Identification of tumor-associated antigens in human hepatocellular carcinoma by autoantibodies

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Abstract. Hepatocellular carcinoma (HCC) is a major cause of death in Asian countries. The false-negative rate with serum α-fetaprotein level alone can reach 40% for early stage HCC patients. Due to the lack of sensitive and specific tumor markers for early diagnosis, it is impossible for HCC patients to receive effective therapy. However, tumor antigens can be recognized by immune cells and be rejected in immune responses. In order to identify antigens which may be used as new markers and immunotherapy targets for HCC, a cDNA expression library derived from an HCC sample was constructed, which was screened with mixed autologous and allogenic serum of HCC patients. Seventeen different HCC antigens were obtained, which are classified as tumor-associated antigens. A panel of allogenic sera from patients with chronic hepatitis, liver cirrhosis, HCC and other tumor entities and sera from health volunteers, was used for frequency analysis of antibody responses. Four of 17 antigens, including eukaryotic translation initiation factor 3, subunit I, lactate dehydrogenase 1, A chain, replication factor C2, 40 kDa and mitochondrial carrier triple repeat 1, reacted predominantly with sera from patients with HCC (31.8, 45.5, 27.3 and 50.0% respectively). Patients (81.8%) with HCC had the antibody against at least one of these four antigens, which indicates that disease-specific humoral response against these antigens was induced in HCC patients and the corresponding antibodies may be used as tumor markers for HCC.

Introduction

Hepatocellular carcinoma (HCC) is major cause of death in Asian countries and is a terminal complication of chronic inflammatory and fibrotic liver diseases (1). The false-negative rate with AFP level alone maybe as high as 40% for patients with early stage HCC. Due to the lack of sensitive and specific tumor markers for early diagnosis, it is impossible for HCC patients to accept an effective therapeutic modality. Presently, surgery still plays a major role in the treatment of HCC, particularly for small HCC (2).

However, tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs) can be recognized by immune cells and be rejected in immune responses. So they may be detected as tumor markers and be used as potential targets for cancer immunotherapy.

Serological analysis of recombinant cDNA expression library (SEREX) is a convenient and effective method to identify tumor antigens (3), which does not require cultured tumor cell lines or tumor-specific cytotoxic T lymphocytes (CTLs), compared with CTLs methods (4). SEREX has been applied to many tumor types, such as melanoma (5,6), esophageal squamous cell cancer (7-9) and prostate cancer (10). A large amount of tumor antigen genes, including HOM-MEL-40/SSX, NY-ESO-1 and MAGE were detected. Vaccine therapies with specific peptides of tumor antigens, have been used for the treatment of patients with various malignant tumors, such as MAGE, NY-ESO-1 and NY-BR-1. Specific $CD8^+$ T cell response was observed in cancer patients (11-16) and the clinical trial treatment resulted in regression or growth suppression of tumors (17-19). Some were suggested to be potential targets in cancer and the corresponding targeted immunotherapy which represents a viable therapeutic strategy (20,21).

A great number of tumor antigen genes have been found in HCC patients using the SEREX technique, such as tat binding protein-1 (TBP-1), ribosomal protein L30 (rpl30), p100, albumin and SEC63 (22-25) and specific CTL responses to hepatoma cells have been observed *in vitro* and *in vivo* (26-28).

Multi-gene expression is one of the molecule characteristics of HCC occurring in development (29), antigen investigation and immunotherapy of HCC remain unsatisfactory, antigens and their coding genes of HCC are not distinct, so it is urgent to search for new antigens and identify their epitopes.

In this study, a cDNA expression library was constructed from the total RNA, which was extracted from well-differentiated HCC tissue. SEREX was applied to screen the

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Table I. Sequences of primers for polymerase chain reaction amplification.

Clone no.	Primer		Product size (bp[a])
1	Forward	5'-AAGGACCCTATCGTCAATG-3',	268
	Reverse	5'-GGTAGCCCATCTGCTTGT-3'	
7	Forward	5'-TGTGCCTGTATGGAGTGG-3',	260
	Reverse	5'-TTATTCCGTAAAGACCCT-3'	
9	Forward	5'-TTCGCCCTTGCTTGTAAT-3',	196
	Reverse	5'-CCTGCCTCATGTCTCCCT-3'	
11	Forward	5'-AGGGTGAAGCATGGTGGC-3',	388
	Reverse	5'-TGGAGTTTGGCAGGATGATAG-3'	

[a]Base pairs.

library with the mixed autologous and allogenic serum of HCC patients. The identified antigens may be used as new markers for the diagnosis and prognosis of HCC.

Materials and methods

Tissues and serum samples. This study was approved by the Ethics Review Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University. A well differentiated HCC surgical resection sample from a man aged 57 years who had hepatitis B virus-related hepatitis, was used for the construction of the cDNA library. The patient had no history of other malignancies, autoimmune diseases, chronic alcoholic liver disease or hyper-γ-globulinemia due to chronic liver injury that may have caused a nonspecific immune response. To examine gene expression of the clones obtained by SEREX analysis, 20 surgically resected HCC specimens and 10 other cancer specimens, and corresponding samples from uninvolved regions were stored at -80˚C. All these tissue samples were comfirmed by pathology.

Serum from 15 healthy volunteers, 22 patients with HCC, 20 patients with liver cirrhosis (LC), 20 patients with chronic hepatitis (CH), 8 patients with gastric carcinoma (GC) and 5 patients with pancreatic carcinoma (PC) were used for analysis of the antibodies. The serum AFP level in patients with HCC was measured by enzyme-linked immunosorbent assay and cut-off value was set at 20 ng/ml.

RNA extraction and construction of the cDNA library. Total RNA were extracted using TRIzol (Invitrogen Company) method and Poly A(+) RNA was obtained by an mRNA Purification kit (Sangon Company, Shanghai, P.R. China). The cDNA library was constructed in a λTriplEx2 express vector using a SMART™ cDNA cloning kit (Clontech Company). The cDNA fragments were packaged into a λTriplEx2 express vector and then transfected into *E. coli*, which resulted in $1.37x10^6$ primary recombinants.

Immunoscreening and sequencing. Immunoscreening for the detection of reactive clones was performed with autologous serum, as described elsewhere (7,30,31) with some modification. *E. coli* transfected with recombinant λTripleX_2 phages were plated onto LB-agar plates and expression of recombinant proteins was induced with isopropyl ß-D-thiogalactoside (IPTG, Promega Company) for 6 h. The membranes were blocked with BSA and incubated with a 1:5000 dilution of the alkaline phosphatase-conjugated antibody specific for human IgG (Southern Biotechnology Associate Company) for 2 h. Reactive clones were visualized by staining with BCIP/NBT (Roche Company) for 0.5 h, the resulting blue dots were marked on the filter with a needle. These premarked membrane were extensively washed with TBS, and then incubated with diluted autologous and allogenic HCC patient serum (1:1000), which had been preabsorbed with transfected *E. coli* phage lysate (Stratagene Company). The filters were stained and processed for visualization in a manner identical with that described above. Only clones that appeared blue and unmarked from the needle in the pre-screening were considered serum positive. These clones were subcloned three times to obtain monoclonality. Positive clones were subcloned into pTriplEx2 phagemid vector (Clontech Company) and the nucleotide sequences of cDNA inserts were determined using a BigDye cycle terminator ready reaction kit and ABI Prism automated DNA sequencer (PE Applied Bio systems, Foster City, CA).

Allogeneic serum screening. Clones that reacted with the autologous serum were purified and then mixed with nonrecombinant phages as negative controls at a 1:2 ratio. This mixture was tested against preabsorbed sera of interest by using the immunoscreening assay described above. In this study, we tested 15 healthy controls, 22 patients with HCC, 20 patients with LC, 20 patients with CH, 8 patients with GC and 5 patients with PC.

Reverse transcription-polymerase chain reaction analysis. The mRNA expression pattern of the selected cDNAs that reacted only with sera from patients with HCC was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA from HCC tissue samples and corresponding samples of uninvolved liver were isolated by TRIzol method, and cDNA was generated from 3 μg of RNA by reverse transcriptase (Fermentas Company, Vilnius, Lithuania) with Oligo-dT (Gibco Company) as primers. The sequences of the PCR primers are indicated in Table I. PCR for 28 amplification cycles (at 95˚C for 1 min, at 55˚C for

Table II. Genes identified by serological analysis of a recombinant cDNA expression library of hepatocellular carcinoma.

Clone	Gene	GenBank access no.	Possible function no.
1	Eukaryotic translation initiation factor 3, subunit I (EIF3SI)	BC003140	Regulation of translational initiation
2	RAB4B, member RAS oncogene family (RAB4B)	BC033081	Signal transduction
3	Heat shock protein 90 kDa α (cytosolic), class A member 1 (HSP90AA1)	AI250920	Molecule champerone
4	Insulin-like growth factor binding protein 2,36 kDa (IGFBP2)	M35410	Insulin-like growth factor binding
5	Heat shock 70 kDa protein 9 (mortalin) (HSPA9)	NM004134	Molecule champerone
6	Melanoma antigen family A, 3 (MAGEA3)	BC005963	Unknown
7	Lactate dehydrogenase 1, A chain (LDHA)	X02152	Anaerobic glycolysis
8	Melanoma antigen family A, 1 (MAGEA1)	BC017555	Unknown
9	Replication factor C2, 40 kDa (RFC2)	AL560344	Cell proliferation
10	Hypothetical protein FLJ25976	AK098842	Unknown
11	Mitochondrial carrier triple repeat1 (MCART1)	BP363489	Binding
12	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 4, 9 kDa (NDUFA4)	AF201077	NADH dehydrogenase activity, oxidoreductase activity
13	Ribosomal protein L36a (RPL36A)	BC070204	Structural constituent of ribosome
14	CCHC-type zinc finger, nucleic acid binding protein (CNBP)	M28372	Positive regulation of cell proliferation
15	Cell division cycle 7 homolog (*S. cerevisiae*) (CDC7)	AF015592	Positive regulation of cell proliferation
16	Signal peptidase complex subunit 1 homolog (*S. cerevisiae*) (SPCS1)	BU070128	Signal peptidase activity
17	Mitogen-activated protein kinase-1 (MAPK1)	BC017832	Signal transduction

Table III. Genes reactivity of allogenic sera against the antigens identified by SEREX.

Clone no.	Gene	Health control (n=15)	HCC[a] (n=22)	LC[b] (n=20)	CH[c] (n=20)	GC[d] (n=8)	PC[e] (n=5)
1	EIF3SI	0	7 (31.8%)	0	0	0	0
7	LDHA	0	10 (45.5%)	0	0	0	0
9	RFC2	0	6 (27.3%)	0	0	0	0
11	MCART1	0	11 (50.0%)	0	0	1	0
5	HSPA9	0	5	0	1	5	2
6	MAGEA3	0	12	2	1	6	4
8	MAGEA1	0	13	1	1	7	5
3	HSP90AA1	0	9	1	0	6	3
2	RAB4B	2	6	-	-	-	-
4	IGFBP2	1	4	-	-	-	-
10	Hypothetical protein FLJ25976	1	5	-	-	-	-
12	NDUFA4	3	7	-	-	-	-
13	RPL36A	9	11	-	-	-	-
14	CNBP	3	8	-	-	-	-
15	CDC7	1	2	-	-	-	-
16	SPCS1	0	1	-	-	-	-
17	MAPK1	0	1	-	-	-	-

[a]Hepatocellular carcinoma; [b]liver cirrhosis; [c]chronic hepatitis; [d]gastric carcinoma and [e]pancreatic carcinoma.

1 min and at 72˚C for 1 min) was carried out on each cDNA sample. Furthermore, mRNA expression of the selected clones in cancer specimens and corresponding uninvolved regions were analyzed by RT-PCR described as above, including tissues of the stomach, colon, pancreas, kidney, bladder, prostate, uterus, testicle, brain and lung.

Results

Thirty-eight positive clones were obtained by immunoscreening of the primary cDNA expression library with autologous sera, representing 17 different cDNA sequences, from 324 to 805 bp, which coded for known or closely related proteins (Table II).

Among the 17 antigens identified, 14 cDNAs were genes with known functions, the other 3 cDNAs were genes with unknown functions, including hypothetical protein FLJ25976, MAGEA3 and MAGEA1.

Antigens of EIF3I (clone 1), LDH-A (clone 7), RFC2 (clone 9) and MCART1 (clone 11) reacted only with sera from patients with HCC (Table III). Their response frequency was 31.8, 45.5, 27.3 and 50.0%, respectively. Antigens of HSPA9 (clone 5), MAGEA3 (clone 6), MAGEA1 (clone 8) and HSPCA (clone 3) reacted with sera from patients with HCC and not with control sera, however, they also reacted with sera from patients with LC, CH, GC and PC. The other 9 antigens (clone 2,4,10,12,13,14,15,16,17) reacted with sera from both health control samples and patient samples.

The expression of HCC-specific antibodies against EIF3SI, LDHA, RFC2 and MCART1 antigens was compared with clinical parameters in 22 HCC samples (Table IV). Although 4 patients were negative for the 4 antibodies, 81.8% of patients with HCC reacted with at least 1 of 4 antigens and serological responses were induced even in patients with HCC tumors that measured <2 cm in greatest dimensions. The presence of the antibodies did not correlate with patient age, gender, tumor size, the grade of differentiation, serum AFP level, HBV infection, or the combination of liver cirrhosis ($p>0.05$).

Although the 4 antigens were immunogenic only in patients with HCC, mRNAs from the 4 antigens were expressed constitutively in all HCC samples and their corresponding, uninvolved liver samples by RT-PCR analyses. They nearly expressed in all the human cancer tissues and corresponding uninvolved regions.

Discussion

In the current study, we applied the SEREX technique to identify tumor antigens in human HCC and found that antibodies against EIF3SI, LDHA, RFC2 and MCART1 were produced specifically in patients with HCC, while the expression of these antigens was not disease-specific.

Table IV. Clinical parameters of patients with HCC and antibody production against EIF3SI, LDHA, RFC2 and MCART1.

Patient	Age	Gender	Tumor size (cm)	AFP (ng/ml)	Differentiation	Virus	EIF3SI	LDHA	RFC2	MCART1
1	52	M	5.4	193	well	HBV	+	+		+
2	11	M	1.9	1275	mod	Negative	+			+
3	49	M	4.0	11.2	well	Negative		+		+
4	61	M	6.5	2.4	mod	Negative			+	+
5	60	M	10.0	17.6	well	HBV	+	+	+	
6	44	M	5.5	2339	mod	HBV		+	+	
7	50	M	6.0	2498	nd	HBV	+	+		+
8	42	M	3.7	1210	nd	HBV			+	+
9	38	M	3.0	1.0	mod	HBV	+			
10	69	F	5.3	2.6	well	Negative		+		
11	60	M	4.5	11.4	well	HBV				
12	57	M	8.6	250.4	nd	HBV	+			+
13	51	M	11.8	63.4	well	HBV		+		
14	49	M	6.5	1636	nd	HBV			+	+
15	49	M	3.2	562	mod	HBV	+	+	+	
16	41	M	4.0	135.7	well	HBV				
17	41	F	5.6	2.8	well	Negative				
18	56	M	5.0	2108	nd	HBV		+	+	
19	40	F	2.6	102.9	mod	HBV				
20	75	F	3.7	2.5	well	HBV		+		+
21	44	M	5.5	1017.5	mod	Negative				+
22	57	M	8.6	2381	mod	HBV				+

eIF3SI is the p36 subunit of eukaryotic translation initiation factor protein complex, which has translation initiation factor activity during the course of protein synthesis. Overexpression of EIF3SI has been reported in HCC (32) and nasopharyngeal carcinoma (33,34).

Other subunits from the same family with EIF3SI, such as EIF3S10 (35,36) and EIF2 (37) have been reported in various kinds of cancers, including squamous cell esophagus carcinoma, lung cancer, melanoma, prostate cancer and ovarian cancer.

LDHA is a subunit of LDH, which converts pyruvate lactate under anaerobic conditions in normal cells. LDHA was recognized by breast cancer patient sera (38) and was reported overexpressed in prostatic carcinoma (39) and renal cell carcinoma (40). Reduction in LDHA activity resulted in diminished tumorigenicity, which was reversed by complementation with the human ortholog LDHA protein, this demonstrates that LDHA plays a key role in tumor maintenance (41).

RFC2, a subunit of RFC, is a DNA binding protein and ATPase that acts as a clamp loader of the proliferating cell nuclear antigen (PCNA) processivity factor for DNA polymerases δ and ε. RFC2 has been reported in glioblastomas (42,43), hydatidiform moles, invasive moles and choriocarcinomas (44).

MCART1 codes for a protein whose function is unknown, possibly it participates in enery metabolism during tumorigenicity.

The frequency of anti-EIF3SI, anti-LDHA, anti-RFC2 and anti-MCART1 was high, among the eight patients who were negative for AFP, two patients had antibodies against EIF3SI, four patients against LDHA, two patients against RFC2 and three patients against MCART1. In addition, antibodies against EIF3SI and MCART1 were found even in patients with small HCC tumors, suggesting that they may be useful as a complementary marker for the diagnosis of HCC.

Antigens identified by SEREX represents extremely diverse proteins, most of which result from overexpression in HCC cells. It is not possible to draw conclusions on how these antigens could be involved in tumorigenesis, while rapid turnover of HCC cells may increase the chance of antigen presentation and result in antibody production against these antigens with HCC. The corresponding vaccine injection is a promising therapy in patients with HCC.

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