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• LIVER CANCER •

Expression of cancer-testis antigens in hepatocellular carcinoma

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Abstract

AIM: To investigate the expression of cancer-testis (CT) antigens *MAGE-1, SSX-1 ,CTp11* and *HCA587* genes in hepatocellular carcinoma (HCC) and the possibility of applying these antigens as targets for specific immunotherapy for HCC.

METHODS: Expression levels of *MAGE-1, SSX-1, CTp11* and *HCA587* mRNA were detected with reverse transcription polymerase chain reaction (RT-PCR) in HCC tissues and corresponding adjacent non-cancerous tissues from 105 HCC patients, 40 samples of cirrhosis and normal liver tissues. Genes of five samples with positive PCR results were sequenced.

RESULTS: Of 105 HCC tissues, *MAGE1, SSX-1 ,CTp11* and *HCA587* mRNA expressions were detectable in 75.2%(79/105), 72.4%(76/105), 62.9%(66/105) and 56.2%(59/105) of HCC samples, respectively. About 93.3%(98/105), 72.4%(76/105), 48.6%(51/105) and 37.1%(39/105) of HCC tissues positively expressed at least one, two, three, and four members of CT antigens, respectively. Conversely, only *SSX-1* could be detectable in 2.9%(3/105) of the corresponding adjacent non-HCC tissues in which no metastatic lesion was found. Of the latter 3 patients, biopsy samples far from tumor were obtained in 2 patients and RT-PCR indicated no expression of S*SX-1* mRNA in these two samples. In addition, none of 40 samples of cirrhotic and normal liver tissues expressed CT antigen gene mRNA. DNA sequences confirmed that the RT-PCR products were true target cDNA. No relationship was found between expression of CT antigens and clinico pathological indicators such as age, gender, tumor size, degree of tumor differentiation, serum α-fetoprotein level and infection of hepatitis B virus or hepatitis C virus (P>0.05).

CONCLUSION: CT antigens genes (*MAGE-1*, *SSX-1*, *CTp11* and *HCA587*) are expressed with high percentage and specificity in HCC and their products are promising targets for antigen-specific immunotherapy of HCC. High frequent co-expression of multiple members of CT antigens in HCC provides possibility of polyvalent vaccinations for HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common tumor with a poor prognosis, irrespective of a variety of treatment options[1,2]. So, there is a need to develop additional therapeutic approaches for the management of this disease. A proposed strategy is immunotherapy, which has the potency to eradicate systemic tumor cells in multiple sites in the body and the specificity to discriminate between neoplastic and non-neoplastic cells[3,4]. Genes or antigens, which are either exclusively or preferentially expressed in malignant tissues, are a prerequisite for antigen-specific cancer immunotherapy[4,5].

Recently, the integration of molecular and immunological techniques has led to the identification of a new category of tumor-specific antigens called cancer-testis (CT) antigens, such as MAGE superfamily, SSX family, GAGE, BAGE, SCP-1, NY-ESO-1, CTp11 and HCA587[6,7]. The CT antigens share several distinct features including: (1) CT antigen mRNA is expressed predominantly in tumors of different origins with various frequencies but not in normal tissues except for testis; and (2) the genes encoding CT antigens locate at the X chromosome[8,9]. The combination of CT antigen peptides and human leukocyte antigen (HLA), when efficiently presented by antigen presenting cells to cytotoxic T lymphocytes (CTL), is capable of eliciting cellular immune responses in cancer patients[10,11]. Also, some members of CT antigens are capable of inducing humoral immunity[12,13]. Both cellular and humoral immune response can kill tumor cells specifically. The testis is an immune privileged organ because spermatogenic cells do not express HLA class I antigen at their surface. Concomitantly, the testis has a so-called blood-testis barrier, which limits contact between testicular and immune cells, in the seminiferous tubuli generated by the Sertoli cells[7,14]. Because of these features, CT antigens have the potential to provide specific antitumor immunity directly to malignant cells without harm to normal cells. At present, the application of tumor antigenic peptide vaccine based on CT antigens for cancer immunotherapy has become a hot spot[15,16].

The screening of tumor antigens recognized by autologous cytotoxic T cells led to the isolation of *MAGE-1*, which has been identified as a member of *MAGE-A* genes family[17]. Clinical trials targeting MAGE-1 antigen are in progress for malignant melanoma and have shown favorable curative effect[18,19]. *SSX* genes and *HCA* genes were originally identified using serological analysis of recombinant cDNA expression libraries (SERAX). SEREX was to define B cell epitopes in antigens that were specifically recognized by antibodies in cancer sera, suggesting that SSX and HCA antigens were immunogenic and capable of inducing an antibody response[6,20]. In a recent report, Li *et al*. have proved HCC patients were able to develop humoral immune response to HCA587 antigen. Based on representational difference analysis of cDNA, Zendman *et al*.[21]

first identified the expression of *CTp11* in melanoma cell lines. In another study (unpublished data), our group predicted HLA-A2-restricted CTL epitopes of *CTp11* by peptide supermotif prediction combined with quantitative motif [22,23] and found the potential HLA-A2-restricted CTL epitopes of CTp11 antigen (*HLA-A2* is the most common *HLA-A* allele in Asian population, especially in Chinese, with an estimated frequency of more than 50%[24,25]), suggesting the possibility of CTP11 antigen to trigger cellular immunity and a new potential vaccine candidate for HCC immunotherapy.

Several studies reported high frequent expression of CT antigens in the HCC tissues, such as MAGE-1[26,27] and SSX-1[4,28]. However, the expression of CT antigens is heterogenous in a variety of human tumors, which may be a way for malignant cells to evade from immunosurveillance[7,29-31]. Screening highly and specifically expressed multiple CT antigens in tumors and using them as targets for immunotherapy are promising means to overcome the heterogenous expression of tumor cells, thus expanding patients' opportunities for cancer specific immunotherapy and establishing the basis of polyvalent vaccinations[29].

In an effort to improve the efficiency of tumour vaccines and prevent immune escape, we investigated the expression of CT antigens MAGE1, SSX-1, CTp11 and HCA587 in HCC tissues.

MATERIALS AND METHODS

Data of patients

From October 2000 to September 2003, 105 patients receiving operation for HCC, including hepatectomy or orthotopic liver transplantation, at the 2nd Hospital of Peking University Health Science Center, were enrolled in this study. There were 90 men and 15 women with a mean age of 47.2±8.4 years (range 18-75). The hepatitis B surface antigen was positive in 98 cases. Among 105 cases, anti-HCV was positive in 5 cases. The serum α-fetoprotein (α-FP) level was normal (<20 ng/mL) or elevated slightly (<40 ng/mL) in 35 patients. Histopathological examination indicated that 18 HCC samples were well differentiated, 61 moderately and 26 samples poorly differentiated, respectively. According to TNM classification of the International Union Against Cancer[32], there were 13 cases at stage I, 30 at stage II, 12 at stage III and 50 at stage IV, respectively. Seventy-nine patients had large-sized tumors (>5 cm), while the tumor size of the other 26 patients was equal to or less than 5 cm (≤5 cm). The control samples included 20 tissues from cirrhosis patients and 20 normal liver tissues from patients without liver disease by surgical biopsy. Testis tissues (kindly provided by Urological Department of the 2nd Hospital of Peking University Health Science Centre) were used as the positive control. Each sample was immediately frozen in liquid nitrogen after a surgical resection and stored at -80 ℃ until the extraction of total RNA. Informed consent was obtained from each patient before the study was conducted. The Ethic Committee of Peking University approved the study protocol.

Total RNA extraction and synthesis of cDNA

Total RNA was extracted from frozen tissue specimens (50-100 mg) using TRIzol reagent (GIBCOL BRL) according to the protocol provided by the manufacturer. Total RNA (2.5 μg) was primed with an Oligo (dT) 15 oligonucleotide (Promega) and reverse-transcribed according to manufacturer's instructions.

PCR amplification of CT antigens

The amplification reaction contained 5 μL of a 1:5 dilution of reverse-transcribed products, 1 μL each of 10 μmol/L specific primers, 1 μL 10 mmol/L dNTP mixture (dATP, dGTP, dCTP, dTTP), 2.5 U *Taq* DNA polymerase (Gibco BRL) and PCR-buffer solution. The total volume was brought to 50 μL using water. The PCR amplifications were performed in a UNO II thermocycler (Perkin-Elmer, USA) under the following conditions: After an initial denaturation for 5 min at 94 ℃, samples were subjected to 35 cycles of amplification, followed by a final extension of 8 min at 72 ℃. The length of PCR products was 421 base pair (bp) (*MAGE-1*), 422 bp (*SSX-1*), 297 bp (*CTp11*) and 238 bp (*HCA587*), respectively. To verify that the RNA had not degraded, a PCR assay with specific primers for the gene β_2-microglobin (B_2-*MG*) was performed on each cDNA sample under the following conditions: After an initial heating for 2 min at 94 ℃, samples were subjected to 30 cycles of amplification, followed by a final extension of 8 min at 72 ℃. The product size was 335 bp. The PCR conditions and specific primer sets for *MAGE-1*[33], *SSX-1*[4], *CTp11*[21], *HCA587*[34] and B_2-*MG*[26] used in this study are shown in Table 1.

At last, 10 μL of reaction product was analyzed by electrophoresis on a 20 g/L agarose gel (Promega, USA), followed by ethidium bromide staining and digital camera photographing (Korda D3.5, USA).

Sequence analysis of PCR products

To confirm the specificity of the RT-PCR products of the CT antigen genes, we performed the sequence analysis. In brief, purified PCR products were cloned into pGEM-T Easy Vector (Promega) by *T4* DNA ligase and amplified in *E. coli*, JM109. Five cases of positive colonies were selected and assessed using *Eco*RI digestion of mini-prepared DNA. The putative *MAGE-1*, *SSX-1*, *CTp11* and *HCA587* cDNA samples were sequenced with T7 sequencing primers in Sangon Co, Shanghai, China.

Statistical analysis

The statistical analysis was assessed using the chi-square test and the Fisher's exact test, and the significant level was set at $P<0.05$.

RESULTS

Expression of CT antigen genes

Among the 105 HCC tissues investigated, *MAGE1*, *SSX-1*, *CTp11* and *HCA587* mRNA was expressed in 75.2%(79/105), 72.4%(76/105), 62.9%(66/105) and 56.2%(59/105) of tumor samples from HCC patients, respectively. At least one CT antigen mRNA was positive in 93.3%(98/105) of HCC tissues. Conversely, only *SSX-1* could be detected in 2.9%(3/105) of the corresponding adjacent non-HCC tissues in which no metastatic lesions were found. Of the latter 3 patients, biopsy samples far from tumor were obtained in 2 patients and RT-PCR indicated no expression of *SSX-1* mRNA was detectable in these 2 samples. No expression of *MAGE-1*, *CTP11* and *HCA587* was detected in the samples of corresponding adjacent non-cancerous tissues. In addition, none of 40 samples of cirrhosis and normal liver tissue expressed each CT antigen mRNA. In consistent with Chen *et al.*'s findings[4], the electrophoretogram analysis revealed the intensities of PCR products varied considerably among different samples, indicating HCC cells expressing tumor antigens at mRNA level are heterogeneous. The typical electrophoresis of RT-PCR products amplified from cDNA of tissue samples of some HCC patients is shown in Figure 1.

Highly synchronous expression of multiple CT antigens in HCC samples

Highly frequent co-expression of multiple members of CT antigens was observed in HCC samples, with synchronous expression of at least two, three, and four of these gene in

Table 1 PCR amplification program

Gene	Temperature, duration of denaturation, annealing, extension	Primer sequence	Product size (bp)
MAGE-1	94 ℃, 45 s	f: 5'-CGGCCGAAGGAACCTGACCCAG-3'	
	65 ℃, 45 s	s r: 5'-GCTGGAACCCTCACTGGGTTGCC-3'	421
	72 ℃, 45 s		
SSX-1	94 ℃, 60 s	f: 5'-CTAAAGCATCAGAGAAGAGAAGC-3'	
	57 ℃, 60 s	r: 5'-AGATCTCTTATTAATCTTCTCAGAAA-3'	422
	72 ℃, 60 s		
CTp11	94 ℃, 45 s	f: 5'-CTGCCCCAGACATTGAAGAA-3'	
	57 ℃, 60 s	r: 5'-TCCATGAATTCCTCCTCCTC-3'	297
	72 ℃, 90 s		
HCA587	94 ℃, 30 s	f: 5'-AGGCGCGAATCA AGTTAG-3'	
	60 ℃, 30 s	r: 5'-CTCCTCTGCTGTGCTGAC-3'	238
	72 ℃, 30 s		
B_2-MG	94 ℃, 45 s	f: 5'-CTCGCGCTACTCTCTCTTTCTGG-3'	
	55 ℃, 45 s	r: 5'-GCTTACATGTCTCGATCCCACTTA-3'	335
	72 ℃, 45 s		

f: Forward primer, r: Reverse primers.

Table 2 Expression of CT antigens and clinico pathological indicators

Group	n^1	MAGE-1		SSX-1		CTp11		HCA587		CT^2(+)	
		+	-	+	-	+	-	+	-	+	-
Total number	105	79	26	76	29	66	39	59	46	98	7
Gender											
Male	90	70	20	66	24	57	33	48	42	85	5
Female	15	9	6	10	5	9	6	11	4	13	2
Tumor diameter											
≤5 cm	26	18	8	16	10	14	12	15	11	22	4
>5 cm	79	61	18	60	19	52	27	44	35	76	3
TNM stage											
I+II	43	29	14	31	12	27	16	22	21	39	4
III+IV	62	50	12	45	17	39	23	37	25	59	3
Hepatitis B virus											
HBV (+)	98	74	24	71	27	62	36	55	43	91	7
HBV (-)	7	5	2	5	2	4	3	4	3	7	0
Hepatitis C virus											
HCV (+)	5	5	0	4	1	4	1	4	1	5	0
HCV (-)	100	74	26	72	28	62	38	55	45	93	7
AFP (ng/mL)											
<40	35	28	7	22	13	20	15	18	17	32	3
≥40	70	51	19	54	16	46	24	41	29	66	4
Differentiation											
Well	18	13	5	12	6	12	6	9	9	16	2
Moderately	61	44	17	44	17	39	22	34	27	58	3
Poorly	26	22	4	20	6	15	11	16	10	24	2

n^1 : Number of samples ; CT^2: At least one of these 4 CT antigen genes; No relationship is found between the expression of CT antigens and clinical indicators such as age, gender, degree of tumour differentiation, serum α-FP level, TNM classification, tumor size, infection of hepatitis B virus or hepatitis C virus (P>0.05).

72.4%(76/105), 48.6%(51/105) and 37.1%(39/105) of HCC tissues, respectively. We also analysed the cases with expression of neither *MAGE-1* nor *SSX-1* genes to determine targets for cancer immunotherapy other than *MAGE-1* and/or *SSX-1*. Among 18 HCC patients with expression of neither *MAGE-1* nor *SSX-1*, 61.1% (11/18) of HCC samples expressed *CTp11* and/or *HCA587*.

Sequence analysis of PCR products

Sequence analysis of PCR products verified the nucleotide sequences of *MAGE-1*, *SSX-1*, *CTp11* and *HCA587* cDNA fragments were identical to that in the database of the GenBank. It confirmed that the RT-PCR products were true *MAGE-1*, *SSX-1*, *CTp11* and *HCA587* cDNA (data not shown).

Expression of CT antigens and clinical indicators

No relationship was found between the expression of CT antigens and clinico pathological indicators such as age, gender, differentiation degree of tumour, serum α-FP level, TNM classification, tumor size, infection of hepatitis B virus or

hepatitis C virus (P>0.05). However, among 35 patients with the normal (<20 ng/mL) or slightly elevated (<40 ng/mL) serum α-FP level, 32 patients had *MAGE-1*, *SSX1*, *CTp11* or *HCA587* gene transcripts detected in their HCC tissues. The results are demonstrated in Table 2.

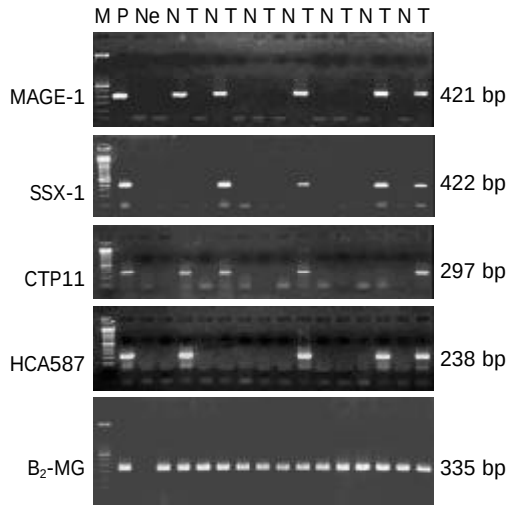

Figure 1 Electrophoresis of RT-PCR products amplified from cDNA of HCC tissues. M: Molecular marker, 100-bp DNA ladder (Gibco); P: Positive control, testis tissues; Ne: Negative control, PCR amplification in the absence of template; B_2-MG: 335-bp cDNA quality control; T: HCC tissues, arrows indicate the RT-PCR products of CT antigens; N: Corresponding adjacent non-tumor tissues. All the samples shown here except for the negative control are positive.

DISCUSSION

Tumor antigens should demonstrate high frequency expression in the tumor tissues and restricted expression in normal tissues, which is the prerequisite for being an ideal candidate antigen for antigen-specific cancer immunotherapy[35]. The growing class of CT antigens is of particular interest for antitumor immunotherapy because of their specific expression on tumor cells. It has demonstrated high frequent expression of *MAGE-1* and *SSX-1* in HCC tissues, but little in nontumor tissues[4,26-28]. However, the heterogeneous expression of MAGE-1 and SSX-1 antigens may cause problems for immunotherapy targeting these antigens, as tumor cases with a low percentage or negative expression of MAGE-1 and/or SSX-1 antigens may be less suitable for immunotherapy targeting these antigens. Furthermore, to our knowledge, so far little information is available on the *CTp11* and *HCA587* expression in HCC tissues. Therefore, we investigated the expression of CT antigens MAGE1, SSX-1, CTp11 and HCA587 in HCC tissues.

In the present study, we found highly positive rate of *MAGE-1, SSX-1, CTp11* and *HCA587* transcripts in HCC tissues (56.2-75.2%). No expression of *MAGE-1, CTP11* and *HCA587* was detected in the samples of corresponding adjacent non-cancerous tissues, while *SSX-1* mRNA could be detected in 2.9%(3/105) of the corresponding adjacent non-HCC tissues where no metastatic lesions were found, similar to Chen *et al.*' s findings[4]. Of these 3 patients, biopsy samples far from tumor were obtained in 2 patients and RT-PCR indicated expression of SSX-1 mRNA was not detectable in these two samples. The sensitivity of RT-PCR assay was much higher than that of conventional methods[36], such as histochemistry and cytology, so we speculate that there might be micrometastasis in the adjacent non-tumor liver tissue. In addition, none of 40 samples of cirrhosis and normal liver tissues expressed each of these 4 CT antigen mRNAs. As *MAGE-1*, *SSX-1*, *CTp11* and *HCA587* were expressed with a high percentage and specificity in HCC, their products might be ideal antigen targets for HCC immunotherapy. A high proportion (93.3%, 98/105) of the HCC tissue samples was positive for at least one of these 4 CT antigen genes, indicating using multiple CT antigens as targets will greatly increase the number of candidates for CT antigen-based HCC immunotherapy.

In the present study, we found relatively high frequency of expression of *CTp11* and *HCA587* mRNA, being 62.9%(66/105) and 56.2%(59/105) of tumor samples from HCC patients, respectively, while no expression of *CTP11* and *HCA587* was detected in the samples of corresponding adjacent non-cancerous tissues and control liver tissues, indicating their products may also be ideal antigen targets for HCC immunotherapy. Though frequency of expression of *CTp11* and *HCA587* mRNA was relative lower than that of *MAGE-1* and *SSX-1*, being 75.2%(79/105) and 72.4%(76/105), respectively, we found that samples with negative expression of *MAGE-1* and *SSX-1* showed expression of *CTp11* and *HCA587*. With respect to these HCC patients, vaccines based on *CTp11* and *HCA587* should be a desirable choice.

We also found a high frequency of synchronous expression of multiple CT antigens in HCC samples, with synchronous expression of at least two, three, and four of these genes in 72.4%(76/105), 48.6%(51/105) and 37.1%(39/105) of HCC tissues, respectively. High frequent co-expression of multiple members of CT antigens in HCC provides possibility of polyvalent vaccinations for HCC. The polyvalent vaccinations with multiple antigens, which avoid the heterogenous expression of tumor cells, might obtain better clinical results than a single antigen-based vaccination in cancer immunotherapy. Patients' opportunities for cancer specific immunotherapy can thus be expanded[37]. However, our results showed no expression of CT antigens in 6.7%(7/105) of HCC tissues. For these patients, it is necessary to screen other CT antigens or tumor specific antigens serving as immune targets for HCC immunotherapy.

Concerning the issues of heterogenous expression of CT antigens and possibility of polyvalent vaccinations for HCC, Tahara *et al*.[27] investigated the 10 CT antigens expression in HCC with RT-PCR and found that 86%(19/22) of HCC tissue samples expressed at least one CT antigen gene. Comparably, Luo *et al*.[28] showed that 81%(17/21) of HCC tissue samples expressed at least one of the 10 CT genes. While in our present study, selecting just 4 CT antigens, we proved that 93.3%(98/105) of HCC patients were detectable of at least one positive CT antigen expression. Notably, the sample number of our study is 105, which is much more than the case number in the study of Tahara *et al*.[27] (22 cases) or Luo *et al*.[28] (22 cases), therefore our results should be more convincing.

Our previous report showed the prognostic value of *MAGE-1* and *MAGE-3* as tumor specific markers to detect blood dissemination of HCC cells after hepatectomy[33]. In this study, our results indicated *SSX-1, CTp11* and *HCA587* were also expressed in HCC tissues with high percentage and sensitivity, and some cases with negative *MAGE* expression were positive for *SSX-1, CTp11* and/or *HCA587* transcripts. Therefore, we can combine *SSX-1, CTp11* and *HCA587* genes with *MAGE* genes and apply them as multiple-markers to detect hepatoma cells in circulation, which may improve the sensitivity of the assay.

In this study, the serum α-FP was normal (<20 ng/mL) or slightly elevated (<40 ng/mL) in 30.0%(35/105) of HCC patients. In these 35 patients, 32 had *MAGE-1, SSX1, CTp11* or *HCA587* transcripts in their HCC tissues, suggesting applying their mRNAs as tumour-specific markers to detect HCC cells in circulation might be an adjuvant diagnostic tool. This assay combined with the examination of serum α-FP level may improve the diagnosis of HCC patients.

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