·TECHNOLOGY AND METHODS·

Human Esophageal Carcinoma Antigens Screened by Serologic Analysis of Recombinant cDNA Expression Libraries (SEREX)

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[ABSTRACT] BACKGROUND & OBJECTIVE: In malignant transformation, mutant gene products and dysregulated proteins can become tumor antigens and activate immunoreactions. Therefore, auto-antibodies exist in sera of cancer patients. Serologic analysis of recombinant cDNA expression libraries (SEREX) using autologous and allogenic patient sera provides a powerful approach to identify tumor antigens. This study was to identify esophageal cancer antigens with SEREX for serologic diagnosis, gene therapy, and immune therapy. METHODS: Expression library of cDNA from esophageal squamous cell carcinoma was constructed. SEREX screened out 21 positive clones from the 1.6×10^6 clones in the established library. The 21 positive clones were subcloned to monoclonality and submitted to *in vivo* excision of pBluescript phagemids. The nucleotide sequences of cDNA inserts were analyzed with DNASIS and BLAST software on EMBL and GenBank. According to the bioinformatics analyses, serologic immunoreactions of 4 colons in 10 samples of esophageal cancer serum and 10 samples of normal control serum were further detected by SADA. RESULTS: Of the 21 positive clones, 4 had no homology to any known genes, 17 were known fragments which were defined as antigens of esophageal cancer for the first time. The serologic immunoreaction rates of 4 selected antigens, including Ribosomal protein S4, and so on, were 40%, 60%, 70%, and 30%, respectively, in cancer sera, and 0%, 10%, 20%, and 20%, respectively, in normal sera. CONCLUSIONS: Antigens, such as Ribosomal protein S4, are frequently involved in serologic immunoreactions of esophageal cancer. The 21 antigens identified by the present study can be used as potential targets for gene therapy and serologic biomarkers of esophageal cancer.

KEYWORDS: Esophageal neoplasm; Tumor antigen; SEREX; cDNA expression library

Introduction

Esophageal carcinoma is one kind of common malignant tumors with high incidence and high mortality. The esophageal carcinoma in China accounts for 53.86% and 49.26% of the world occurrence and mortality, respectively. Its mortality was the fourth in all tumors. In malignant transformation, mutant gene products and dysregulated protein can become tumor antigens and activate immunoreactions, and thus auto-antibodies can be detected in sera of cancer patients. Study has found that auto-antibodies in sera between cancer patients and normal persons exist significant difference, which also indicates that auto-antibodies may be markers of early diagnosis of breast carcinoma. Serological analysis of recombinant cDNA

expression libraries (SEREX), which has identified about one thousand kinds of tumor antigens, provides a powerful approach to identify tumor antigens by using sera from autologous and allogenic patients. This research adopted SEREX technology to screen and identify the relevant antigens of esophageal cancer, and thus to offer some candidate molecule targets and serum molecule markers for serum diagnosis and immunization therapy of esophageal carcinoma.

Materials and Methods

Materials

The tissues were taken from a 48-year-old male patient, with moderately differentiated squamous cell esophageal carcinoma, T3N1M0, from Cancer Hospital in Linzhou City, Henan Province. The cancer tissues and sera of the patient were preserved at -80℃ for use right after the operation. Ten cases of preoperative sera were taken from esophageal squamous carcinoma patients of Cancer Hospital of the Chinese Academy of Medical Sciences, of which 4 cases were well-differentiated, 5 were moderately differentiated and 1 was poorly differentiated. There were another 10 cases of sera from healthy volunteers of pre-marriage check-up. The sera were stored at -80℃ for use after being collected.

Methods

mRNA extraction from esophageal cancer tissues

500mg of esophageal cancer tissues were extracted according to the instruction of mRNA extraction kit, mRNA PolyATtract System 1000; the extracted mRNAs were dissolved in RNase-free deionized water; the value of A_{260}/A_{280} was measured, and 1% agarose gel electrophoresis (AGE) was performed; 6μ g mRNA was selected for the construction of cDNA library.

Construction of cDNA expression library

ZAP-cDNA@Library Construction Kit purchased from Stratagene Company was used to construct cDNA expression library; thermoscript RT hyperthermia reverse transcriptase (Invitrogen) was used for the synthesis of the first-strand cDNA; and the procedure was performed according to the instruction of Library Construction Kit; the library was stored at 4℃ , without pre-screening multiplication.

Preparation of escherichia coli / bacteriophage lysate

Monoclonal XL1-Blue-MRF host bacterium was selected from the tetracycline plate, and cultivated in LB medium with $MgSO_4$ and maltose until A600 was between 0.4 and 1.0; lactobacillus was collected after 10 min of centrifugation at 3000r/min, and re-suspended and precipitated with 10mmol/L $MgSO_4$ until A600 was about 0.5. Bacteria fluid of 600μ l was selected and added into 7×10^3 pfu λ -ZAP idler bacteriophage, incubated at 37℃ for 15 min, added to 8ml NZY/agarose surface gel, mixed, and spread onto a φ 18cm plate with NZY/Agar as bottom gel and cultured overnight at 37℃ for amplification; then 10ml couple balanced solution (0.1 mol/L $NaHCO_3$, pH 8.3) was added onto the plate, shaken and eluted at 4℃ for 16 h. The eluate was collected, among which, incompletely decomposed *escherichia coli* was broken by ultrasound, and the protein quantity was determined by ultraviolet spectrophotometry.

Coupling between the lysate of escherichia coli and bacteriophage and CNBr-Sepharose 4B column material~

The coupling process was proceeded according to the instruction provided by Amersham Company. After being coupled, the gel medium was balanced with TBS containing 0.02% sodium azide, and then preserved at 4℃ for use. The coupling rate of column material was initially calculated by the following formula: coupling rate = (protein amount in the pre-coupling lysate －protein amount in the post －coupling lysate)/ total protein amount in the pre-coupling lysate.

Absorption of serum specimen

The antibodies that would react with the bacteria and bacteriophage needed to be excluded from the serum specimen before the immunological screening of the library. CNSr-Sepharose 4B column material~coupled with XL1-Blue-MRF host bacteria protein and infected with λ -ZAP idler bacteriophage was used for the pre-absorption of the sera. The antibodies that reacted with the proteins of escherichia coli / bacteriophage were exterminated. The sera were 1:10 diluted with 1 × TBS (with 1% BSA), mixed with the column material coupled with the decomposed proteins of escherichia *coli* and bacteriophage

by the volume ratio of 1:1, slightly shaken and stored at 4℃ overnight, and then the sera were collected. The sera were repeatedly collected for 3 times. The post-absorption sera were collected and diluted 1:50 by 1 × TBS containing 1% BSA. Then 0.02% sodium azide was added to avoid corrosion and the sera were stored at 4℃ for later use.

Testing of the absorptive effects on the serum specimens

The pre- and post-absorption sera were diluted 1:50, then reacted respectively with the nitrocellulose membranes transferred with protein expressed from λ-ZAP idler bacteriophage to test the absorptive effect of the sera. The λ-ZAP idler bacteriophage infected by the XL1-Blue-MRF host bacteria was spread onto a 90mm plate with NZY/agar bottom gel, cultivated at 37℃ until the bacteriophage plaques were barely visible, and transferred onto the nitrocellulose membranes soaked in 10mmol/L IPTG （Merck）at 37℃ to induce expression for 8h; the plate was placed at 4℃ for 2h, the nitrocellulose membrane was taken off, rinsed for 5 times in TBS/T balanced solution (TBS balanced solution with 0.1% tween- 20) and sealed in 1% BSA/TBS at 4℃ over night; then, the nitrocellulose membrane was divided into two parts to react with the sera respectively before and after absorption at room temperature for 1h. The membranes were rinsed with TBS/T for 5 times, and incubated together with diluted 1:2500 goat anti- human IgG（Sigma）secondary antibody marked with alkaline phosphatase at room temperature for 1h; rinsed with TBS/T buffer solution for 5 times afterwards and with TBS for the last time. The membranes were put into the prepared NBT/BCIP substrate solution for color development in the dark room and the background reactions of nitrocellulose membranes before and after absorption of sera were compared.

Library screening

7×10^3 pfu recombinant phage was added into 600μl XL1-Blue-MRF bacteria fluid, incubated for 15min at 37℃, mixed with 8ml NZY gelose surface gel, spread onto a φ 18cm plate with NZY/agar as the bottom gel, cultivated at 37℃ until the bacteriophage plaques became visible, transferred to the nitrocellulose membranes soaked in 10mmol/L IPTG at 37℃ to induce expression for 8h; then the plate was placed at 4℃ for 2h. The autologous sera of nitrocellulose membranes reacted for 2h at room temperature. Other steps were the same as those of testing the absorptive effects on the serum specimens.

The bacteriophage plaques corresponding to the positively stained plaques were the positive bacteriophage clones screened out from cDNA libraries for the first round. The positive clones screened for the first round were mixed and then the second round screenings were carried on. Two nitrocellulose membranes were put on one plate, one was for patients autogenic serum reaction and the other was for the reaction with goat anti-human IgG-AP. The two nitrocellulose membranes underwent simultaneous coloration. The bacteriophage plaques that reacted positively with the auto sera and negatively with goat anti human IgG-AP secondary antibodies were the positive clones screened from libraries in the second time.

Inner cutting of positive clones

The plasmids with insertion sequence were transformed into pBluescript phasmid in the form of single-strand filobactivirus according to the instruction provided by Stratagene Company. Plasmids were extracted by alkaline lysis method after infected with SOLR bacteria, double digested by *EcoR* I and *Xho* I, and then the length of the inserted fragment was initially identified.

Nucleotide sequencing and bioinformatics analysis

Sequencing was completed by Shanghai Boya Biotechnology Company. Analyses of nucleotide sequences obtained from the sequencing were conducted using the data banks of various nucleotides and proteins, mainly including the analysis of the homology of nucleotide sequence, reading frame analysis, the analysis of the homology of protein sequence, SEREX date bank analysis, Locuslink analysis, UniGene analysis and SAGE analysis, etc.

Detection of reaction between antigen clones and serum antibodies by serum antibody detection array (SADA)

Various positive clone bacteriophage and idler control bacteriophage were dotted on the LB agar plate containing 5mmol/L IPTG and 0.8% agrose according to the density of 0.5μl/dot

(500pfu); then the plate was placed upside down at 37℃ over night for amplification; the clones were transferred onto the nitrocellulose membranes to induce expression at 37℃ for 4-5 h. The plate was placed at 4℃ for 2h and then left to react with the foreign post-absorption sera at room temperature for 1h.

Results

The construction of human esophageal carcinoma cDNA expression library

Identification of mRNA of human esophageal carcinoma tissues

About 7.5μg mRNA was obtained from 300mg of esophageal carcinoma tissues, of which the values of A260/A280 and A260/A230 were 2.01 and 1.98 respectively measured by ultraviolet spectrophotometer. The results showed that the purity of mRNA was qualified. An electrophoretic lagging band of purified mRNA was found from the sampling hole to the position of 0.1kb band in the 1% agarose gel, which centered around 0.5-2.0kb, indicating that the mRNA had good quality without obvious degradation (Fig.1).

Construction of cDNA expression library of esophageal carcinoma tissues of 1× 106pfu

5μg mRNA extracted from esophageal carcinoma tissues was used as the template, and 32P was randomly incorporated when synthesized the first-strand and second-strand cDNA. After electrophoresis, autoradiography showed that the length of both the first-strand and the second-strand cDNA was between 0.5-5.0kb, and was mainly centered from 1-3kb (Figure 2). The obtained double- strand cDNA had two adhesive ends of *EcoR* I and *Xho* I after end-filling. The obtained double -strand cDNA fragments by this method were collected after centrifugation according to the molecular size at Sepharose CL-2B gel column. Non-denaturant polyacrylamide gel electrophoresis and autoradiography of the collected cDNA fragments from each tube showed that the length of the fragments of the 5^{th}-11^{th} tubes were more than 500bp (Figure 3). The fragments of the 5^{th}-11^{th} tubes were put together, precipitated, dissolved in 5μl deionized water, and quantitatively measured as100ng/μl by the EB agar plate.

Samples of 100ng were packaged in package protein provided by GigapackⅢ Gold Cloning

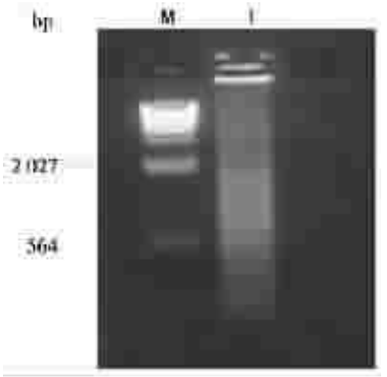

Figure 1 mRNA of esophageal cancer tissue

Lane M: marker digested by λ/*Hind*Ⅲ; lane 1: pure esophageal cancer mRNA.

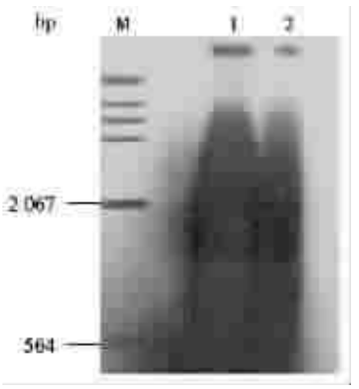

Figure 2 X-ray autoradiograph of first-strand and second-strand cDNA of esophageal carcinoma tissue

Lane M: marker digested by λ/*Hind*Ⅲ; lane 1: first-strand cDNA; lane 2: second-strand cDNA.

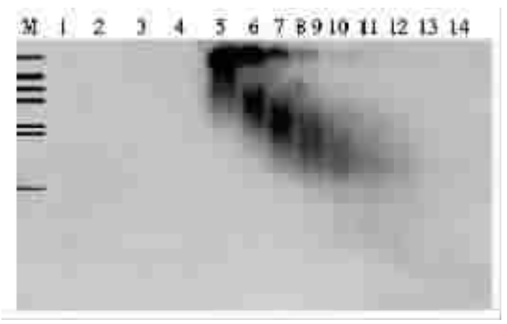

Figure 3 X-ray autoradiograph of cDNA fragments fractionated by sepharose CL-2B gel filtration medium

Lane M: marker digested by λ*Hind*Ⅲ; lanes 1-14: cDNA fragments fractionated by Sepharose CL-2B gel filtration medium and collected after centrifugation.

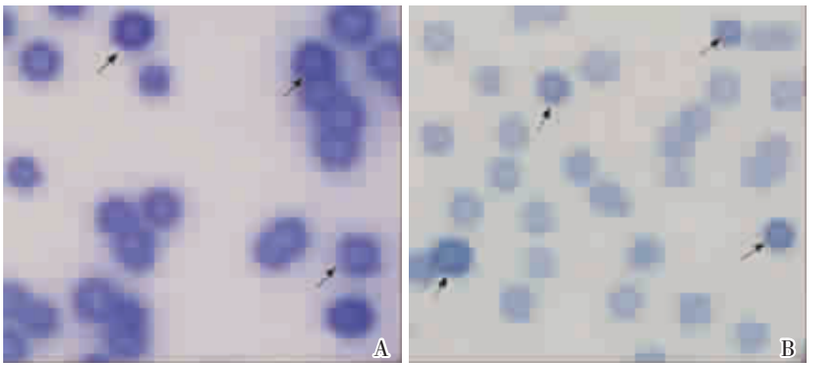

Figure 4 Immunoreaction of library clones with autologous serum

A: Immunoreaction of autologous serum without absorption by proteins of Bacterial Host Strain and wild-type lambda phage.

B: Immunoreaction of autologous serum absorbed by proteins of Bacterial Host Strain and wild-type lambda phage.

The arrows point to positive clones.

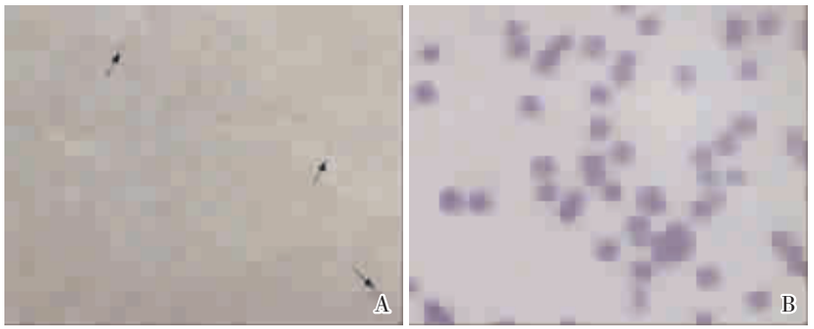

Figure 5 Positive clones of the library screened by autoantibodies in autologous serum

A: primary screening; B: second round screening.

The arrows point to positive clones during the primary screening.

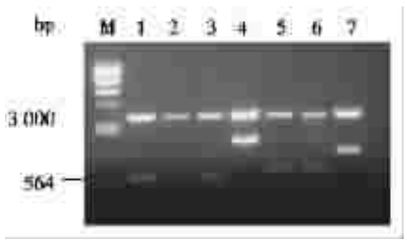

Figure 6 Seven positive clones digested with *Eco*R Ⅰ and *Xho* Ⅰ

Lane M: marker; lanes 1-7: positive clones.

kit after the conjugation with ZAP Express carrier. Original cDNA expression library of human esophageal carcinoma tissue with the size of 1× 106pfu was consequently obtained. The expression library was directly used for antigen screening without amplification.

Screening of the clones reactive to patients sera from cDNA expression library of esophageal carcinoma tissues

Absorption of reacted sera, and exclusion of antibodies reactive to bacterium and bacteriophage

The adopted autologous and foreign sera were both pre-absorbed by CNBr-Sepharose 4B column chromatography coupled with XL1-Blue-MRF host bacteria protein infected with λ -ZAP idling bacteriophage. Figure 4 is a representative figure showing the reactive effects between patients autologous sera with and without absorption. After absorbing, the color intensity of the background plaque was obviously reduced and plaques of positive clones were clear to distinguish.

Screening of positively reactive clones using serum immunology

1×10^6 clones were screened from the post-absorption autologous sera of patients with esophageal carcinoma and 184 positive clones were obtained. Figure 5A shows the staining of the nitrocellulose membranes on which the positive clones

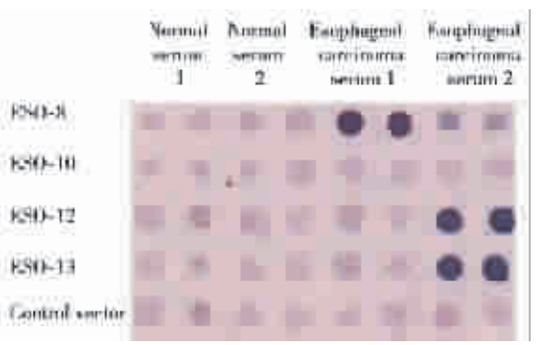

Figure 7 The immunoreactions of 4 positive clones with heterologous sera detected by SADA

Each serologic reaction was parallel detected with two same clones. Blue-black dots represent positive immunoreactions. Vector without insert was used as a negative control.

were initially screened. Seventy-five positive clones were obtained after excluding the artificial positive clones. After being mixed and cultured, the clones were transfer onto the new membranes for the second round of screening and those autologous IgG reaction clones were excluded. Fig. 5B is a representative figure of the positive clones stained in the membranes after the second round. Twenty-one positive clones were obtained in the second round screening.

Homological analysis of representative positive clone genes

Double digestion of positive clones

Internal splicing was performed on these 21 positive clones. The length of the inserted fragments was identified by *EcoR I* and *Xho I* digestion. Figure 6 shows the results of 1% agarose gel electrophoresis of part of the positive clones digested with *EcoR* I and *Xho* I. All of the 21 positive clones contained externally inserted fragments, with the length of 0.5-2.0kb.

Identification and homological analysis of tumor antigenic gene sequence represented by *positive clones*

Positive clones were sequenced and the results showed that the 21 clones represented different antigenic molecules. EST homology comparison in GenBank revealed that 17 clones had higher homology with the EST in GenBank, as shown in Table 1. Among them, 5 cDNA clones contained the total length of cDNA sequences of 5 genes respectively, which were ESO-8-co- transcription factor 4 of human activated RAN polymerase, ESO-10 -human ribosomes protein S4, ESO-12- human *hepatocyte cytochrome C*, oxidase sub-unit Ⅶ, polypeptide 2, ESO-13 -Gluysyhione S-transferase 2 of human microsome, and ESO-15 (receptor 7of interleukin). Among them, the cDNA sequence of ESO-15 was newly found and its registered number was AF373204 in GeneBank, which was subsequently identified as receptor 7 of interleukin. The remaining 4 sequences had no obvious homology with the reported EST or exon sequences of genes in GeneBank.

Serum reactivity between positive clones and normal controls

The reaction between 4 clones with the full length of cDNA sequence and sera of 10 esophageal carcinoma patients and 10 normal people were performed by SADA method. Fig. 7 shows the reaction between 2 patients of esophageal carcinoma and 2 normal controls. The serum of patient 1 reacted with clone

Table 1 BLAST outcome of inserts from positive clones

Clone	Gene	Length of identified nucleotide sequence (bp)	EST homology analysis in GenBank (%)	Function
ESO-1	Homo sapiens desmin (DES)	635	96	Cell structure
ESO-2	Homo sapiens CDP-diacylglycerol—inositol 3-phosphatidyltransferase (phosphatidylinositol synthase) (CDIPT)	595	99	Metabolism
ESO-3	Homo sapiens ferritin heavy chain subunit	573	98	Metabolism
ESO-4	Homo sapiens glutathione S-transferaes pi (GSTP1)	336	100	Metabolism
ESO-7	Homo sapiens cathepsin B (CTSB)	651	99	Metabolism
ESO-8	RNApolymerase Ⅱ transcription cofactor 4	381	99	RNA synthesis
ESO-10	Ribosomal protein S4	802	100	Ribosome protein
ESO-11	Homo sapiens RAN, member RAS oncogene family	703	98	Signal transduction
ESO-12	Homo sapiens similar to cytochrome c oxidase subunit Ⅶa polypeptide 2 (liver) (H. sapiens)	408	100	Metabolism
ESO-13	Homo sapiens microsomal glutathione S-transferase 2 (MGST2)	541	100	Metabolism
ESO-14	Homo sapiens singed (Drosophila)-like (sea urchin fascin homolog like) (SNL)	482	99	Cell mobility
ESO-15	Interleukin 7 receptor (IL-7R)	701	100	Signal transduction
ESO-16	Human calmodulin-Ⅰ (CALM1)	656	97	Signal transduction
ESO-18	Homo sapiens transgelin (TAGLN)	544	100	Cell structure
ESO-19	Homo sapiens fibronectin (FN precursor).	684	98	Cell adhesion
ESO-20	Homo sapiens collagen, type Ⅰ, alpha 1 (COL1A1)	663	94	Cell adhesion
ESO-21	Homo sapiens Humanin (HN)	605	99	Signal transduction

ESO-8, and the serum of patient 2 reacted with clones ESO-12 and ESO-13; while the sera of the 2 normal controls did not react with these 4 clones. The reaction between 4 clones and 20 cases of heterologous sera is shown in table 2. The positive rate of reactions of the 4 clones with esophageal carcinoma patient was higher than that of normal controls. Among them, the positive rates of reactions between No. 8, 10 and 12 clones and the sera of esophageal carcinoma patients were 3 times higher than that of the normal control.

Table 2 Reactions of 4 positive clones with 10 samples of esophageal cancer serum and 10 samples of normal control serum (cases)

Clone	Esophageal cancer sera	Normal control sera
ESO-8	4	0
ESO-10	6	1
ESO-12	7	2
ESO-13	3	2

Discussion

The study on immunologic recognition of tumor focuses more on T cell mediated immunity, especially the effect of CTL. Cell immunity and humoral immunity synergistically exert anti-tumor immune response, thus tumor antigens can induce not only cellular immunologic response, but also specific antibody *in vivo*. It has been confirmed that tumor antigens screened by SEREX, such as MAGE, can not only induce cellular immunologic response, but also stimulate the body to produce antibodies. We applied the technology to study the tumor antigens of esophageal carcinoma, and screened out 21 antigen molecules, among which 17 coding genes had comparatively high homology to known genes, and they could stimulate the immune system of patients with esophageal carcinoma to produce special antibodies. Meanwhile, 4 fragments without any homology to known EST or gene exons were screened out. It has been reported that the peptide expressed by these fragments may be identified by tumor auto-antibodies, because they simulate epi-position of some antigen molecules [7].

As indicated by bioinformatics analysis, among the known antigens we identified, there were types as follows:

(1) Involved in the relevant antigen of esophageal cancer of carcinogenic metabolism. We screened one clone- *cytochrome C*, oxidase sub-unit Ⅶa, polypeptide 2, which may relate to the activation of carcinogen. We screened an esophageal cancer antigen with high homology to GST π, which may paly a role in detoxifying the carcinogen activated in the body. GST- π is an early marker before the esophageal tissues are cancerated and a marker of increased carcinogens that the esophageal mucous membranes are contacted with [8].

(2) Relevant antigen of signal pathway. By adopting SEREX technology, we screened the clone ESO-11, which belongs to the member of ras cancer gene family, and the predicted amino acid sequence of this cDNA contained a GTP binding domain. Ran was initially screened from cDNA library of teratoma [9], which involved in signal transduction of various kinds of tumors. When ran molecule binds with GTPase Activated Protein (GAPs), GTPase activity of ran is induced and binds with GTP, and the signal pathway is activated. If ran permanently lies in ran-GTP binding status, abnormal mitosis would occur in the cells which are relevant to the formation of tumor. Therefore, the high homological protein to ran screened from cDNA expression library of the metastatic sites of esophageal cancer indicates that, the occurrence of esophageal cancer may have the similar mechanism [10]. In addition, ESO-16 clone which belongs to human calmodulin, also called phosphorylase kinase delta sub-unit (PHKD), has involved in the cell growth and the regulation of cell cycle [11].

(3) Relevant antigen of cell adhesion molecules. The key difference between benign tumor and malignant tumor is that, malignant tumor possesses invasive and metastatic capacity. The current researches show that adhesion molecules of cell surface are directly involved in the invasion and metastasis of tumor cells. In our research, we found two clones were cell adhesion molecules, human fibronectin and human type Ⅰ collagen 1. There are many reports of fibronectin on the occurrence and development of tumors. High

n of fibronectin has been found to be
o esophageal cancer and fibronectin
ved in the activation of Erk pathway
cells and accelerating the progress of
eal cancer [12]. One Japanese
group reported that, the type Ⅰ
increased in the sera of patients with
al cancer, and it can be taken as the
arker for esophageal cancer diagnosis
herefore, as a relevant antigen of
type Ⅰ collagen can stimulate the
immunity and at the same time
he corresponding antibody in the sera
s with tumors.

levant antigen of human ribosome
We screened one positive clone
from the cDNA library of the
c sites of esophageal cancer.
k analysis indicated that it has 100%
with human Rps4 mRNA. Some
how that human ribosome protein
closely related to the occurrence and
ent of tumors. For example, rPS2
in the human hepatocarcinoma is up
[14], and RPS3 is involved in the
of cell apoptosis [15]. Therefore, it
nferred that, rPS4 may play an
role in the occurrence of esophageal

A method, we further verified the
ent of these clones, which were
from autologous sera of patients, in
logous tumor antigen reaction in
al cancer patients and normal
Four clones positively reacted with
esophageal cancer patients, and the
ate of ESO-12 was up to 70%, which
that this antigen is universally
in the pathological progress of
al cancer. Besides ESO-13, the
ates of the other three clones in sera
ageal cancer patients were obviously
an those of normal control. This
dicates that antigen may act as the
marker for the diagnosis of
al cancer. In 2005, Wang et al [7]
that 22 prostate cancer antigens were
by a similar method, and the
was 81.6% and the specificity was
when they were used to diagnose
cancer. This was superior to the
PSA diagnosis. Their researches
en selected as the most important
for the molecular diagnosis marker by

New England Journal of Medicine in recent years [16]. In the next step, we will carry on systematic research on these 21 clones. The sample size will be enlarged to probe into the possibility of these antigens as the serologic diagnostic marker for clinical esophageal cancer.

In a word, we obtained tumor antigen relevant to esophageal carcinoma in our study. Because these tumor antigens can induce immune reaction in human body, they may be used as the molecular targets in the immunotherapy of esophageal carcinoma, or as serologic molecular markers in the diagnosis of esophageal carcinoma.

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