice with 50 μl of 60% acetonitrile/1% trifluoroacetic acid. After removal of acetoneitrile by centrifugation in a vacuum centrifuge, the peptides were concentrated by using pipette tips C18 (Millipore, Bedford, MA).

Analyses were performed primarily using a PerSeptive Biosystems matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) Voyager-DE mass spectrometer (Framingham, MA), operated in delayed extraction mode. Peptide mixtures were analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in acetonitrile containing 1% trifluoroacetic acid (Sigma). Peptides were selected in the mass range of 800–4000 Da. Spectra were calibrated using calibration mixture 2 of the Sequazyme peptide mass standards kit (PerSeptive Biosystems, Framingham, MA). The search program MS-Fit, developed by the University of California at San Francisco (prospect.uchc.edu), was used for searches in the NCBi data base. Search parameters were as follows: maximum allowed peptide mass error of 400 ppm, consideration of one incomplete cleavage per peptide, and a pI range between 4 and 8.

**RESULTS**

**Occurrence of Autoantibodies in Sera from Patients with HCC**—The presence of autoantibodies in sera from patients with HCC was initially investigated using two hepatoma cell lines as antigen sources. Proteins extracted from the two cell lines (PLC-PRF5 and Huh7) were separately subjected to 2-D PAGE and transferred onto Immobilon-P membranes. Initially, sera from 37 patients with HCC (see Table I) and from 24 healthy subjects were screened individually by Western blot analysis, in which each serum was incubated with one blot of PLC-PRF5 and a separate blot of Huh7 proteins. A secondary anti-IgG antibody was utilized to detect reactive proteins. In general, a greater number of reactive proteins were detected with sera from patients with HCC than with control sera (Fig. 1). Proteins that exhibited selective reactivity with sera from HCC patients (p < 0.05) were targeted for further characterization. This set consisted of 13 protein spots that exhibited reactivity with at least four (11%) of HCC sera (Table II). Fig. 2 shows the position of the 13 protein spots in 2-D patterns of Huh7 and PLC-PRF5 cells. Among the 13 proteins, eight were common to both PLC-PRF5 and Huh7 cell lines. Two reactive proteins were observed only in PLC-PRF5 and three only in Huh7 extracts, reflecting differences in the protein expression profiles between the two cell lines. In all cases, sera that reacted to a protein in one cell line also reacted with the same protein in the other cell line, if detected in the silver-stained 2-D patterns. In all, 27 of the 37 HCC sera (73%) showed reactivity against at least one of the 13 proteins, and 17 of the 37 sera (46%) showed reactivity against at least two of the 13 proteins.

**Identification of Reactive Proteins**—For protein identification, additional 2-D gels were produced with Huh7 and PLC-PRF5 lysates and were silver-stained as described under “Experimental Procedures.” The 13 proteins of interest were then excised from the gels, digested with trypsin, and subsequently analyzed by MALDI-TOF mass spectrometry. The resulting spectra were used to identify the proteins, using the MS-FIT search program. Of the 13 spots excised from the gels, 11 were identified (Table II). The proteins identified were members of diverse groups consisting of chaperones (hsp60 and calreticulin), structural proteins (cytokeratin 8, cytokeratin
and \( \beta \)-tubulin), and enzymes (creatine kinase-B, \( F_{1}-\text{ATP synthase} \) \( \beta \)-subunit, and nucleoside phosphate kinase A (NDPKA)). The protein in spot 12 with an estimated molecular mass of 32 kDa and pI of 4.1 was identified as corresponding to a calreticulin isoform by mass spectrometry. Calreticulin is a protein with a molecular mass of 48 kDa and a pI of 4.3. We recently identified and characterized in dendritic cells a truncated form of calreticulin with a molecular mass of 32 kDa, which we designated Crt32 (24). To confirm the identity of

![FIG. 1. Detection of autoantibodies in sera from patients with HCC.](image)

The proteins from the hepatoma cell line PLC-PRF5 were separated by 2-D electrophoresis and subsequently silver-stained (a) or transferred on polyvinylidene difluoride membranes for Western blotting experiments using sera from a healthy individual (b) or from two patients with HCC (c and d) as a first antibody.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Recurrence (%)</th>
<th>Identification</th>
<th>Accession No.</th>
<th>Matching peptides (%)</th>
<th>% of the protein covered</th>
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<tbody>
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<td>1</td>
<td>27</td>
<td>Calreticulin</td>
<td>4757900</td>
<td>7/13 (53%)</td>
<td>23</td>
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<tr>
<td>2</td>
<td>27</td>
<td>Calreticulin</td>
<td>4757900</td>
<td>4/6 (66%)</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>( \beta )-tubulin</td>
<td>2119276</td>
<td>6/6 (100%)</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>Calreticulin</td>
<td>4757900</td>
<td>10/13 (76%)</td>
<td>31</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>hsp60</td>
<td>129379</td>
<td>20/45 (53%)</td>
<td>42</td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>Cytokeratin 18</td>
<td>4557888</td>
<td>5/9 (55%)</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>Cytokeratin 8</td>
<td>87303</td>
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<td>9</td>
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<td>Creatine kinase-B</td>
<td>180570</td>
<td>14/24 (58%)</td>
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<tr>
<td>10</td>
<td>11</td>
<td>( F_{1}-\text{ATP synthase} ) ( \beta ) subunit</td>
<td>114549</td>
<td>18/45 (40%)</td>
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<td>14</td>
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<tr>
<td>12</td>
<td>27</td>
<td>Crt32</td>
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<td>4/6 (66%)</td>
<td>19</td>
</tr>
<tr>
<td>13</td>
<td>14</td>
<td>NDPKA</td>
<td>35068</td>
<td>6/11 (54%)</td>
<td>34</td>
</tr>
</tbody>
</table>

**TABLE II**

*Identification of reactive proteins*

Analysis by MALDI-TOF-MS of the tryptic profiles followed by search in NCBI database. ND, not determined.
spot 12 as Crt32, additional enzymatic digestions were performed, and peptides that exhibited high intensities were analyzed further by electrospray ionization mass spectrometry to obtain amino acid sequence information. Altogether, the results indicated that the protein in spot 12 represented a truncated form of calreticulin identical to Crt32 and corresponding to the C-terminal end (amino acids 157–400) (Fig. 3A). Identification of the protein in spot 12 as the C-terminal portion of calreticulin was confirmed further by Western blotting using two specific antibodies against calreticulin, SPA-600 and T-19 antibodies, produced against a C-terminal and a N-terminal peptide, respectively. The protein spot reacted with the SPA-600 antibody but not with the T-19 antibody (Fig. 3B). These antibodies also confirmed the identification of spots 1, 2, and 5 as other calreticulin isoforms (Fig. 3B). Calreticulin proteins, including Crt32, exhibited the highest frequency of autoantibodies (27%) in sera from patients with HCC.

Frequency of Autoantibodies against the Identified Proteins in Patients with Chronic Hepatitis—Autoantibodies against calreticulin, β-tubulin, cytokeratin 8, creatine kinase-B, F1-ATP synthase β-subunit, and NDPKA were detected in sera from HCC patients with or without HBV and/or HCV infection. In contrast, hsp60 autoantibodies were detected only in sera from HCC patients with HBV and/or HCV infection, and autoantibodies against cytokeratin 18 were detected only in sera from HCC patients with HBV infection. To determine the association between certain autoantibodies and hepatitis virus
infection, we investigated the occurrence of autoantibodies in sera from 31 subjects infected with HBV or HCV (21 and 10, respectively) and showing chronic hepatitis but without HCC. Reactivity against calreticulin proteins, cytokeratin 8, F1-ATP synthase β-subunit, and NDPKA was observed in 2, 1, 1, and 0 subjects, respectively (Table III), at a lower frequency than in patients with HCC. Reactivity against β-tubulin, creatine kinase-B, hsp60, and cytokeratin 18 was observed at a comparable frequency in chronic hepatitis subjects and HCC patients (Table III). Remarkably, reactivity against cytokeratin 18 was restricted to subjects infected with HBV, with or without HCC.

**Specificity of Calreticulin and Crt32 Autoantibodies in HCC**—Calreticulin has been shown to be antigenic in certain autoimmune diseases such as SLE (25). The epitopes eliciting a humoral response have been located in the N-terminal part of the molecule (25). Therefore, we compared autoreactivity to the different forms of calreticulin between sera from HCC patients and sera from three patients with active SLE. Interestingly, reactivity against full-length calreticulin but not against Crt32 was observed in patients with active SLE (Fig. 4), indicating a difference in epitopes that induce autoreactivity in HCC and in SLE.

Autoantibodies against calreticulin and Crt32 were largely restricted to liver cancer patients among the different cancer sera we have analyzed (Table IV). Crt32 autoantibodies were found in sera of two of 24 patients with breast cancer and in two of 52 patients with lung cancer. None of the sera from 16 patients with brain tumor, seven with melanoma, and 17 with esophageal cancer exhibited autoantibodies against calreticulin proteins.

**Crt32 Expression in Liver Tumors**—Given that the occurrence of autoantibodies to Crt32 was restricted to patients with HCC, we examined Crt32 expression in liver tumor and adjacent non-tumor tissue obtained from five patients with HCC. Crt32 levels were measured by 2-D PAGE and silver staining, using a computerized quantitative approach. Interestingly, Crt32 levels were increased in tumor relative to non-tumor counterpart tissue (3.9 ± 1.3-fold; means ± S.E.) (Fig. 5).

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**Table III**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Calreticulin</th>
<th>Crt32</th>
<th>β-Tubulin</th>
<th>Hsp60</th>
<th>Cytokeratin 18</th>
<th>Cytokeratin 8</th>
<th>Creatine kinase-B</th>
<th>F1-ATP synthase β subunit</th>
<th>NDPKA</th>
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<tr>
<td>HCC patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HBV+ (n = 24)</td>
<td>6</td>
<td>7</td>
<td>3</td>
<td></td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
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<tr>
<td>HCV+ (n = 9)</td>
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<td>4</td>
<td>2</td>
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<td>2</td>
<td>1</td>
<td>2</td>
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<tr>
<td>HBV+ HCV+ (n = 11)</td>
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<td>1</td>
<td>0</td>
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<td>0</td>
<td>2</td>
<td>1</td>
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<tr>
<td>Total (n = 37)</td>
<td>10</td>
<td>9</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
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<tr>
<td>Chronic carriers without HCC</td>
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<td>HCV+ (n = 10)</td>
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<td>2</td>
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<tr>
<td>Total (n = 31)</td>
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<td>5</td>
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<td>3</td>
<td>1</td>
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**Table IV**

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<th>Positive/total sera</th>
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<td>Breast</td>
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</tr>
<tr>
<td>Lung</td>
<td>2/52</td>
</tr>
<tr>
<td>Brain</td>
<td>0/16</td>
</tr>
<tr>
<td>Melanoma</td>
<td>0/7</td>
</tr>
<tr>
<td>Esophagus</td>
<td>0/17</td>
</tr>
</tbody>
</table>

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**Fig. 4. Calreticulin autoantibodies in SLE patients.** Close-up sections of Western blots, following 2-D electrophoresis of total PLC-PRF5 protein extract, using sera from patients with HCC or SLE. The different isoforms of full-length calreticulin and Crt32 are indicated by a black line and an arrow, respectively.

**Fig. 5. Crt32 expression in liver tumor tissues.** A, close-up sections of silver-stained 2-D gels of tumor tissue and non-tumor counterpart obtained from a patient with HCC. B, quantitation of c32 expression level from silver-stained 2-D gels after digitalization.
have utilized has identified a diverse set of antigens, with substantial heterogeneity between patients. We identified eight proteins that elicited a humoral response in sera from 70% of the 37 patients investigated, with a frequency for individual proteins ranging from 11 to 27%. Occurrence of autoantibodies against β-tubulin, creatine kinase-B, hsp60, and cytokeratin 18 was also observed in sera of subjects chronically infected with HBV and/or HCV, whereas autoantibodies against calreticulin, cytokeratin 8, F1-ATP synthase β-subunit, and NDPKα were largely restricted to HCC patients. Some of the eight antigenic proteins we have identified were associated previously with autoantibodies in various conditions. Interestingly, the protein F1-ATP synthase β-subunit was reported previously to be antigenic in patients with HCC, by SEREX (20). Three of the reactive proteins with autoantibodies in patients with HCC we have identified represented cytoskeletal proteins, namely β-tubulin and cytokeratin 8 and 18. Anti-tubulin autoantibodies were found previously in sera of patients with neuroectodermal tumors (26), acquired demyelinating polyneuropathies (27), and SLE (28, 29). Circulating anti-cytokeratin 8 antibody immune complexes were reported in sera of patients with pulmonary fibrosis (30). The presence of autoantibodies directed against cytokeratin 18 has been observed in sera from patients with gastric cancer (31). Interestingly, transgenic mice that express a mutant form of cytokeratin 18 develop chronic hepatitis with a disruption of hepatocyte keratin filaments (32). In human, it has been suggested that defects in cytokeratin 18 predispose to cryptogenic cirrhosis (33), and alteration of cytokeratin 18 expression has been reported during tumor transformation in hepatoma (34, 35). The presence of autoantibodies against hsp60 has been reported in sera of patients with Lyme disease or rheumatoid arthritis patients (36, 37), as well as in patients with osteosarcoma (38).

The proteomic approach we have utilized has allowed identification of several forms of calreticulin including Crt32, a novel truncated form, all of which were recognized by autoantibodies in sera of patients with HCC. The protein calreticulin has been identified as an autoantigen in various rheumatic diseases such as rheumatoid arthritis, SLE, Sjögren’s syndrome, celiac disease, congenital heart block, and connective tissue disease (25). However, whereas the epitopes eliciting a humoral response in patients with autoimmune diseases have been reported to be located in the N-terminal part of the molecule, the epitopes eliciting a humoral response in patients with HCC in our study are located in the C-terminal portion. This suggests a specific mechanism of calreticulin processing during hepatocarcinogenesis. Calreticulin is a component of major histocompatibility complex class I peptide loading complex (39), and it has been reported recently that calreticulin elicits tumor- and peptide-specific immunity (40). Calreticulin was reported to be abundant in the nuclear matrix fraction of hepatocellular carcinoma but not in nonmalignant liver tissue (41). We have shown that Crt32 was up-regulated in HCC tumor tissue as compared with the non-tumor counterpart. This overexpression of Crt32 may contribute to the humoral response observed against calreticulin and Crt32 in liver cancer patients.

The proteomic approach we have utilized has uncovered a distinct repertoire of autoantibodies that characterize the humoral response in HCC. The detection of autoantibodies directed against HCC-associated antigens we have identified may have value for HCC screening, diagnosis, or follow-up. Subjects at a particularly high risk for HCC are the chronic HBV and HCV carriers. The identification of autoantibodies associated with the development of HCC will have substantial utility in monitoring this high risk group. Additionally, identification of tumor-associated antigens in HCC may also have utility in antigen-based immunotherapy.

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