Original Research

Identification and differential expression of human carcinoma-associated antigens in hepatocellular carcinoma tissues

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Abstract

This study was designed to identify and verify hepatocellular carcinoma (HCC)-associated human carcinoma antigens (HCAs) that may be useful as tumor markers for HCC. We found that BCE075 and BCD021 anti-HCA antibodies were immunostained in the liver tissue samples and showed specific staining. Their expression was increased in HCC compared with normal liver tissues ($P = 0.008$). Immunoprecipitation and mass spectrometry analyses of the proteins precipitated by these two antibodies were identified to be cytoskeleton-associated protein 4 (CLIMP63) and brain-type glycogen phosphorylase (PYGB). This study demonstrated that HCC tissues expressed specific HCA glycoproteins, suggesting that our mouse monoclonal anti-HCA antibodies could be useful for immunohistochemical analysis of HCA expression as potential biomarkers for HCC diagnosis.

Keywords: human carcinoma-associated antigen, hepatocellular carcinoma, monoclonal antibody, CLIMP63

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Introduction

Liver cancer is one of the most prevalent malignant diseases worldwide, with an estimated 711,000 new cases occurring in 2007, 55% of which reside in China.[1] Approximately 680,000 patients worldwide were shown to have died from liver cancer in 2007.[1] Of these primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological type, and accounts for up to 85% of cases.[1] HCC treatment is mainly through surgery, followed by radiation and chemotherapy, while liver transplantation[2] may offer the only curative procedure; however, organ donors are limited. The risk factors for causing HCC are associated with chronic infection of hepatitis B virus[3,4] or hepatitis C virus (HCV)[5] followed by alcohol consumption, aflatoxin, cirrhosis and type 2 diabetes. These risk factors cause HCC through multifactorial and multistage pathogenesis that leads to the dysregulation of cellular homeostasis and gene mutations and then eventually to HCC development. To date, although many HCC-associated genes and molecular markers have been identified in a study of genomics and proteomics of liver cancers,[6] a search for and identification of novel HCC-associated proteins may offer useful insight into HCC early detection or the study of HCC mechanisms.

Human carcinoma antigens (HCAs)[7] are cell surface mucin-like proteins expressed primarily in cancers of epithelial origin, but generally not present on normal human cells. Thus, identification of HCA may be useful in HCC early detection or predication of prognosis. HCA was discovered in the sera of cancer patients by its cross reactivity with antibodies specific for the murine cell surface mucin epiglycanin (EPGN).[7] Although HCA is similar to EPGN, which is a glycoprotein with a very large molecular weight (up to 750 kDa), the amino acid composition of HCAs isolated from different sources varies significantly, i.e. they are related but not identical.[8] So far, HCA has been shown to be highly expressed in cancers of the bladder, prostate and other organs.[9,10] However, to the best of our knowledge, expression of HCA in HCC has not been reported. In the present study, we isolated different HCAs and then generated monoclonal antibodies against

human HCA with high affinity and specificity. We then used these anti-HCA antibodies to immunostain HCC tissue specimens and identified several HCC-specific HCAs in HCC tissue specimens.

Materials and methods

Patient samples

HCC tissue specimens were recruited from the Pathology Department of Eastern Hepatobiliary Surgery Hospital at the Second Military Medical University (Shanghai, China) between January 2007 and December 2008. Seventy-six HCC specimens and 20 control tissue samples were obtained and the control specimens were from patients who received surgical resection of hepatic hemangiomas. Of the 76 HCC patients, 51 were men and 25 women with a mean age of 63 ± 10.1 years (ranged from 25 to 78). The HCC diagnosis was confirmed using pathological evaluation of surgical tissue specimens. This study was approved by the Eastern Hepatobiliary Surgery Hospital Ethics Committee and the written informed consent was obtained from all the patients prior to enrollment into the study. All tissue samples were collected immediately following resection, transported in liquid nitrogen and stored at -80°C until further study.

Production of anti-HCA IgG antibodies

Immunizations

Ten-week-old female Balb/c mice were immunized using 5–10 subcutaneous injections of 100 μL volumes of approximately 70 μg each of the three HCA glycoprotein compounds (American Egenix Company, Millbrook, NY, USA) emulsified 1:1 with Freund's completed adjuvant for the primary injection, and with Freund's incomplete adjuvant for all subsequent booster injections. Injections were administered every four weeks, and polyclonal antisera were collected at week 5 for immunoassay screening. The blood was then collected from tail veins of the mice and allowed to clot at 4°C for 20 min, and then centrifuged at 4500 *g* for 10 min to sediment the cellular fraction. The serum was collected and frozen at -20°C until assayed using an enzyme-linked immunosorbent assay (ELISA) according to a previously described protocol.[11] During the experiments, only those mice from which the antibody potency exceeded 1:8000 after two immunizations were repeated for booster injections.

Production of hybridoma cell lines

Mice with highly positive HCA antibody titer following ELISA analyses were sacrificed and aseptically splenectomized. Spleenocytes isolated from the mice were used for cell fusion experiments with myeloma cells. The fused cells (300 cells per well) were then plated in 24-well sterile culture plates with 5 mL HAT medium containing hypoxanthine, aminopterin and thymidine, and grown at 37°C under 95% air and 5% CO_2. Two to three weeks later, HAT-resistant cultures were isolated and screened for production of IgG that was specific for HCA. After multiple screening, positive cultures were identified and subsequently cloned, expanded and frozen. The cloning was performed with a limiting dilution (1 cell per well) and microscopically scored to assure monoclonality. Those clones that were screened positively were selected and expanded in RPMI-1640 with 10% fetal bovine serum.[12,13]

Large-scale production of anti-HCA antibodies

To large-scale produce these anti-HCA antibodies, we purchased 10, 6–8-week-old female Balb/c mice weighing 20 g, and intraperitoneally injected 0.5 mL paraffin oil into these mice. One week later, these mice were inoculated with an intraperitoneal injection of 1×10^6 hybridoma cells and left for 10–14 days. Ascitic fluid from the abdominal cavities of the mice was then collected and centrifuged at 2000 rpm for 10 min, and the antibodies were collected and analyzed.

Serological screening of mouse subclones of HCA antibodies

We performed an ELISA to screen mouse serum IgG specific for HCA production in mouse serum and the supernatant of hybridoma subclones according to a previous study.[6] Briefly, each well of flat-bottom 96-well plates for ELISA was coated with 100 μL HCA (1 μg/mL) with the coating buffer that contained 15 mmol/L sodium carbonate and 35 mmol/L sodium bicarbonate in distilled water. The plates were then wrapped with plastic wrap and aluminum foil, stored at 4°C overnight and used within one week. For ELISA, the plates were first blocked with 200 μL/well of blocking buffer for two hours at room temperature. Blocking buffer contained 5% (w/v) skimmed milk powder in Tris-buffered saline (TBS). The plates were then incubated with 100 μL/well of independent triplicate dilutions of animal sera for 30 min at room temperature. After extensive washing with the washing buffer containing 0.1% Tween-20 in TBS using a plate washer, the plates were incubated with 100 μL/well of the secondary antibody for two hours. The secondary antibodies used were alkaline phosphatase-conjugated goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b} or IgG_3 with a dilution at 1:1000 in TBS with 1% bovine serum albumin.[12] After washing, the substrate (0.1 mg/well of *p*-nitro phenyl phosphate) diluted with coating buffer was added into the plates and then the plates were incubated for 20 min in the dark. The reaction was terminated by the addition of 25 μL/well of stopping buffer (5 *N*-sodium hydroxide in distilled water) and absorbance at 450 nm was read using an ELX800 micro plate reader (Maryport, Cumbria, UK).

Protein extraction and Western blotting

Total cellular protein was extracted from cell and tissue samples with lysis RIPA buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate [SDS]). The protein concentration of these samples was measured using the bicinchoninic acid method. Protein lysates were added with or without dithiothreitol (the reduced or non-reduced

state, respectively) then resolved using SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes. The membranes were then blocked in 5% non-fat milk in PBST (phosphate-buffered saline [PBS] + 0.05% Tween-20) and incubated with anti-HCA antibodies with a dilution at 1:1000 in 5% milk in PBST at 4°C overnight. The next day, following three washes in PBST for about five minutes each in triplicate, the blots were incubated with a secondary antibody (goat anti-mouse IgG 1:2000) for 90 min and then washed with PBST in triplicate, and the positive bands were developed using an Odyssey® Infrared Imaging System (Lincoln, NE, USA).

Immunohistochemical staining of HCA protein

Human HCC tissue specimens were obtained surgically, and then fixed in 4% PBS-buffered paraformaldehyde, embedded in paraffin and 2-μm thick sections were stained with hematoxylin–eosin for tumor classification. After microwaving for 30 min for antigen retrieval, tissue sections were incubated with an anti-HCA antibody diluted at 1:100 for 60 min at room temperature, followed by incubation for 30 min with the secondary antibody (RE7112, Novolink Polymer, Wetzlar, Germany). Next, the color was developed in 3,3-diaminobenzidine solution under microscopic observation and counterstained with hematoxylin. The sections stained without the primary antibodies were used as a negative control. The stained sections were reviewed under a light microscope and scored based on the percentage of staining and staining intensity (0, 1+, 2+ and 3+, respectively). The data were recorded as positive (more than 5% tumor cells stained for a staining intensity of 2+ or 3+) and negative only (fewer than 5% tumor cells stained for a staining intensity less than 2+).

Immunoprecipitation analysis of anti-HCA antibodies

Total cellular protein was extracted from HCC tissue specimens and cells and then quantified. For immunoprecipitation (IP), 200–500 μg of total cellular proteins was mixed with 1–2 μg anti-HCA antibodies (clone #BCE075 or BCD021) and incubated at 4°C overnight with continuous rotation. The next day, the antibody–antigen complexes were precipitated using protein G-linked Sepharose (Pharmacia, Stockholm, Sweden) for 30 min and after washing, the beads were re-suspended in a 60 μL sample buffer, and boiled at 95°C for five minutes. The precipitated protein samples were centrifuged at 10,000 *g* for 15 s in a microcentrifuge and loaded onto SDS-PAGE. We then prepared and ran two identical gels under the exact same conditions and after that, one was used for Western blot analysis and another for Coommassie staining and mass spectrometry. These experiments were repeated in triplicate with identical results.

Mass spectrometry and bioinformatical analyses

The proteins immunoprecipitated by these antibodies were the subjected to Mass spectrometry and bioinformatical analyses as described previously[14] with some modifications. Briefly, protein spots were first cut out from the Coommassie-stained gels according to the results of Western blot, distained for 20 min in 30 mmol/L KCN/100 mmol/L $Na_2S_2O_3$ 1:1 (v/v) and washed with Milli-Q water until the gels became clear. The gel with background-only was cut out and used as a negative control. These gel spots were kept in 0.2 mol/L NH_4HCO_3 for 20 min, lyophilized and digested overnight with 12.5 ng trypsin/μL in 0.1 mol/L NH_4HCO_3. The peptides were extracted three times using 50% acetonitrile with 0.1% trifluoroacetic acid and lyophilized. Then 0.5 μL of peptides was mixed with the a-cyano-4-hydroxycinnamic acid matrix (Sigma, St Louis, MO, USA), applied onto the target, air dried and analyzed using a Bruker REFLEX III MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). Protein identification using peptide mass fingerprint was performed with a Mascot search (www.matrixscience.com; MatrixSicence Ltd, London, UK) against the NCBI non-redundant protein database. Errors in peptide masses were in the range of 0.01–0.1%. One missed tryptic cleavage sites per peptide were allowed during the search. Cysteines including carboamidomethylated and methionines were considered as oxidation. Proteins matching more than four peptides and Mascot scores higher than 63 were considered significant ($P < 0.05$).

Quantitative reverse transcription polymerase chain reaction

Total RNA from cells and HCC tissue samples was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was then reversely transcribed into cDNA using a kit from Taqman Company and the cDNA was then subjected to polymerase chain reaction (PCR) analyses of CLIMP63 expression. The primers for CLIMP63 were 5′ TTTTCTCGAGATCTTCACAGAAGTCCAG 3′ (sense) and 5′ TTTTGAATTCTTAGACCTTTTCGTGAATC 3′ (antisense). Primers for the internal control 18S gene were 5′ TAT GGT TCC TTT GGT CGC TC 3′ (sense) and 5′ CTT GGA TGT GGT AGC CGT TT 3′ (antisense). Expected product sizes were 273 and 200 bp, respectively. PCR conditions were an initial 95°C for 4 min and then 45 cycles of 95°C for 40 s, 60°C for 30 s and 68°C for 70 s with a final extension at 72°C for 10 min. In addition, the PCR products were also analyzed in 1% agarose gel electrophoresis followed by ethidium bromide staining for specificity.

Statistical analysis

Descriptive analyses of different variables were performed using SPSS software, version 13.0. (Chicago, IL, USA). Univariate analyses were performed using the χ^2 for categorical variables, and independent samples *t*-test for discrete variables. $P < 0.05$ was considered statistically significant. For each risk factor, the odds ratios of HCC and their 95% confidence intervals were computed as estimates of the

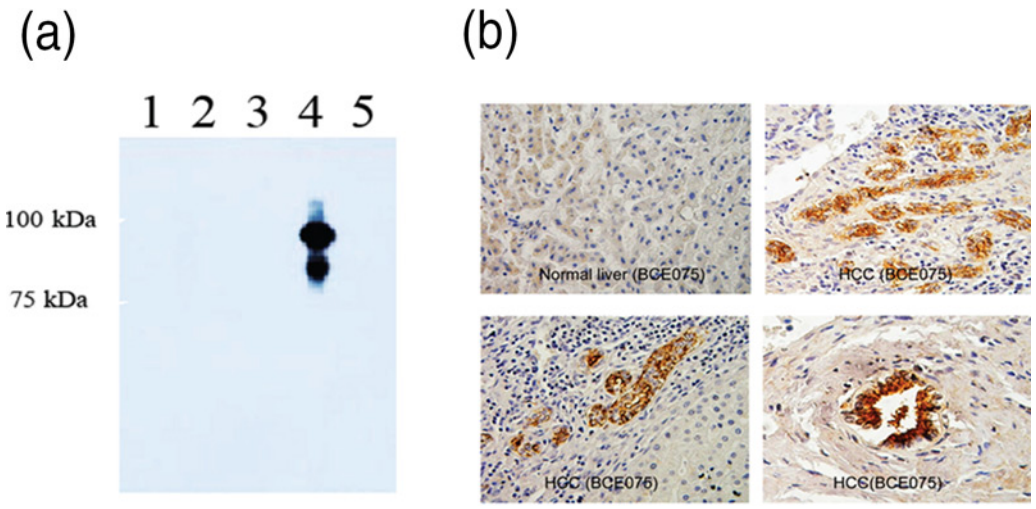

Figure 1 Detection of HCC protein using BCE075 mAb in HCC tissues. (a) Western blot analyses of liver tissues using BCE075 antibody. Western blot data represented one of three experiments with similar results. Lane 1, normal liver (reduced); lane 2, normal liver (non-reduced); lane 3, HCC tissue (reduced); and lane 4, HCC tissue (non-reduced). lane 5, L-02 (b) immunohistochemical staining of HCC tissue sections with BCE075 mAb. HCC, hepatocellular carcinoma. (A color version of this figure is available in the online journal)

relative risks by unconditional logistic regression analyses, using the maximum-likelihood estimates. A Fisher's exact test was used to analyze immunohistochemical data.

Results

Isolation of HCA and production of monoclonal anti-HCA antibodies

In this study, we first isolated and purified three different HCA glycoprotein complexes (American Egenix Company) using HAE_3 affinity chromatography. We then used these gylycoproteins as antigens to generate polyclonal and monoclonal anti-HCA antibodies. A total of 161 hybridomas were obtained, of which 62 were positive against HCAs, and these positive clones were then expanded. The results from ELISA showed that titers of these antibodies against HCAs were more than 1:1000 (representative data shown in Figure S1). Most antibodies tested could recognize both reduced and non-reduced antigens in Western blot gels, while a few antibodies could only recognize non-reduced antigens. Our data indicate that these antibodies can recognize linear and spatial epitopes and that some antibodies recognize deglycosylated proteins, whereas others recognize different epitopes (saccharide or peptide). We also used different glycoproteins as antigens (such as CA199, CA153 and BSM) to perform ELISA analyses using these antibodies. We found that four different clones of these antibodies, i.e. BFB077, BDA025, BCD025 and BEG025 can cross react with these five tumor markers (Supplementary Figure S1).

Using the monoclonal anti-HCA antibodies, we screened our collections of HCC and normal liver tissue specimens and found that some of them are quite specific and the expression was increased in HCC compared with the normal liver tissues (Figures 1 and 2) (Table 1)

Detection of HCA expression in HCC tissues with anti-HCA antibodies (clone #BCE075 and #BCD021)

We chose clone #BCE075 and #BCD021 for immunohistochemical analyses of HCC tissues and found that the proteins recognized by the BCE075 mAb significantly increased in HCC tissues and that the positive immunostaining is localized in the cholangiole and the interlobular bile duct. The proteins recognized by BCD021 mAb also increased in HCC tissues and positive immunostaining was localized in the cytoplasm of HCC cells. The difference in positive staining of HCC versus adjacent normal liver tissue was $P = 0.008$. The difference of HCA expression in these tissues was statistically significant. Also, we found that expression of HCA (BCD021) is associated with tumor size and cancer thrombus, wherever it (BCE075) is associated with HCC differentiation (Tables 2 and 3).

We then performed Western blot analyses using these two antibodies in HCC cell lines and found that HCC HepG2 cells were positive for BCD021 mAb, but L-02 cells were negative. We also immunoprecipitated HCA protein using these two antibodies (i.e. BCE075 and BCD021) for mass spectrometer and bioinformatical analyses. We found that the anti-HCA antibody (clone #BCD021) can recognize a protein with a molecular weight of 60 kDa after the tissue protein isolated from HCC was immunoprecipitated with this antibody in its non-reduced state, while the anti-HCA antibody (clone #BCE075) recognized proteins with the size of 100 kDa and more than 173 kDa (Figures 3a and b). We therefore excised these protein bands from the duplicate gels following Coomassie brilliant blue staining and performed in-gel trypsin digestion and mass spectrometry fingerprinting analysis. We then searched our data against the protein database and found that the mouse monoclonal anti-HCA antibody (clone #BCD021) recognized CLIMP63 protein

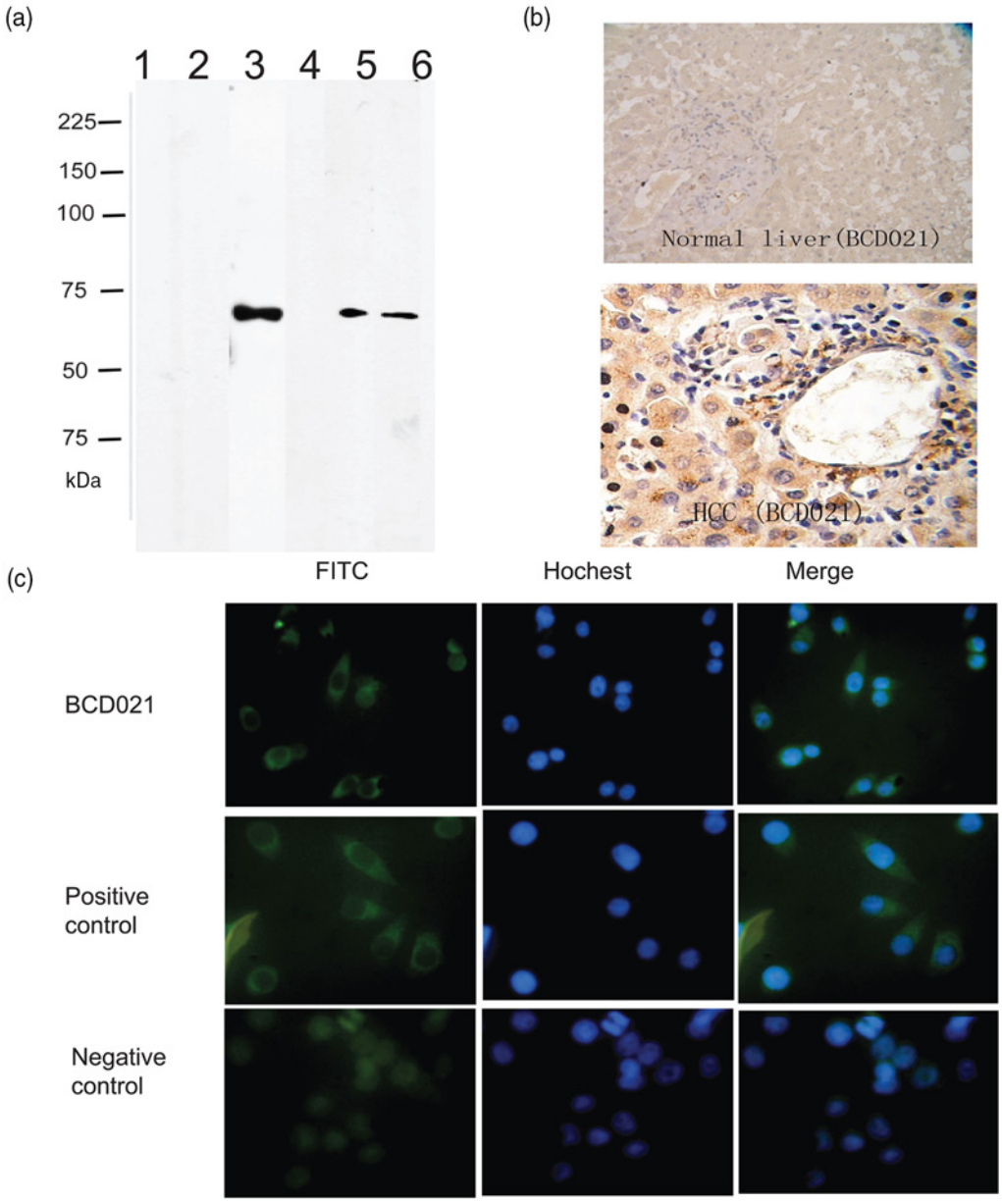

Figure 2 Detection of HCC protein using BCD021 mAb in HCC tissues. (a) Western blot analyses of liver tissues using BCD021 antibody. The experiments were repeated in triplicate with similar results. Lane 1, normal liver (reduced); lane 2, normal liver (non-reduced); lane 3, HCC tissues (reduced); lane 4, HCC tissues (non-reduced); lane 5, HepG2 cells (reduced); lane 6, HepG2 cells (non-reduced); (b) immunohistochemical staining of HCA in liver cancer tissue sections. BCD021 mAb recognized proteins in HCC. (c) Localization of BCD021 in HepG2 cells. HCC, hepatocellular carcinoma. (A color version of this figure is available in the online journal)

and clone BCE075 antibody also bound to CLIMP63 with the brain-type glycogen phosphorylase (glycogen phosphorylase brain, PYGB) complexes.

Detection of CLIMP63 protein by the mouse monoclonal anti-HCA antibody (clone #BCD021)

Since the clone #BCD021 antibody was bound to CLIMP63, we detected levels of CLIMP63 mRNA in liver tissues and HCC cell lines. The data showed that CLIMP63 mRNA was indeed increased in HCC compared with normal liver tissues (Figure 4a). We further examined whether the BCD021 antibody can recognize CLIMP63 by performing three different experiments. First, we co-immunolocalized CLIMP63 protein using the clone #BCD021 antibody and a commercial anti-CLIMP63/p63 antibody in HCC tissues. As expected, both antibodies can co-localize CLIMP63 protein in the cytoplasm of HCC tissues (Figure 4b). CLIMP63 protein is usually presented in the endoplasmic reticulum (ER). In addition, we performed immunohistochemistry using both the anti-CLIMP63 and BCD021 mAbs in HCC tissues and found that 20 cases of HCC tissues were strongly and diffusely stained with both antibodies, whereas the normal liver tissues and bile ducts were negative or weak (Figure 4c). Secondly, co-immunoprecipitation data showed that anti-CLIMP63 antibody-recognized protein could be identified using the BCD021 antibody (Figure 4d). Finally, a plasmid carrying

Table 1 List of mAbs that can recognize normal liver, HCC and HepG2

	Normal liver		HCC		HepG2	
Anti-HCA mAbs	**Reduced**	**Non-reduced**	**Reduced**	**Non-reduced**	**Reduced**	**Non-reduced**
BGG066	+/115 kDa	+/115 kDa	+/115 kDa	+/115 kDa	–	–
BAE086	–	+/200 kDa	–	+/200 kDa	–	–
BCD021	–	–	+/70 kDa	–	+/70 kDa	+/70 kDa
EFC116	–	–	–	+/140 kDa	–	–
BAF112	–	–	–		–	–
BEE055	–	–	–	+/140 KDa/>225 KD	–	–
BEH083	–	–	–	+/140 kDa/>225 KDa	–	–
BBF101	–	–	–	+/140 Kda/>225 KDa	–	–
BCE075	–	–	–	+/140 kDa/>225 KDa	–	–
BEB103	–	–	–	+/140 kDa/>225 KDa	–	–
EDD03	–	–	–	+/140 kDa/>225 KDa	–	–
BCB071	–	–	–	+/140 kDa/>225 KDa	–	–
BFD113	–	–	–	+/140 kDa/>225 KDa	–	–
BFB117	–	–	–	+/140 kDa/>225 KDa	–	–
BAD812	–	–	–	+/140 kDa/>225 KDa	–	–
BBB108	–	–	–	+/>225 kDa	–	–
BAB032	–	–	–	+/>225 kDa	–	–
BAD119	–	–	–	+/>225 kDa	–	–
DAA032	–	–	–	+/>225 kDa	–	–
BDD048	–	–	–	+/>225 kDa	–	–
BFD011	–	–	–	+/>225 kDa	–	–

Table 2 Profiles of patients with BCD021 antibody on HCC

Clinicopathological Variables	BCD021 (−/+)	BCD021 (++/+++)	χ^2	*P* value
Sex			0.92	$P > 0.05$
Male	5	4		
Female	20	41		
Age (year)			0.58	$P > 0.05$
<50 ($n = 11$)	12	4		
≥50 ($n = 49$)	19	35		
HBsAg			0.05	$P > 0.05$
Absent	4	5		
Present	21	40		
Liver cirrhosis			0.18	$P > 0.05$
Absent	5	11		
Present	20	34		
Tumor size (cm)			6.91	$P < 0.01$
≤5	9	2		
>5	20	39		
Tumor number			0.16	$P > 0.05$
Single	28	35		
Multiple	2	5		
Prooperative AFP (ng/mL)			1.61	$P > 0.05$
Negative	10	13		
Positive	28	19		
Cancer thrombus			3.89	$P < 0.05$
Negative	5	16		
Positive	25	26		
TNM			0.33	$P > 0.05$
I and II	5	16		
III and IV	15	34		

TNM, tumor node metastasis; HBsAg, hepatitis B surface antigen; AFP, alphafetoprotein
Fisher's exact tests and χ^2 tests for all the other analysis

CLIMP63 cDNA was constructed and transiently transfected into 293-T cells and the expression of CLIMP63 protein can be detected by both the anti-CLIMP63 and BCD021 antibodies (Figure 4e).

Table 3 Results of immunohistochemical study of liver specimens with BCE075

	HCA (BCE075)	Total
Normal	2	27
Low-differentiation HCC	6	21
Moderatie/high-differentiation HCC	15	21

HCC, hepatocellular carcinoma; HCA, human carcinoma antigen
$\chi^2 = 22.10$, $P < 0.01$

Brain-type glycogen phosphorylase, another protein recognized by both anti-CLIMP63 and BCE075 antibodies

To confirm IP, spectrometer and bioinformatical data, we immune-coprecipitated proteins isolated from HCC tissues with anti-brain-type glycogen phosphorylase (BGP), anti-CLIMP63 and anti-BCE075 antibodies, respectively. Our data showed that a 95-kDa protein was recognized by all three antibodies (Figure 5). These data suggest that BGP and CLIMP63 proteins could be two different components of HCC-associated HCA.

Discussion

To date, there are several methods used to detect HCA expression in HCC cells and tissues. For example, immunohistochemical studies using monoclonal anti-HAE_3 antibody constitute one way to analyze HCA. This antibody has a low affinity and specificity against mouse epiglycanin due to its IgM properties, which makes it less popular in the field of HCC research. Moreover, antibodies against different HCAs could be used as biomarkers for tumor diagnosis.

There are no reports on HCC-associated HCA in the literature. The present study described a strategy to generate monoclonal IgG antibodies for detection of HCA in HCC

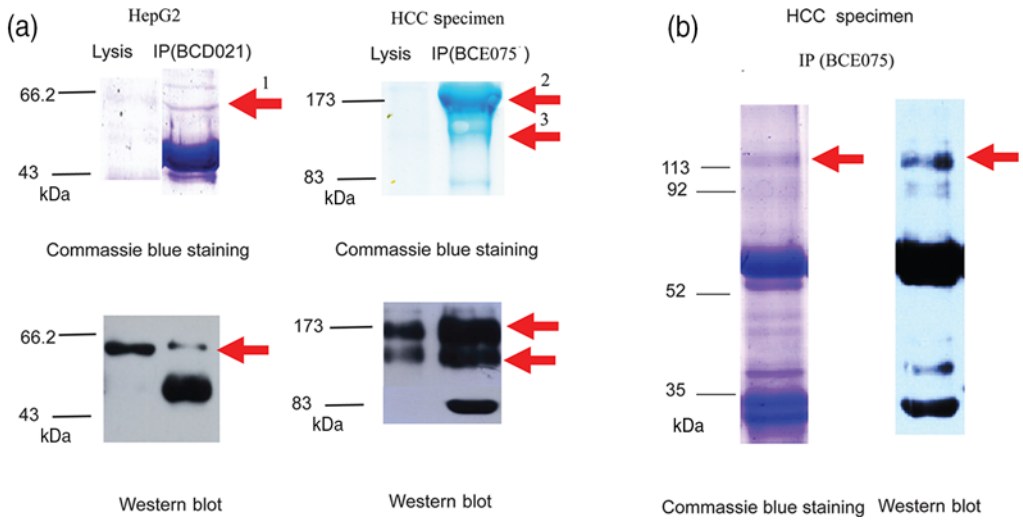

Figure 3 Immunoprecipitation of proteins from HCC tissue and cells using BCD021 and BCE075 mAbs. (a) Immunoprecipitation of proteins from HCC tissue and cells using BCD021 and BCE075 mAbs in a non-reduced state. The left panel shows immunoprecipitation with BCD021 antibody in HepG2 cells. Arrows points to proteins about 44–62 kDa. The right panel shows immunoprecipitation with BCE075 antibody in liver tissues. Arrows point to proteins above 173 band and approximately 83–173 kDa. (b) Immunoprecipitation of proteins from HCC tissue and cells using BCE075 mAbs in reduced state. Arrows indicate that the size of protein is approximately 92–113 kDa. HCC, hepatocellular carcinoma. (A color version of this figure is available in the online journal)

tissue samples. Some of these antibodies against HCA, especially clone #BCE075 and BCD021, were confirmed using Western blot, ELISA and immunohistochemistry and showed high affinity and specificity. The proteins detected by these antibodies were over-expressed in HCC tissues compared with the corresponding normal tissues. We also identified the proteins recognized by two antibodies (i.e., clone #BCE075 and BCD021) were CLIMP63 and PYGB proteins.

There are few serological markers available for early detection of HCC, such as AFP. However, a significant number of patients with HCC do not have elevated alpha-fetoprotein (AFP) levels although AFP does have the value to detect HCC in patients with elevated AFP levels. Thus, additional biomarkers are really needed to increase the sensitivity of HCC detection.[15,16] AFP has only shown the limited sensitivity (41–65%) in the early diagnosis of HCC.[17] In this study, we found that HCA was elevated in 65% patients (clone #BCD021) and 67% patients (clone #BCE075). It may provide a better marker in the early detection of HCC. Moreover, high expression of HCA associated with tumor size and metastasis (clone #BCD021) and the degree of tumor differentiation (clone #BCE075). In addition, if these HCA, in combination with other existing serological biomarkers, can be valuable in the diagnosis of HCC. Based on our results, we suggest HCA with AFP measurements will provide a much better tool for HCC diagnosis than the use of AFP alone.

Why the proteins recognized by the BCE075 mAb are localized in cholangiole and interlobular bile duct remains unclear. Bile capillary is a hollow space formed by two adjacent part of the liver cell membrane, with the diameter of bile capillary variable. It enlarges when exuberant, wherever it collapses when the activity is reduced. The bile produced by liver cells is excreted into the capillary, and then gathered at the interlobular bile duct and hepatic tube. The secretions of multiple hepatoma cells may be easier to detect using these antibodies. Apart from this, Golgi bodies have no strictly accurate control system. Polysaccharides of glycoproteins with defects even severely abnormal glycosylation of glycoproteins can also be output. Thus, glycoprotein in different cells has different structures and composition. These monoclonal antibodies prepared in this study may recognize mature glycoprotein not unmature glycoprotein.[18]

CLIMP63 is a 63-kDa non-glycosylated type II protein that localizes in the ER membrane with an extracytoplasmic segment of 474 amino acids and an NH2-terminal cytoplasmic segment of 106 amino acids.[19] It can be reversibly palmitoylated and phosphorylated to link the ER to the cytoskeleton. Previously, CLIMP-63 protein was identified as a cell surface receptor for tissue plasminogen activator[20] and surfactant protein.[21] It is also a major substrate of the palmitoyl–acyltransferase DHHC2, a putative tumor suppressor, and a receptor for the frizzled-8 protein-related anti-proliferative factor (APF) from interstitial cystitis patients.[22] The role of APF protein was shown to inhibit tumor cell proliferation, migration, and adhesion and to regulate expression of genes involved in cell migration and adhesion.[22,23] However, these changes in cellular behaviors by APF are mediated through high-affinity binding of APF to CLIMP63. After CLIMP63 gene knockdown, it leads to abrogating APF signaling and cellular behavior changes.[22,23] Therefore, over-expression of CLIMP63 in HCC tissues may result in more aggressive tumors. Indeed, a previous study demonstrated that CLIMP63-positive breast cancers were poorly differentiated, hormone receptor-negative neoplasm with a high proliferation rate. CLIMP63 expression also correlated with advanced pathological stage, tumor size and the expression

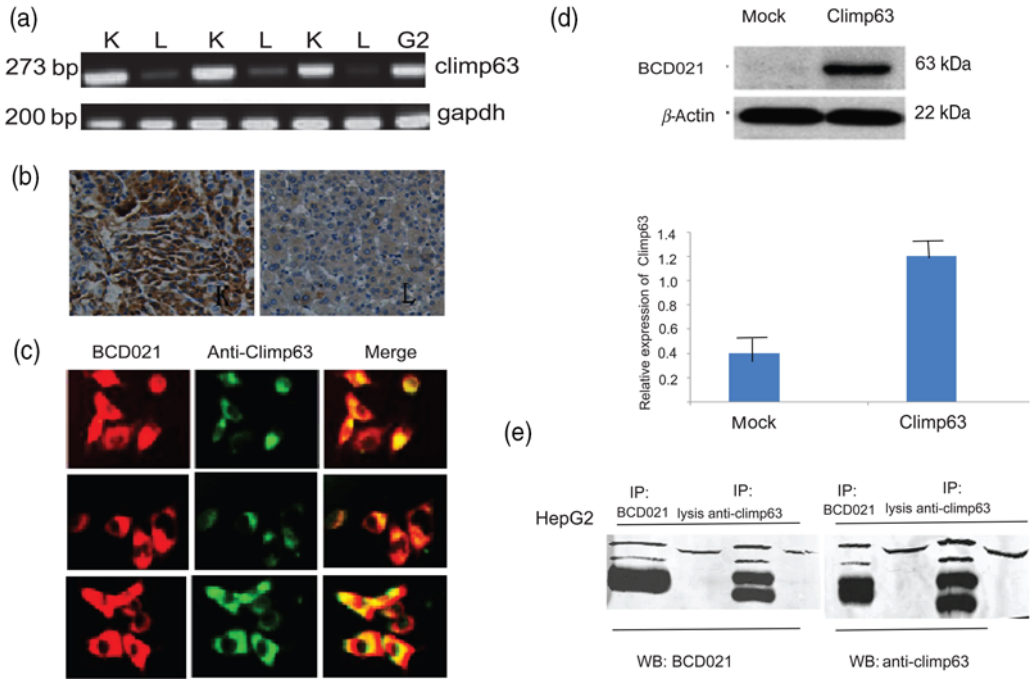

Figure 4 BCD021 can recognize CLIMP63 in liver cancer tissues. (a) CLIMP63 PCR detection on PLC, K: HCC tissues; L: normal liver tissues. G2: HepG2 cells. (b) Immunohistochemical staining of CLIMP63 in liver cancer tissue sections. (c) Confocal microscopy of anti-CLIMP63 and BCD021 in HepG2 cells. Normal HepG2 cell explants were incubated with anti-CLIMP63 and BCD021, and followed by FITC-labeled secondary antibodies. Lane 1, binding of BCD021 followed by FITC-labeled anti-mouse antibodies; lane 2, binding of anti-CLIMP63/p63 followed by FITC-labeled anti-rabbit antibodies; lane 3, overlap of images lanes 1 and 2. Arrows indicate binding of HCA and CLIMP63/P63 at the cell cytoplasm. Expression of CLIMP63 in liver cells in HCC. (a–c) in HCC, CLIMP63 are mainly expressed in liver cancer cells (×400, scale bar 50 μm). CLIMP63 expression was observed staining higher than that in normal liver (d). (d) At 72 h post-transfection, cells were lysed with SDS-PAGE loading buffer. Proteins were analyzed on 10% SDS-PAGE, followed by immunoblotting with antibodies against BCD021 and beta-actins. (b) The 293-T cells were seeded in six-well plates and transfected with CLIMP63 plasmid. At 48 h post-transfection, cells were harvested with TRIzol reagent. Realtime PCR amplifications were performed and the mRNA expression of CLIMP63 was standardized by the amounts of b-actin. Realtime PCR signals were analyzed with LightCycler 3.5 software (Roche Diagnostics, Shanghai, China). (e) Demonstration of human CLIMP63 in HepG2 cell using co-immunoprecipitation. Lane 1, Western blot analyses of immunoprecipitates of total lysates of HepG2 cells by BCD021, primary antibody: BCD021; lane 2, Western blot analyses of HepG2 cells, primary antibody: BCD021; lane 3: Western blot analyses of immunoprecipitates of total lysates of HepG2 cells by anti-climp63, primary antibody: BCD021; lane 4, Western blot analyses of HepG2 cells, primary antibody: anti-climp63; lane 5, Western blot analyses of immunoprecipitates of total lysates of HepG2 cells by anti-climp63, primary antibody: anti-climp63.1; lane 6, Western blot analyses of immunoprecipitates of total lysates of HepG2 cells by BCD021, primary antibody: anti-climp63. PCR, polymerase chain reaction; HCC, hepatocellular carcinoma; FITC, fluorescein isothiocyanate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis. (A color version of this figure is available in the online journal)

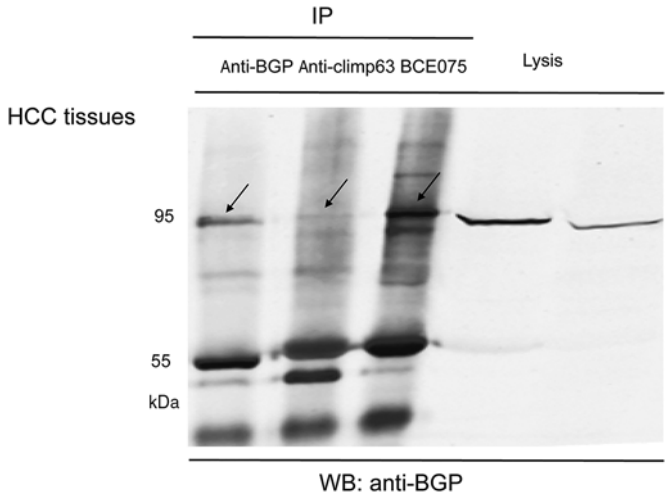

Figure 5 BGP and CLIMP63 can be shown to two components of HCC-associated HCA. HCC, hepatocellular carcinoma; HCA, human carcinoma antigen; BGP, brain-type glycogen phosphorylase

of human telomerase reverse transcriptase, tissue inhibitor of matrix metalloproteinase 1 and vascular endothelial growth factor.[24] In addition, tissue plasminogen activator (tPA) binding to CLIMP63 on the plasma membrane regulates the response of vascular smooth muscle cells to a variety of blood vessel injuries. This is in accordance with the function of HCA suppressing E-cadherin cell adhesion.[25]

In addition, the results from mass spectrometry analysis of proteins immunoprecipitated by BCE075 and BCD021 also includes glycogen phosphorylase and heat shock protein. Hsp70[26] has been reported to bind several proteins involved in energy metabolism, including glycogen phosphorylase, and is associated with glandular intermediate filaments in an ATP-dependent manner. BGP[27] is the major isoform of glycogen phosphorylase found in fetal and neoplastic tissues, and is generally thought to induce glucose supply during an ischemic period. BGP expression[28] in non-small cell lung carcinoma was associated with poorer survival of the patients. In colorectal cancer, expression of BGP[29] is a potentially novel early biomarker.

Glycogen phosphorylase isoenzymes from hepatoma 3924A and from a non-tumorigenic liver cell line express a phosphorylase isoform different from the liver type. These data indicate that expression of these proteins identified by these two antibodies may be associated with HCC development or tumor progression.

In summary, this study demonstrated that HCA compounds identified by our newly produced monoclonal anti-HCA antibodies are identical to CLIMP63 and BGP. However, BCD021 recognizes the protein in HCC tissues in the reduced state but in HCC cells. Like HepG2, BCD021 recognizes the protein in both reduced and non-reduced states. Our further studies will identify proteins in HCC tissues using these 62 antibodies and hopefully, some of them could be used as tumor markers for early detection and prediction of HCC.

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