Screening Serum Biomarkers for Early Primary Hepatocellular Carcinoma Using a Phage Display Technique

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Hepatocellular carcinoma (HCC) occurs mainly in chronically diseased livers following hepatitis B virus (HBV) or hepatitis C virus (HCV) infection. Early detection and diagnosis of HCC would be of great clinical benefit. In this study, we used a random phage display peptide library and sera from early-stage primary HCC patients ($n = 30$) to screen potential serum biomarkers for early primary HCC. Age- and sex-matched patients with HBV and/or HCV infection were used as controls. In the screening phase, 19 out of 20 randomly selected phage clones exhibited specific reaction with purified sera IgG from early primary HCC patients, among them 14 coming from the same phage clone with inserted peptide

sequence RGWCRPLPKGEG (named HC1). In the validation phase, phage ELISA results showed that the positive reaction rate of the HC1 phage clone was 91.4% with the early HCC group ($n = 70$), significantly higher than that with the HBV infection group (20.0%) ($n = 70$), the HCV infection group (12.9%) ($n = 70$), the HBV + HCV infection group (24.3%) ($n = 70$), the cirrhosis group (17.1%) ($n = 70$), and the healthy control group (10.0%) ($n = 70$). In conclusion, the HC1 mimic peptide showed high diagnostic validity for early primary HCC, and thereby could be a candidate serum biomarker for early primary HCC. *J. Clin. Lab. Anal.* 25:402–408, 2011.

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Key words: hepatocellular carcinoma; phage display peptide library; biomarker; diagnostic validity; hepatitis B virus; hepatitis C virus; cirrhosis

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, some 630,000 new cases being diagnosed each year (1). More than 80% of HCC cases are from the Asian and African continents, of which more than 50% cases are from mainland China (2). HCC is the dominant form of primary liver cancer and is histologically and etiologically distinct from other forms of primary liver cancer (3). Approximately 70–90% of patients with HCC have an established background of chronic liver disease and cirrhosis. The primary risk factor for developing HCC is cirrhosis, the major causes of which are hepatitis B virus (HBV) and hepatitis C virus (HCV) infection (4). Indeed, there is evidence to show that 50% of all cases of HCC worldwide are associated with HBV infection, with a further 25% associated with HCV (5).

The prognosis of patients with HCC is generally very poor with a 5-year survival rate of less than 10–15%,

because most of them are diagnosed at their late stage using current strategy (6,7). Surgical resection is still the most used treatment option for the diagnosed HCC patients (8). Therefore, early detection and diagnosis of HCC would be of great clinical benefit. Although ultrasonography and conventional tumor markers such as α -fetoprotein (AFP) and DCP are widely used and important for HCC detection in clinical scenes (9), they still do not provide an entirely satisfactory solution to detect HCC at the early stage. More effective biomarkers for the detection of early HCC are yet to be discovered.

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In 1985, Smith et al. established a method for displaying polypeptides on the surface of filamentous M13-derived bacteriophage (phage) (10). This phage display technique was originally developed to map epitope-binding sites of antibodies by panning random peptide–phage libraries on immobilized immunoglobulins. Since then, phage display has been used as a powerful method to register polypeptide binding with a diverse range of basic biological, as well as technical and medical applications (11,12). In this study, we used a random phage display peptide library and sera from early-stage primary HCC patients to screen potential serum biomarkers for early primary HCC.

MATERIALS AND METHODS

Patients

From August 2008 to February 2011, 100 early HCC patients admitted to Xiangya Hospital of Central South University were recruited to this study and divided into two groups. The first HCC group consisted of 30 primary HCC patients (22 males and 8 females; mean age 52.5 ± 10.2 years). Controls groups for this group of HCC patients included: (1) 30 patients with only chronic HBV infection; (2) 30 patients with only chronic HCV infection; and (3) 30 patients with chronic HBV and HCV dual infection. All control groups were age and sex matched to the HCC group. The first early HCC group and its control groups were used to screen potential early HCC serum biomarkers with a random phage display peptide library. The second HCC group consisted of 70 other primary HCC patients (48 males and 22 females; mean age 50.9 ± 9.3). A new set of age- and sex-matched control groups were recruited for the second group of HCC patients, including: (1) 70 patients with only chronic HBV infection; (2) 70 patients with only chronic HCV infection; (3) 70 patients with chronic HBV and HCV dual infection; (4) 70 cirrhosis patients with chronic HBV or HCV infection; and (5) 70 healthy volunteers. The second early HCC group and its control groups were for validation of the potential early HCC serum biomarkers obtained from screening the first groups. Primary HCC was diagnosed according to the Chinese Society of Liver Cancer criteria (13) and staged according to the Barcelona Clinic Liver Cancer (BCLC) staging system (14). Only BCLC stage A HCC (i.e., early-stage HCC) was included in this study. Pregnant women and patients with other malignant diseases were excluded. The study was approved by the Ethical Committee of Xiangya Hospital, Central South University. After obtaining informed consent, blood samples were obtained from all participants.

Reagents

The phage display peptide library and its host strain *E. coli* ER 2738 were purchased from New England

Biolabs (Ipswich, MA). The library, which is based on a combinatorial library of random peptide 12-mers fused to a minor coat protein (pIII), consists of 2.7×10^9 electroporated sequences and the phage titer is 1.5×10^{13} plaque forming units (pfu)/ml. The QIAprep Spin M13 kit was purchased from Qiagen (Valencia, CA). Serum AFP levels were analyzed with an ELISA kit purchased from GenWay Biotech (San Diego, CA).

Preparation of Sera and Biopanning and Amplification of Phages

Blood samples were allowed to clot and then centrifuged at 4000 rpm for 10 min. All sera were filtrated with microcell filter (Φ 0.22 μ m) to eliminate red blood cell fragments and bacteria, and then frozen at -70°C immediately. The serum pools were obtained from the first group of early HCC patients and its control groups, respectively. IgG from the pools were purified with the ammonium sulphate precipitation method (50–33–33%). Then, the supernatant was dialyzed against phosphate-buffered saline (PBS; pH 7.2). Microtiter wells were respectively coated overnight at 4°C with 100 μ l of purified IgG (100 μ g/ml) from each of the control groups. The plates were blocked with 3% nonfat milk for 2 hr at 37°C , and then washed five times with 0.05% Tween-20 in Tris-buffered saline (PBST). One hundred μ l of diluted random 12-peptide phage display library with a titer of 1.5×10^{11} pfu/ml was added to coated plates. After sequential incubation for 1 hr at room temperature with each of the control IgG-coated plates, the unbound phages were collected and added 100 μ l/well into the early HCC-IgG-coated plate. After incubation for 1 hr at room temperature, the bound phages were eluted with 100 μ l of 0.2 mol/l glycine-HCl (pH 2.2) and neutralized with 1 mol/l Tris-HCl (pH 9.1). The eluted phages were then amplified in the host strain and purified by precipitation for about 4 hr using one-sixth volume of polyethanol glycol (PEG)/NaCl. Another two rounds of affinity selection were carried out in the same way, except that 1:200 and 1:400 sera dilution was added to 100 μ l of diluted phages from the last round. The percentage of enrichment was calculated using the following formula: percentage enrichment of phage clones (%) = (eluted phages/added phages) $\times$ 100%.

Phage Enzyme-Linked Immunosorbent Assay (Phage ELISA)

Ten μ l of eluted phages from the third round of biopanning were added to 200 μ l of the host strain cultured overnight, and incubated for 20 min at 37°C . The transformed host strain cells were then transferred to a culture tube with agarose, quickly vortexed, and immediately poured onto a prewarmed LB plate. After

cooling for 5 min, the plates were inverted and incubated overnight at 37°C. Twenty phage clones were randomly picked the next day. Each picked clone was amplified, purified, and tittered as previously described (15). The ELISA wells were coated with 2×10^{11} phage particles and then blocked with 2% bovine serum albumin in PBS. One hundred microliters of a 1:100 dilution of purified sera IgG from early HCC patients were subsequently added and allowed to incubate for 1 hr at 37°C. The wells were then washed three times with PBS containing 0.05% Tween-20 (PBST). HRP-conjugated goat antihuman IgG antibody was added, and the specifically bound antibodies were visualized by adding 3,3',5,5'-tetramethylbenzidine (TMB). The absorbance value at 450 nm was determined. With the original phage library used as the negative control, phage clones were considered positive when the absorbance value was more than two times that of the negative control.

Phage DNA Sequencing

Positive phage clones in phage-ELISA were precipitated with PEG/NaCl. The phage DNA was extracted for sequencing with the QIAprep Spin M13 kit and sequenced with an ABI PRISM 377 sequencer using the -96 g111 sequencing primer (5'-CCC TCA TAG TTA GCG TAA CG-3') from the Phage Display Peptide Library kit.

Determination of Optimal Reaction Concentration of the Positive Phage and the Early HCC Sera

Chessboard titration was performed with diluted positive peptides (10^{10} pfu/well, 10^{11} pfu/well, 10^{12} pfu/well, 10^{13} pfu/well, and 10^{14} pfu/well) and purified sera IgG from the early HCC patients (1:10, 1:100, 1:200, 1:400, 1:800) to determine the optimal reaction concentrations for the positive phage and the early HCC sera IgG. The plates were coated with 100 μ l of diluted phages overnight at 4°C, and then washed and blocked with 2% BSA. Diluted sera IgG were subsequently added and incubated for 1 hr at 37°C. After incubation with HRP-conjugated goat antihuman IgG antibody for 1 hr at 37°C, TMB was added to each well, and the enzymatic reaction was allowed to proceed for 15 min at room temperature. The absorbance values were determined at 450 nm with a microplate reader. The original phage library and the undiluted purified sera IgG from early HCC patients were used as the negative and positive control, respectively. Each dilution had triplicates in this experiment.

Diagnostic Validity of the Positive Peptide-Phage Clone

Sera from the second early HCC group and its control groups were used to react with the positive

peptide-phage clone using phage ELISA method. The ELISA wells were coated with 10^{11} pfu/well phage particles and incubated with 1:100 diluted sera IgG. Then 1:2,000 diluted HRP-Staphylococcal Protein A (SPA) was added, with TMB as the substrate for coloration. Absorbance values at 450 nm were recorded. With the original phage library used as the negative control, the phage ELISA results were considered positive when the absorbance value was more than two times that of the negative control. Diagnostic validity indicators were calculated using the following formulas: sensitivity = true positive/(true positive + false negative), specificity = true negative/(true negative + false positive), positive predictive value (PPV) = true positive/(true positive + false positive), negative predictive value (NPV) = true negative/(true negative + false negative), accuracy = (true positive + true negative)/(true positive + false negative + true negative + false positive).

Statistical Analysis

Statistical analyses were performed using SPSS for Windows version 13.0. All values were expressed as Mean $\pm$ SD. One-way ANOVA was employed to analyze group difference in means, followed by the least-significance differences method for post hoc pairwise comparisons of means. Correlations were evaluated to identify association between AFP and the absorbance value of the HC1 peptide phage clone in phage ELISA. Categorical variables were compared with Chi-square test. The statistical significance level of this study was set at two-sided $\alpha = 0.05$.

RESULTS

The early HCC groups were age and sex matched with the control groups. There was no significant difference in age, sex ratio, and body mass index among the early HCC groups and its control groups (Tables 1 and 2). As shown in Table 1, all patients in the first early HCC group had HBV (83.3%) and/or HCV infection (23.3%), and 86.7% of the patients had cirrhosis. The cirrhosis rate in the disease control groups was as follows: HBV infection group, 63.3%; HCV infection group, 53.3%; and HBV+HCV infection group, 80.0%. There was no statistically significant difference in the serum AFP level among the first early HCC group and its control groups. Similar results were seen with the second early HCC group and its control groups (Table 2), except that the healthy control group showed significantly lower serum AFP level than the early HCC group and the disease control groups.

As shown in Table 3, the percentage enrichment of phage increased from $2.1 \times 10^{-5}\%$ to $2.2 \times 10^{-2}\%$ after three rounds of biopanning, an enrichment of nearly 1,000 folds. The result indicates a successful affinity

TABLE 1. Baseline Characteristics of the First Group of Early HCC Patients and Its Control Groups

Characteristics (Group 1) ^a	Early HCC	HBV infection	HCV infection	HBV+HCV infection	P
N	30	30	30	30	N/A
Male N (%)	22 (73.3)	22 (73.3)	22 (73.3)	22 (73.3)	1.0
Age years (Mean±SD)	52.5±10.2	52.4±10.2	52.8±10.4	52.7±10.1	1.0
BMI kg/m ² (Mean±SD)	18.0±4.7	19.5±4.2	19.1±4.0	18.6±4.5	0.58
HBV infection N (%)	25 (83.3)	30 (100)	0 (0)	30 (100)	N/A
HCV infection N (%)	7 (23.3)	0 (0)	30 (100)	30 (100)	N/A
Cirrhosis N (%)	26 (86.7)	19 (63.3)	16 (53.3)	24 (80.0)	0.02
Serum AFP µg/l (Mean±SD)	16.5±7.6	12.4±4.5	13.2±5.0	15.9±6.3	0.51

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α -fetoprotein.

^aGroup 1 of early HCC patients and controls was used to screen potential early HCC serum biomarkers with a random phage display peptide library. The *P*-values were derived from one-way ANOVA or χ^2 R $\times$ C contingency table analysis.

TABLE 2. Baseline Characteristics of the Second Group of Early HCC Patients and Its Control Groups

Characteristics (Group 2) ^a	Early HCC	HBV infection	HCV infection	HBV+HCV infection	Cirrhosis	Healthy control	P
N	70	70	70	70	70	70	N/A
Male N (%)	48 (68.6)	48 (68.6)	48 (68.6)	48 (68.6)	48 (68.6)	48 (68.6)	1.0
Age years (Mean±SD)	50.9±9.3	51.2±9.5	50.7±9.4	51.0±9.2	50.8±9.2	50.9±9.2	1.0
BMI kg/m ² (Mean±SD)	18.2±4.3	20.5±3.8	19.9±4.1	18.7±4.2	18.5±4.2	23.4±5.6	0.27
HBV infection N (%)	58 (82.9)	70 (100)	0 (0)	70 (100)	60 (85.7)	0 (0)	N/A
HCV infection N (%)	16 (22.9)	0 (0)	70 (100)	70 (100)	13 (18.6)	0 (0)	N/A
Cirrhosis N (%)	56 (80.0)	43 (61.4)	37 (52.9)	24 (80.0)	51 (72.9)	0 (0)	N/A
Serum AFP µg/l (mean±SD)	15.9±6.6	12.1±4.3	12.8±4.9	13.7±5.5	14.5±6.2	5.8±1.7	0.02

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α -fetoprotein.

^aGroup 2 of early HCC patients and controls were for validation of the potential early HCC serum biomarker obtained from screening group 1. The *P*-values were derived from one-way ANOVA.

TABLE 3. Biopanning Enrichment of Phages

Biopanning	Phages added (pfu/ml)	Phages eluted (pfu/ml)	Enrichment (%) ^a
1	5.8×10^{11}	1.2×10^5	2.1×10^{-5}
2	2.5×10^{12}	1.5×10^8	6.0×10^{-3}
3	1.2×10^{11}	2.6×10^7	2.2×10^{-2}

^aPhage clone enrichment (%) = (eluted phages/added phages) $\times$ 100%.

selection of phage that could specifically bind the sera IgG from early HCC patients. As shown in Figure 1A, purified sera IgG from the early HCC patients had positive reaction with 19 out of 20 randomly picked phage clones. The positive percentage was 95%. The single-strand DNA from the positive phage clones was sequenced. It turned out that 14 clones with high absorbance values (H1, H3, H4, H5, H6, H10, H11, H12, H13, H14, H17, H18, H19, and H20) bear the same inserted DNA sequence 5'-CCT CCC CCA GCA CCT AGG CAA AGG CTT CCC CTC CCC-3' (Fig. 1B). The corresponding peptide sequence was RGWCRPLPKGEG (named HC1). We analyzed the sequence with Nucleotide Blast Software of NCBI and found that it had no significant similarity with other

peptides (score < 50). Chessboard titration was applied to determine the optimal reaction concentrations for the HC1 phage clone and sera IgG from early HCC patients. The optimal coating concentration of the HC1 peptide phage clone was 10^{11} pfu/well and the optimal dilution for sera IgG from early HCC patients was 1:100.

To evaluate the diagnostic validity of HC1, the second early HCC group and its age- and sex-matched control groups were used to react with the HC1 phage clone using phage ELISA. The original phage library was used as the negative control. When the cutoff absorbance value was set at two times to that of the negative control value at 450 nm, phage ELISA showed that the positive reaction rate of the HC1 clone was 91.4% with the early HCC group, significantly higher than that with the HBV infection group (20.0%), the HCV infection group (12.9%), the HBV+HCV infection group (24.3%), the cirrhosis group (17.1%), and the healthy control group (10.0%) (Table 4). The mean absorbance value in the early HCC group was significantly higher than those in the control groups (Table 4).

In comparison with the healthy control group, the sensitivity of the HC1 clone to detect early HCC patients was 91.4%, specificity 90.0%, PPV 90.1%, NPV 91.3%,

Fig. 1. (A) Detection of positive phage clones with phage ELISA. After three rounds of biopanning, 20 phage clones were randomly picked and reacted with sera from early HCC patients in phage ELISA. Phage clones were considered positive when their absorbance values were above the cutoff value, which was set at two times to that of the absorbance value of the negative control value at 450 nm. H1-H20, phage clones; NC, negative control (the original phage library). (B) Inserted DNA sequence in the positive phages. H1, H3, H4, H5, H6, H10, H11, H12, H13, H14, H17, H18, H19, and H20 positive phage clones bear the same inserted DNA sequence 5'-CCT CCC CCA GCA CCT AGG CAA AGG CTT CCC CTC CCC-3'. HCC, hepatocellular carcinoma.

and accuracy 90.7%. In comparison with the disease control groups, the diagnostic validity values of HC1 for detecting early HCC were as the following: when compared with the HBV infection group, sensitivity 91.4%, specificity 80.0%, PPV 82.1%, NPV 90.3%, accuracy 85.7%; when compared with the HCV infection group, sensitivity 91.4%, specificity 87.1%, PPV 87.7%, NPV 91.0%, accuracy 89.3%; when compared with the HBV+HCV infection group, sensitivity 91.4%, specificity 75.7%, PPV 79.0%, NPV 89.8%, accuracy 83.6%; when compared with the cirrhosis group, sensitivity 91.4%, specificity 82.9%, PPV 90.1%, NPV 91.3%, accuracy 90.7%.

In China, the cutoff value of serum AFP level for HCC diagnosis is set at 400 µg/l, according to the guidelines of clinical diagnosis and staging criteria for primary HCC by the Chinese Society of Liver Cancer (13). None of the patients in the first and second early HCC groups showed an AFP level ≥ 400 µg/l. Therefore, in comparison with any of the control groups, the sensitivity of serum AFP to detect early (BCLC-stage A) HCC patients was 0% in this study. The absorbance value of the HC1 clone showed no statistically significant correlation with the level of serum AFP ($r = 0.187$, $P = 0.335$).

DISCUSSION

HCC occurs mainly in chronically diseased livers following HBV or HCV infection (16). Approximately

TABLE 4. Phage ELISA Detection of Binding Between the HC1 Phage Clone and Sera IgG From the Second Group of Early HCC Patients and Its Control Groups

Experimental group	Total number	Absorbance value (mean $\pm$ SD)	Positive number	Positive rate (%)
Early HCC	70	0.839 $\pm$ 0.256	64	91.4 ^b
HBV infection	70	0.335 $\pm$ 0.150 ^a	14	20.0 ^c
HCV infection	70	0.304 $\pm$ 0.122 ^a	9	12.9 ^d
HBV+HCV infection	70	0.352 $\pm$ 0.169 ^a	17	24.3 ^e
Cirrhosis	70	0.316 $\pm$ 0.138 ^a	12	17.1 ^f
Healthy control	70	0.283 $\pm$ 0.115 ^a	7	10.0 ^g

Absorbance values between the early HCC group and the control groups were compared. One-way ANOVA was employed to analyze group difference in means ($F = 45.827$, $P < 0.0001$), followed by the least-significance differences method (LSD) for post hoc pairwise comparisons of means. HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus.

^a $P < 0.05$ compared with the early HCC group.

^bComparison of the positive rates among all groups ($\chi^2 = 160.971$, $P < 0.0001$).

^c $\chi^2 = 72.374$, $P < 0.0001$ compared with the early HCC group.

^d $\chi^2 = 68.588$, $P < 0.0001$ compared with the early HCC group.

^e $\chi^2 = 64.712$, $P < 0.0001$ compared with the early HCC group.

^f $\chi^2 = 77.829$, $P < 0.0001$ compared with the early HCC group.

^g $\chi^2 = 92.848$, $P < 0.0001$ compared with the early HCC group.

80% of all HCC cases throughout the world occur in developing countries, with 55% occurring in China alone (17). In China, 73% of patients with chronic

hepatitis and 71% of those with HCC, are infected with HBV (18). In addition, China alone reportedly has more HCV infections than all of Europe or the Americas (19). Indeed, all the early HCC patients enrolled in this study were infected with HBV and/or HCV (Tables 1 and 2). Thus, in the screening phase of the study, HBV and/or HCV-infected patients were used as disease controls. To increase the screening specificity, microtiter wells were respectively coated with purified sera IgG from the disease control groups to absorb nonspecific phages from the phage random peptide library, and the unbound phages were then incubated with sera IgG from early HCC patients. Because a large portion of patients in the disease control groups had cirrhosis, we did not include a cirrhosis group in the screening phase.

Phage display peptide library technique involves specific screening and affinity selection of a certain phage displaying peptide that is ligand for particular proteins. A major advantage of this technique is the extensive control of selection conditions, which allowed preservation of the three-dimensional structure of the peptide (20,21). In the screening phase, we identified a novel early HCC mimic peptide (HC1) with phage display peptide library technique, using sera from early primary HCC patients and its disease controls.

The field of HCC biochemical markers is steadily advancing, leading to progressive unfolding of the potential roles of HCC biomarkers in basic research, clinical studies, and clinical practice. However, few of the currently available conventional markers provide an entirely satisfactory solution to detect early HCC. Liu et al. reported that none of BCLC-stage A HCC patients showed a serum AFP level $\geq 400 \mu\text{g/L}$, the cutoff value for HCC diagnosis in China (22). Indeed, at this cutoff value, the sensitivity of serum AFP to detect early (BCLC-stage A) HCC patients was 0% in this study. Liu et al. also reported that at a cutoff value of 300 ng/mL, glypican-3 (GPC3) could detect 50% of BCLC-stage A and 39.1% of BCLC-stage B HCC patients. Combined serum GPC3 and AFP could increase the sensitivity of serum AFP from 18.9 to 54% for diagnosis of BCLC-stage A+B HCC (22). In comparison, the HC1 mimic peptide identified in this study showed a 91.4% sensitivity in detecting early HCC, and could differentiate early HCC patients from HBV- and/or HCV-infected patients, cirrhosis patients and healthy controls with high sensitivity, specificity, and accuracy (Table 4). The results indicate that HC1 can specifically react with the sera of early HCC patients. Because HC1 shows no significant similarity with other peptides and no significant correlation with the serum AFP level, further studies

are needed to explore the origin and role of HC1 in early HCC.

In conclusion, the HC1 mimic peptide showed high diagnostic validity for early primary HCC, and thereby could be a candidate serum biomarker for early primary HCC.

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