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• LIVER CANCER •

Construction of human liver cancer vascular endothelium cDNA expression library and screening of the endothelium-associated antigen genes

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Abstract

AIM: To gain tumor endothelium associated antigen genes from human liver cancer vascular endothelial cells (HLCVECs) cDNA expression library, so as to find some new possible targets for the diagnosis and therapy of liver tumor.

METHODS: HLCVECs were isolated and purified from a fresh hepatocellular carcinoma tissue sample, and were cultured and proliferated *in vitro*. A cDNA expression library was constructed with the mRNA extracted from HLCVECs. Anti-sera were prepared from immunized BALB/c mice through subcutaneous injection with high dose of fixed HLCVECs, and were then tested for their specificity against HLCVECs and angiogenic effects *in vitro*, such as inhibiting proliferation and inducing apoptosis of tumor endothelial cells, using immunocytochemistry, immunofluorescence, cell cycle analysis and MTT assays, *etc*. The identified xenogeneic sera from immunized mice were employed to screen the library of HLCVECs by modified serological analyses of recombinant cDNA expression libraries (SEREX). The positive clones were sequenced and analyzed by bio-informatics.

RESULTS: The primary cDNA library consisted of 2×10^6 recombinants. Thirty-six positive clones were obtained from 6×10^5 independent clones by immunoscreening. Bio-informatics analysis of cDNA sequences indicated that 36 positive clones represented 18 different genes. Among them, 3 were new genes previously unreported, 2 of which were hypothetical genes. The other 15 were already known ones. Series analysis of gene expression (SAGE) database showed that *ERP70*, *GRP58*, *GAPDH*, *SSB*, *S100A6*, *BMP-6*, *DVS27, HSP70* and *NAC alpha* in these genes were associated with endothelium and angiogenesis, but their effects on HLCVECs were still unclear. *GAPDH*, *S100A6*, *BMP-6* and *hsp70* were identified by SEREX in other tumor cDNA expression libraries.

CONCLUSION: By screening of HLCVECs cDNA expression library using sera from immunized mice with HLCVECs, the functional genes associated with tumor endothelium or angiogenesis were identified. The modified SEREX, xenogeneic functional serum screening, was demonstrated to be effective for isolation and identification of antigen genes of tumor endothelium, and also for other tumor cell antigen genes. These antigen genes obtained in this study could be a valuable resource for basic and clinical studies of tumor angiogenesis, thus facilitating the development of anti- angiogenesis targeting therapy of tumors.

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INTRODUCTION

Angiogenesis is a critical event in solid tumor growth, invasion, and metastasis. Recently, more attractive targets are thought to be vasculature of tumor compared with tumor cells themselves in the therapy of solid tumor[1]. Tumor endothelium is a key mediator during the complex process of tumor angiogenesis. There will not form new blood vessels in tumor if tumor vascular endothelia are lacking of the functions of proliferation, activation, adhering, migration and vessel formation. To date, the morphology, phenotype, functional aspects and gene expression observed in tumor-derived endothelial cells (TEC) were proven to be different from normal-derived endothelial cells (NEC)[2,3]. Virtually, the therapeutic strategy of solid tumors targeting for tumor vasculature makes use of these differences. Various methods have been developed to identify the differences between TEC and NEC, such as serial analysis of gene expression (SAGE)[4], suppression subtractive hybridization (SSH)[5], antibody target[6], immunohistochemical analysis of known endothelial adhesion molecules[7] phage display peptide library[8], and cDNA microarray[9], *etc*. Due to the difficulty of isolating highly purified TEC, most studies now selected activated endothelial cells as a substitute, however, the activated endothelial cells cannot completely represent TEC.

SEREX has recently emerged as a powerful method for serological identification of tumor associated antigens (TAAs) and/or tumor rejection antigens (TRAs). Up to date, more than 1 000 candidate tumor antigens in various cancers have been identified[10,11]. Tumor antigens identified by SEREX could provide valuable targets for cancer diagnosis, therapy and the study of cancer vaccines. Similarly, the proliferation-associated antigens on tumor endothelial cells may be more useful candidates for antiangiogenic therapy/vascular targeting therapy of tumor. However, up to the present, no data are available that associated antigen genes have been isolated from TEC.

The goal of this study was to define tumor endothelium associated antigen genes by the method of modified SEREX.

Therefore, we constructed and screened the HLCVECs cDNA expression library with murine immunosera of anti-HLCVECs, and identified 18 HLCVECs associated antigen genes. These genes may not only provide a valuable tool for study on the roles of endothelial cells in tumor angiogenesis, but also some potent candidate targets for antiangiogenic therapy of cancer. Our results in this report also indicated that the approach of screening cDNA expression library with functional xenogeneic sera, a modified SEREX, could be an effective strategy for isolation and identification of tumor endothelium associated antigen genes.

MATERIALS AND METHODS

Tumor tissue samples and cells

Human tumor tissue was obtained from therapeutic surgical resection of one patient with hepatocellular carcinoma (HCC) at the Cancer Hospital of Peking Union Medical College. After surgical removal, the tissue sample was immediately transferred to the laboratory in cold culture medium (DMEM, GIBCO) with penicillin (400 U/mL) and EDTA (1 g/L) and was isolated for 2 h. Human umbilical vein endothelial cells (HUVECs) were isolated as described[12]. HUVECs were stimulated to generate activated HUVECs with endothelium growth medium (Clontech) containing EC growth factors and tumor tissues homogenate prepared in our laboratory.

Isolation, purification and culturing of HLCVECs

Isolation, purification and culturing of HLCVECs were performed by previously described method[13,14] with some modifications. Briefly, the liver cancer tissue from patient with HCC was finely minced with curved scissors into approximately 2 mm ×2 mm×2 mm pieces, then was re-suspended in 20 mL of 1 g/L trypsin (type II, Sigma) in DMEM containing 1 g/L EDTA and incubated for 10 min at 37 ℃. After digestion, the whole suspension was filtered through a 200 μm melt mesh sieve and the filtrate was washed twice in DMEM by centrifugation at 450 r/min for 5 min at room temperature. The pellet was re-suspended in 5 mL of DMEM + 100 g/L FCS (Hyclon) added to 25 mL of 200 g/L percoll (Pharmacia Biotech) in DMEM and centrifuged at 1500 g for 15 min at 4 ℃. Again, the cell pellet was washed twice. The isolated cells were resuspended in complete culture medium (DMEM containing 200 mL/L FCS, 2 mmol/L L-glutamin (GIBCO), 100 μg/mL antibiotic (penicillin/streptomycin), 100 μg/mL endothelial cell growth supplement and 40 U/mL heparin). The cells were seeded into a 20 g/L gelatin-coated 6-well plate (FALCON), and cultured at 37 ℃ in 50 mL/L CO_2 incubator and the medium was changed every 3 d, the purification could be carried out by the way of sub-cell colonies after 1 wk. The cells were cultured and proliferated in gelatin-coated T75 plastic tissue culture flasks (Falcon), and passaged by 1 g/L typsin (1 g/L EDTA). The purified endothelial cells were identified by immunocytochemistry for von Willebrand factor (vWF), CD31 and uptake of Ac-LDL. Fenestration was demonstrated by transmission electron microscopy.

Preparation of the anti-HLCVECs immunosera

Female BALB/c mice (6-8 wk) were purchased from Experimental Animal Center of Peking Union Medical College. Six mice were immunized with HLCVECs (immunized), and 4 mice were treated with PBS alone (non-immunized). All studies on mice were approved by the institute's Animal Care and Use Committee.

Sera were obtained as described[15]. For the generation of immunosera, mice were immunized subcutaneously with 1-6×10^6 HLCVECs fixed with 30 g/L paraformaldehyde in PBS or PBS alone once weekly for 8 continuous weeks. Serum was obtained from each mouse of immunized and non-immunized on d 21, 28, 35, 42, 49 and 56 after the first immunization. Serum from each mouse of immunized and non-immunized groups was serially diluted, and the reactivity against HLCVECs was examined by immunocytochemistry.

Immunofluorescence

To determine the reactivity of sera from immunized and non-immunized mice reacting to different endothelium, HLCVECs, HUVECs and activated HUVECs were seeded onto glass coverslips in 6-cm plates, then fixed in cold acetone and incubated with serially diluted sera isolated from immunized or non-immunized mice at 37 ℃ for 1 h, fluorescein-conjugated goat-anti-mouse IgG (H+L) (Sigma) was subsequently applied to them and incubated for another 1h, then to be restained by 0.1 g/L Evens Blue and washed 3 times by PBS. The results were observed under fluorescence microscope (Nicon)[16].

MTT assay

Approximately 8×10^3 cells in 200 μL DMEM were seeded in triplicate into each well of the 96-well tissue culture plates, and immunized or non-immunized sera diluted at 1:30, 1:90, 1:270, 1:810, 1:2 430, 1:7 290 and 1: 21 870 were added to corresponding wells. After 72 h of incubation , 10 μL MTT (100 mg/mL) reagent was added to each well (5 g/L), and incubated for 4 h at 37 ℃, 50 mL/L CO_2. Subsequently, 180 μL medium was pipetted out from each well and 50 μL DMSO was added to it. The absorbency A_{570}, which correlates to the number of cells, was measured with micro-plate reader (Model 450, Bio-Rad)[17].

Cell cycle analysis by flow cytometry

HLCVECs were serum starved for 24 h and then treated with immunized and non-immunized sera diluted at 1:100 for 6 h. Cells were trypsinized, washed twice in PBS. Totally 1×10^6 cells were resuspended in 500 μL PBS and stained with 500 μL propidium iodide (10 μg/mL, Sigma) for 30 min. Flow Cytometry (Becton) was performed to determine DNA content and apoptosis[18].

Construction of cDNA expression library

The mRNA was directly extracted from HLCVECs with mRNA extraction kit (mRNA Poly(A) Tract System 1 000, Promega, Madison, WI), Oligo (dT)-primed double-stranded cDNA was synthesized from 6 ug purified mRNA and ligated into the ZAP phage expression vector DNA according to the user's manual (including THERMO Scripit™ RT-PCR System Kit, ZAP-cDNA systhesis kit, ZAP-cDNA Gigapack III Gold Mrna Cloning kit, Stratagene).

Screening the HLCVECs cDNA expression library with immunosera

Immunoscreening of the HLCVECs cDNA expression library was performed as described previously[19] with the following modifications. Sera from immunized mice were diluted 1:10 and preabsorbed with lysate from *Escherichia coli* (*E.coli*) strain XL-1 coupled to Sepharose 4B to remove antibodies reacting with *E.coli* components. X-L1 infected with recombinant phage vectors containing HLCVECs cDNA were plated onto NZY-tetracycline-agar plates. After induction of protein synthesis in *E.coli*, we transferred the expressed polypeptides onto nitrocellulose membranes (Gelman) and incubated them with 1:500 diluted pre-absorbed sera overnight at 4 ℃ in the first screening. After being washed, the filters were incubated with a 1:10 000 dilution of alkaline phosphatase-conjugated goat-anti-mouse IgG (H+L) (Secondary Ab, Sigma) for 1-2 h at room temperature. Reactive clones were visualized with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium tablets (BCIP/NBT, Sigma). Only clones that appeared blue were considered serum positive. The positive clones were picked out and plated on NZY-tetracycline agar plates for secondary screening with

1:5 000 dilluted pre-absorbed immunosera. Subsequently, positive clones were subcloned 2 times to obtain monoclonality.

Sequence analyses of the reactive clones

Identified and subcloned positive clones were converted to pBluescript phagemide forms by *in vitro* excision, plasmid was purified and subjected to *Eco*R I and *Xho*l I restriction enzyme digestion. Clones representing different cDNA inserts were sequenced with T3 primers by the dideoxy chain termination method using the Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster city, CA) and an ABI PRISM automated DNA sequencer (Perkin-Elmer, Norwalk, CT). DNA and predicted amino acid sequences were compared with sequences in the GenBank and other public databases by using the BLAST program.

RESULTS

Identification of HLCVECs

The heterogeneity of human TEC is detectable at different levels and differentiates the behavior between different tumor tissues. HLCVECs here belong to micro-vascular ECs, and they grow in a monolayer and exhibit contact inhibition properties. Using electron microscopy, surface fenestrations and Weibel-Palade (W-P) bodies were observed in HLCVECs. Immunofluorescence staining showed that these cells expressed vWF, CD31 and took up large amounts of Ac-LDL (Figure 1). These results demonstrated that isolated and purified HLCVECs expressing specific markers of endothelial cells, especially fenestration, a tumor endothelium-specific structure, were also found in HLCVECs.

Detection of antibodies against endothelial cells in immunosera

The antibody titer in sera from immunized mice with HLCVECs are shown in Table 1. Titer of antibody reached 1:1 000 at 21 d after the first immunization, 1:9 000 at 42 d and 1:27 000 at 49 d as well as at 56 d. In contrast, the sera from non-immunized mice were negative for anti-HLCVECs response. A similar antibody response, with a minimal variation in titer, was seen in all six immunized mice.

To determine whether differences existed in reactivity of sera from mice immunized with HLCVECs with human TEC versus NEC, immunofluorescence was applied to detect the reaction of anti-HLCVECs sera with HLCVECs, HUVECs and activated HUVECs. The results indicated that 1:500 dilution of the immunized serum showed the same degree fluorescence staining in HLCVECs and activated HUVECs, but the staining in HUVECs was distinctly weaker than that of the other 2 kinds of cells. The 1:5 000 dilutions of immunoserum showed markedly stronger staining with HLCVECs than with activated HUVECs (Figures 2A and 2B), whereas the staining of HUVECs was completely negative in this dilution (Figure 2C). By comparison, the staining of all the 3 kinds of cells was negative when reacting with sera from non-immunized mice. Simultaneously, the fluorescence staining was observed in the membrane of positive endothelial cells. These data indicated that the murine anti-HLCVECs sera contained the specific antibodies with a high titer against TECs.

Table 1 Titer of anti-HLCVECs antibody in immunized animals

	D21	D28	D35	D42	D49	D56
HLCVEC	1:1 000	1:3 000	1:9 000-	1:9 000	1:27 000	1:27 000+

1 : 9 000- indicates that the titer is lower than 1:9 000, 1:27 000+ indicates that the titer is 1:27 000 or higher.

Identification of the effect of immunosera on ECs growth

To investigate whether the immunosera have any effects on the proliferation of HLCVECs, HLCVECs were treated with variously diluted sera isolated from mice immunized or non-immunized with HLCVECs. MTT assay demonstrated that the growth and proliferation of HLCVECs were apparently inhibited by the sera from immunized mice with HLCVECs, and there was

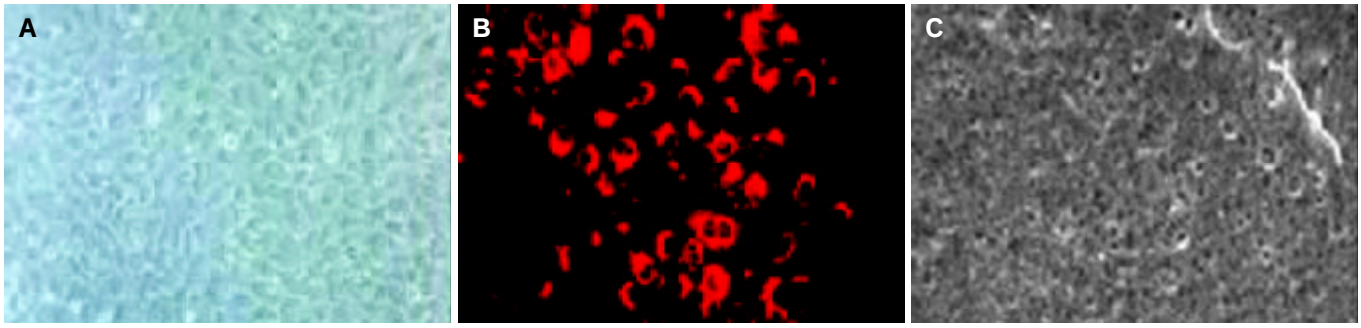

Figure 1 Cultured human liver cancer vascular endothelia. A: Morphology of cultured human liver cancer vascular endothelial cells; B: Uptake of Ac-LDL; C: Electron microscope for fenestration.

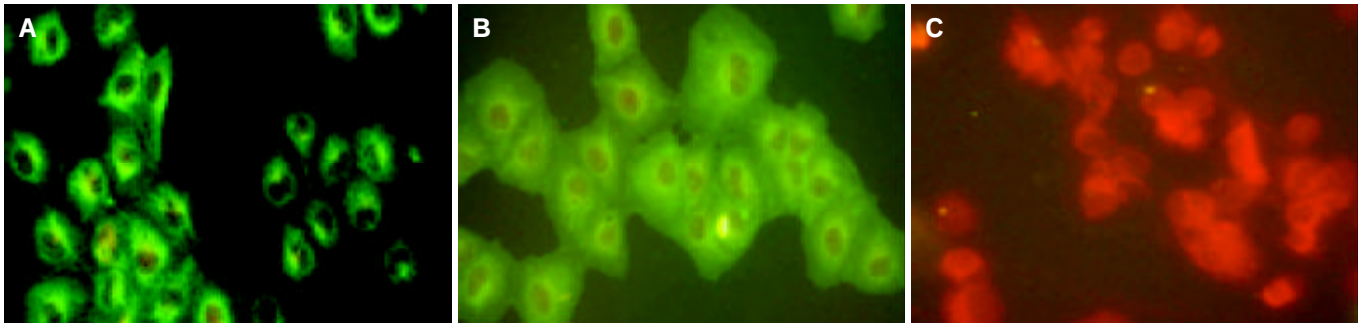

Figure 2 Immunofluorescence analysis of endothelial cells stained with the sera diluted 1:5 000 isolated from the mice immunized with HLCVECs . A: HLCVECs; B: Activated HUVECs; C: HUVECs.

Table 2 Construction of HLCVECs libraries in λ-ZAP expression vector and the number of SEREX-identified antigens

Patient	Age (years)	Primary size	Screening serum	Clones screened	Positive clones	cDNA fragment	Known genes	Unknown gene
HCC	56	2×10^6	immunized	6×10^5	36	18	15	3

Table 3 Results of immunoscreening of HLCVECs cDNA expression library by immunized BALB/C sera screening

Clone	Size (bp)	Gene	Localization	Identity	Accession number
EC19	2 100	HS.Protein disulfide isomerase related protein	7q35	99%	NM 004911.2
EC24	700	HS.Glucose regulated protein	15q15	98%	NM 0O5313.3
EC26	1 000	HS.Chemokine ligand 1	4q12-q13	99%	BC 011976
EC29	1 600	HS.Inner membrane protein	2p11.2	99%	NM 006839.1
EC30	1 800	HS.Hypothetical protein Loc283241	11q12.3	99%	XM 208579.1
EC31	1 700	HS.genomic DNA, clone	11q	98%	AP 002340.3
EC35	700	HS.BMP-6	15q13-q15	98%	NM 013372.1
EC36	2 200	HS.Glyceral dehyde-3-phosphate dehydrogenase	12p13.31	98%	BC 004109
EC38	1 900	HS.Replication factor C5	12q24.2	99%	NM 007370.2
EC39	2 100	HS.BAC, CloneRP11-1591120		99%	AC 010974.9
EC40	800	HS.Sjogren syndrome antigen B	2q31.1	98%	BC 020818
EC42	2 400	HS.X-ray repair complementing defective repair in Chinese	12q23	99%	AY 034001.1
EC51	400	HS.S100 calcium-binding protein A6	1q21	100%	BC 009017
EC52	1 500	HS.Protein kinase C	19p1.1	98%	NM 002743.1
EC53	1 700	HS.Heat shock 70 ku protein4	5q31.3	99%	XM 114482.2
EC59	1 900	HS.DVS27 related protein	9p24.1	99%	BC 04785.1
EC62	1 500	HS.NAC alpha mRNA	12q2	99%	AY 034001.1
EC63	1 500	HS.Hypothetical protein DJ1042k10.2	2 213.1	100%	HS1042k10

a dose dependent response from 1:30 to 1:2 430 dilution of the murine immunosera, while no inhibitory effects were observed in sera from non-immunized mice. Figure 3 shows the results of serum from one of six immunized mice and one of 4 non-immunized mice.

Using MTT assay, we observed that human TEC growth was inhibited when they were incubated with the immunosera, however, the underlying mechanism was unknown. We therefore studied further the effects of the sera on cell cycle of human TEC with Flow Cytometry. The results showed that the amount of apoptotic cells were increased in the group of immunoserum compared with the group of non-immunoserum at 6 h after treatment (Figure 4). Approximately 20.1% apoptotic cells were seen in the group of immunized serum and only 4.9% apoptotic cells were observed in the group of non-immunized serum. The results further indicated that, in terms of specificity and the effects on human TEC, the immunized sera were suitable to screen the HLCVECs cDNA expression library to obtain endothelium associated antigen genes.

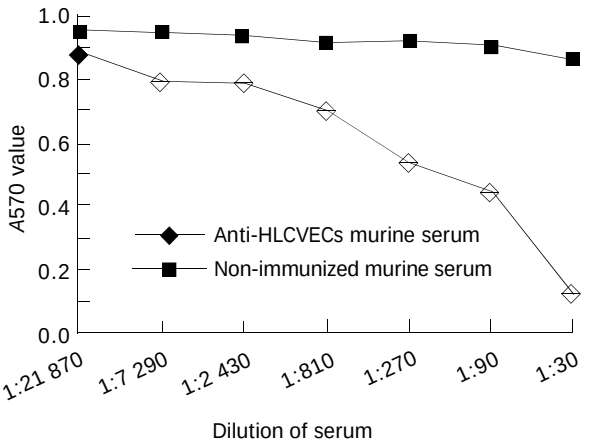

Figure 3 Inhibition of the HLCVECs proliferation by HLCVECs immunized murine serum from 1 of 6 immunized mice and 1 of 4 non-immunized mice. Points are the average of three wells.

Isolation of HLCVECs associated antigens genes by modified SEREX

HLCVEC cDNA expression library with 2×10^6 primary clones was established (Table 2). The 6×10^5 clones were immunoscreened with pooled sera from six immunized BALB/c mice collected on d 56 after inoculation of HLCVECs. Primary screening with 1:500 diluted pooled immunosera yielded 153 positive clones (named EC1 to EC153). After secondary screening with 1:5 000 immunosera and subcloning, a total of 36 positive clones were obtained (Figure 5). These 36 clones were then excised, to phagemide forms and purified *in vitro*. The size of inserts of these positive clones were determined by restriction enzyme digestion with *Eco*R I and *Xho*l I, which yielded inserts sized from 900 bp to 3 600 bp, with an average of about 1 500 bp (Figure 6).

Sequence analysis of the positive clones

Nucleotide sequences of cDNA inserts of 36 positive clones were sequenced. Sequence alignments were analyzed with DNASIS and BLAST software on EMBL and GeneBank. These 36 positive clones represented 18 different antigen genes (Table 3). EC36 was represented by 11 overlapping clones, EC42 by 6 overlapping clones, EC 62 by 4 overlapping clones and the others by a single clone. Of these 18 clones, 3 clones were new unknown genes, 2 of which may be functional genes encoding hypothetical proteins. The other 15 genes were known. However, all of them were first isolated and reported in the endothelial cells of human HCC here. By SAGE database analysis, the 15 genes can be grouped into different classes: (1) 9 of these genes are associated with endothelium and angiogenesis, such

as EC26, EC52, EC59, *etc*, (2) 4 of these genes have been reported previously as tumor antigen genes, such as NAPDH, S100A6, BMP-6 and hsp70, (3) The other genes are involved in genes transcription, protein translation and cell mitosis, such as inner membrane protein gene (IMMT).

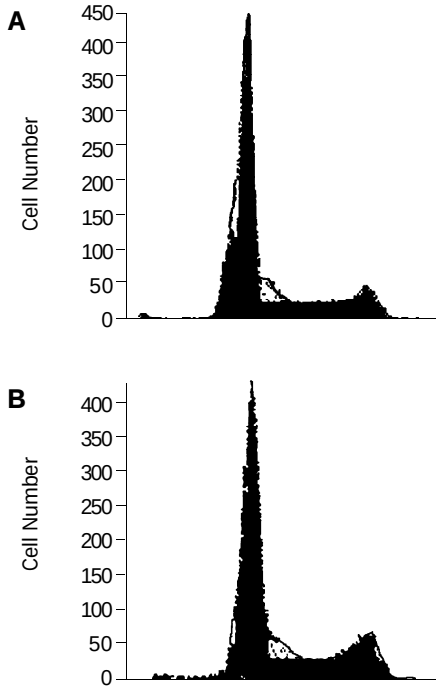

Figure 4 Cell cycle analysis of HLCVECs after treatment with immune sera or non-immune sera. A: HLCVECs treated with immune sera for 6 h; B: HLCVECs treated with non-immune sera for 6 h.

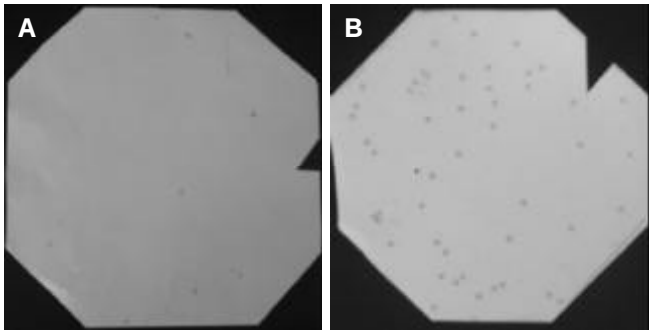

Figure 5 Positive dots of phage clone of screening by immune sera. A: The first cycle of screening by immune sera; B: The second cycle of screening by immune sera.

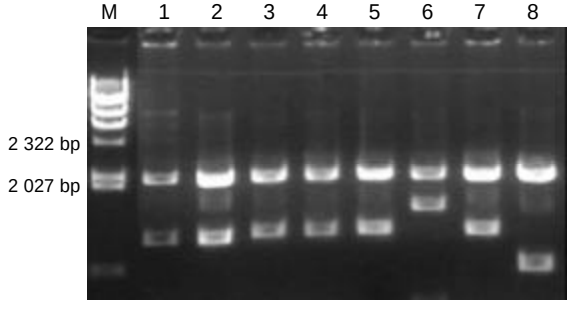

Figure 6 Electrophoresis analysis of enzymatic digestion of SEREX positive clones. M, λ*Hin*d III marker, from above to below: 20 130, 9 416, 6 557, 4 316, 2 322, 2 027, 564 bp; Lanes 1-8, positive clones digested with *Eco*R I and *Xho*l I.

DISCUSSION

Tumor angiogenesis is dependent on biochemical processes mediating the formation and development of the blood capillary network that supplies the tumor. In recent years, increasing evidence has indicated that targeting tumor vasculature is a very promising strategy for the therapy of solid tumor. Identifying molecular markers, target genes and antigen on endothelial cells of tumor vessels, in turn, is critical for the antiangiogenic therapeutical strategy of tumor. A number of researchers have gained some endothelia associated genes from activated HUVECs with cell growth factors and supernatant of cultured tumor cells. But it is difficult to determine tumor specific endothelial genes by these ways, because activated endothelium cannot completely represent tumor endothelium. In 2000, Croix[4] first reported in *Science* that they successful isolated tumor endothelium from human colorectal cancer and gained tumor endothelium associated genes by the method of SAGE. In the present study, to obtain specific endothelium genes of human liver cancer vascular endothelial cells, we isolated and purified endothelial cells from liver tumor tissue of the patients with HCC. These endothelial cells were confirmed to have characteristics of endothelial cells with expressing vWF, CD31, W-P bodies and taking up high level of Ac-LDL, *etc*, and also have the structure of fenestrations found only in tumor endothelial cells. From cDNA expression library constructed with above purified HLCVECs, we isolated 18 endothelia associated genes. Some of them were related to human TEC. Up to date, there have been no reports about the successful isolation of human liver cancer vascular endothelial cells and their specific genes.

Over the past years, efforts have been made to isolate and identify the proliferating TEC genes, including the methods of phage display peptide libraries, SSH and SAGE analysis, *etc*. However, the genes identified using these methods, in terms of their specificity and functions were rarely known. Therefore, in this study, to isolate and identify functional TEC associated antigen genes, we modified traditional SEREX and used it to screen cDNA expression library of HLCVECs. SEREX has recently emerged as a powerful method for identification of human tumor-associated antigen. The identification of tumor antigens with SEREX was based on the existence of autoantibodies in sera of patients[10]. It is not unexpected that the method is applicable to only a limited number of patients because autoantibodies against most antigens can only be detected in 10-30% patients who bear tumors expressing the corresponding antigens. Therefore, a number of modifications have been made since the introduction of SEREX methodology to expand the range of antigens identified. These modifications include using established cell lines instead of tumor specimen to construct the cDNA library, and using allogeneic sera or xenogeneic sera instead of autologous sera as the antibody source. Hideho *et al*., introduced the "cytokine-assisted SEREX (CAS)", which resulted in an enhanced capacity to identify glioma tumor associated antigens (TAAs) with characteristics similar to TAAs identified by traditional SEREX[19-21]. Despite extensive screening of various tumor cDNA libraries with sera from tumor patients, however, identification of human tumor endothelium associated antigens by SEREX have not been reported up to date. To develop a method to screen the endothelium associated functional genes, we first immunized BALB/c mice by injection subcutaneously with high dose purified HLCVECs. Sera from the immunized mice were identified with several fixed methods for their specificity against HLCVECs and the effects on endothelial cells. For example, detection by immunocytochemistry and immunofluorescence showed that the ability of immunosera to bind HLCVECs were higher compared with activated HUVECs when sera were diluted at 1:5 000, whereas the staining of unactivated HUVECs were completely negative. These data

indicated that the immunosera still contained specific antibodies directed against HLCVECs in this dilution. By MTT assay and cell cycles analysis, we have also demonstrated that the growth and proliferation of HLCVECs were significantly inhibited when treated with anti-HLCVECs immunosera in range of 1:30-1:2 430 dilution, while this inhibitory effect of sera from non-immunized (only injected by PBS) mice was not observed. To identify the possible mechanism about anti-HLCVECs proliferation activity of immunosera, we employed Flow Cytometry to analyse cell cycle of HLCVECs treated with the immunoserum. The results showed that there was a 4-fold increase in the amount of apoptotic cells compared with the group of non-immunoserum. These findings suggested that the anti-sera obtained from mice immunized with high dose of fixed HLCVECs presented some functional immunoglobulin with potent antiangiogenic activity. Similar results were also observed by Scappaticci *et al*.[18], who recently demonstrated that vaccination of rabbits with murine endothelial cells yielded immunized sera with antiangiogenic effects *in vitro* and that the mechanism of antiangiogenic effect was provn to be through induction of apopotosis of ECs by polyclonal immunoglobulin in this serum. Furthermore, Wei *et al*.[15] reported also that vaccination of mice with human ECs could induce a specific antiangiogenic immune response with broad anti-tumor activity. In our study, using xenogeneic functional anti-sera from mice immunized with HLCVECs to screen cDNA expression library of HLCVECs, a modified xenogeneic SEREX, we first isolated endothelium associated antigen genes from human liver cancer vascular endothelium.

To isolate TEC associated functional antigens genes, we immunoscreened HLCVECs cDNA expression library by a modified xenogeneic SEREX. Thirty-six positive clones were identified after screening of 6×10^5 clones. Sequencing analysis for homology with the GeneBank and other public databases indicated that these clones represented 18 different genes which were first isolated and identified to be the endothelial genes from human HCC tissues. Three of them were previously not reported new genes, 2 of which may be functional gene encoding hypothetical proteins. There other 15 genes were known. SAGE analysis revealed that 9 of the 15 genes, have been reported as endothelium associated genes and some of them were involved in the proliferation, migration of endothelia cells and the process of angiogenesis. For example, EC26 has 99% homology with chemokine ligand 1(CXCL1), which was implicated having effects on endothelial cells in angiogenesis[22]. EC35 has 99% homology with bone morphogenetic protein-6 (BMP-6), which stimulates angiogenesis and induces migration[23,24]. EC52 may be one of the factors that up-regulate VEGF gene expression during hypoxia[25-27]. The expression of EC59 gene was mostly highly up-regulated in cerebral arteries[28]. Camby *et al*. found that the level of EC51 expression differed markedly in the blood vascular walls according to whether these vessels originated from low- or high-grade astrocyte tumors[29,30]. EC53 had 99% homology with heat shock 70 ku protein (HSP70) and is expressed in human microvascular endothelial cells. The expression of HSP70 is known to increase endothelial cells survival and growth[31-33]. These data indicated that the genes identified in this study using specific and functional antiserum from mice immunized with human TEC, may be functional genes for human endothelial cells and angiogenesis. Furthermore, this approach through screening cDNA expression library with xenogeneic serum containing specific antibodies may serve as an effective strategy for isolation and identification of human TEC genes, which will provide useful marker and targets for tumor anti-angiogenesis therapy.

In summary, the screening of HLCVECs cDNA expression library by using murine immunosera of anti-tumor endothelium yielded 18 functional antigen genes associated with tumor endothelium. These antigen genes may be related to the proliferation, migration and vessel formation of tumor vascular endothelium. Further exploration of these genes and their relationship with tumor angiogenesis would provide a valuable resource for basic and clinical studies of anti- angiogenesis targeting therapy of tumor.

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