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The construction of cDNA library and the screening of related antigen of ascitic tumor cells of ovarian cancer

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Summary

Objective: To construct the cDNA library of the ascites tumor cells of ovarian cancer, which can be used to screen the related antigen for the early diagnosis of ovarian cancer and therapeutic targets of immune treatment. *Materials and Methods:* Four cases of ovarian serous cystadenocarcinoma, two cases of ovarian mucinous cystadenocarcinoma, and two cases of ovarian endometrial carcinoma in patients with ascitic tumor cells which were used to construct the cDNA library. To screen the ovarian cancer antigen gene, evaluate the enzyme, and analyze nucleotide sequence, serological analysis of recombinant tumor cDNA expression libraries (SEREX) and suppression subtractive hybridization technique (SSH) techniques were utilized. The detection method of recombinant expression-based serological mini-arrays (SMARTA) was used to detect the ovarian cancer antigen and the positive reaction of 105 cases of ovarian cancer patients and 105 normal women's autoantibodies correspondingly in serum. *Results:* After two rounds of serologic screening and glycosides sequencing analysis, 59 candidates of ovarian cancer antigen gene fragments were finally identified, which corresponded to 50 genes. They were then divided into six categories: (1) the homologous genes which related to the known ovarian cancer genes, such as BARD 1 gene, etc; (2) the homologous genes which were associated with other tumors, such as TM4SFl gene, etc; (3) the genes which were expressed in a special organization, such as ILF3, FXR1 gene, etc; (4) the genes which were the same with some protein genes of special function, such as TIZ, C1D gene; (5) the homologous genes which possessed the same source with embryonic genes, such as PKHD1 gene, etc; (6) the remaining genes were the unknown genes without the homologous sequence in the gene pool, such as OV - 189 genes. *Conclusion:* SEREX technology combined with SSH method is an effective research strategy which can filter tumor antigen with high specific character; the corresponding autoantibodies of TM4SF1, ClD, TIZ, BARDl, FXRl, and OV - 189 gene's recombinant antigen in serum can be regarded as the biomarkers which are used to diagnose ovarian cancer. The combination of multiple antigen detection can improve diagnostic efficiency.

Key words: Ovarian tumors; CA - 125 antigens; Double hybrid system technology; Gene library; Serological test.

Introduction

Among all ovarian neplasias epithalial ovarian tumor is the first cause of death. It can produce initial curative effect by adopting surgery to reduce the tumor cells and platinum chemotherapy. However, 70% of the advanced patients may result in treatment failure due to the drug resistance which be caused by chemotherapy [1, 2]. It is of important significance to discover the marker for screening early ovarian cancer and the targets of immunotherapy for improving postoperative treatment [3]. This research adopts the technology of serological analysis of recombinant tumor cDNA expression libraries (SEREX) and suppression subtractive hybridization technique (SSH) to screen and identify the ovarian cancer antigen with higher sensitivity and specific degree. Recombinant expression-based serological mini-arrays (SMARTA) methodology was used to detect the positive reaction of corresponding antigen [4-6]. These studies lay the foundation for the search of a new biomarker of ovarian cancer and targets of immunotherapy.

Materials and Methods

Materials

The folloiwng materials were utilized in this study: activation of agarose gel 4B column material of potassium bromide; isopropyl -β-D and IPTG; the extraction kit (Poly real mRNA AT Tract System Prime - a – 1000) and mark System(Prime-a-Gene); CDNA synthesis kit (ZAP – cDNA), cloning kit (ZAP - cDNA Gigapack III Gold) and coli phage cracking liquid; CDNA cut kit (PCR – Select), chromatography laurel (CHROMA SPIN); IgG of alkaline phosphatase sheep tag anti human (Fc), the IgM of alkaline phosphatase people (μ chain), and 5Br,4chlorine,3indole phosphate/nitrogen BcIP/NBT.

The source of specimen

(1) The specimen, which was used to construct the cDNA library, was from the ascitic tumor cells of four cases of ovarian serous cystadenocarcinoma (two cases of Stage IIc, two cases of Stage IIIa, two cases of ovarian mucous cystadenocarcinoma (Stage IIIa), and two cases of ovarian endometrial carcinoma (Stage IIIb) in patients. (2) The mRNA, which was used to prepare the SSH probe, originated from the cancer tissues and the contralateral normal ovarian tissue which was chosen from one case of ovarian serous cystadenocarcinoma (Stage Ia) of patient. (2) The mRNA, which was used

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to prepare the SSH probe, originated from one case of cancer and the contralateral normal ovarian tissue of the patient with ovarian serous cystadenocarcinoma (Stage Ia). (3) The serum for screening the cDNA library originated from eight cases of autologous serum which were constructed to cDNA library, and other five cases of allogeneic serum with the high of IgG which diagnosed the ovarian cancer by pathological examination. (4) The allogeneic serum, which was used for SMARTA, originated from 105 patients with ovarian cancer (not including the allogeneic serum of screening cDNA library). The patients were aged 14-75 years and the average was 47 years. Among them, epithelial carcinoma cases were 85 (54 cases of serous carcinoma, 25 cases of mucinous carcinoma, five cases of endometrial carcinoma, one case of undifferentiated carcinoma); non- epithelial carcinoma were 20 cases (nine cases of germ cell tumor, 11 cases of sex cord stromal tumor); I - II period were 35 cases, III ~ IV period were 70 cases; well-differentiated carcinoma were 24 cases, poorly differentiated carcinoma were 63 cases, and differentiated unknown were 18 cases. All the aforementioned specimens were collected from the untreated patients who were confirmed by pathological examination in People's Hospital. (5) The serum of 105 cases of healthy women, who underwent a physical and without history of tumorigenesis, were considered the normal controls. Their age was 20 ~ 70 years, and the average age was 43 years. The blood serum was stored at 4°C. The entire process lasted two hours. The serum was stored at -80°C partially after it was frozen completely and centrifuged for 15 minutes at 2,000 r/min. Specimen collection was occurred after signed informed consent of the patients.

The construction of cDNA library

The ascites were collected under aseptic conditions. The cells were inoculated in culture flasks after centrifugation. Then the nucleated cells of the non-tumor were removed under gradient centrifugation. After more than 90% of tumor cells were confirmed by cytological examination, Poly AT Tract System 1000 kits were used to extract mRNA according to the manufacturer's instructions. The synthesis of cDNA's first chain used the ZAP - cDNA kit, which contained the enzyme loci of Xho I. The construction and amplification of CDNA library adopted the cloning kits of ZAP - cDNA Gigapack III cold, according to th e manufacturer'sinstructions. The original library was titrated and the storage capacity was calculated according to the number of plaques. The original library was amplified and stored at -70°C.

cDNA library screening through SEREX technology

(1) The adsorption of serum: the serum which was used to screen the cDNA library, was absorbed by the nitrocellulose membrane (NC) which was soaked with E. coli phage cracking liquid. The nonspecific antibody, which reacted to the host bacterium and serum protein phage, was then eliminated. (2) The screening of cDNA library: a diameter of 150 mm flat plaque was spread to prepare bacteriophage plaque after 600 μ host bacteria was infected by 8 x 104 pfu phage. The library was induced and expressed by using the NC which was disposed by the IPTG. The Nc membrane was sealed with a closed liquid of 1% of BSA. Then the c reacted to serum of 1:50 dilution at home temperature two hours after adsorption. It reacted to the goat-anti-human IgG and IgM which were marked by 1:10 000 dilute alkaline phosphatase for one hour at room temperature after washing membrane.Then they were put in the BCIP/NBT liquid to conduct developing in a dark place.The positive plaque was picked and stored at 4°C. According to the above method, the obtained positive plaque was screened with subcloning to obtain monoclone. When the subclone was screened, 2 NC membranes with the liquid of IPTG were used to induce and transfer membrane one after another. The first membrane was induced four hours and the second membrane was induced for eight hours, with the second membrane reacted with IgM2 directly. The bacteriophage plaque, which had a positive reaction to the serum of patients and a negative resistance reaction to IgM2, was positive for the second screen of cDNA library. The positive plaque was picked and stored at 4°C. (3) The internal shear and plasmid extraction of bacteriophage: the host E. coli XL1 - Blue - MRF phage was infected by positive monoclonal phage and auxiliary phage ExAssist together and was sheared inwards. The product was centrifuged. The filamentous phage pBluescript, which completed the internal shear existed in the supernatant fluid. (3) The pBluescript phagocytos was transferred into SOLR and spreaded in the AGAR plate of 37 °C overnight. In order to enlarge cultivation, single colonies were selected, and the plasmid DNA was extracted to identify the the size of the screened antigen gene (exogenous insert fragments) after it was cut by EocR I and Xho I.

Screening of differentially expressed genes with SSH method

The ovarian cancer tissue which was used in SSH and the first and second chain of the matching of normal ovarian tissue were compounded by the cut kit of PCR - Select cDNA. Firstly, the connection of cDNA in the tumor tissue with the joint was treated as the detector to perform the subduction, and then the gene segments that expressed specifically by the tumor were obtained, which indicated that the subduction belonged to positive subduction. Finally, when the negative subduction was conducted by the connection of cDNA in the normal ovary with the joint, the gene segments that expressed specifically by the normal ovarian tissue were achieved. The cDNA fragments, which were obtained by SSH, were purified via CHROMA SPIN chromatography column. After purification and quantitative analysis of ultraviolet spectrophotometer, it was marked by the Pime - a - Gene marker system.

Spot hybridization method and screening of ovarian cancer antigen with nucleotide sequence analysis

The positive plasmid DNA, which was screened by SEREX technology, was applied to the nylon membrane after the denaturation and it was hybridized with the gene fragment, which was prepared by method of SSH, by using the high performance liquid of hybridization kit. Hybridization resulted as positive when it was hybridized with the normal gene fragment. Conversely, the plasmid, whose hybridization resulted as negative, was the one that owned the nucleotide sequence in the differentially expressed ovarian cancer. The plasmid DNA was evaluated by EocR I and Xho I double enzyme. The plasmid with the fragment was detected by nucleotide sequence. The results were analyzed through similarity comparisons and bioinformatic analysis so that the related ovarian cancer antigen gene could be obtained.

The detection of allograft cancer antigen and autoantibody in serum by using the SMARTA method

(1) The adsorption of serum: the λ-ZAP of light phage was transfected to XLl - Blue - MRF7 of host bacterium. When they were amplified and cultivated overnight at 37°C, ten-ml coupling buffer was added. After 12 hours of oscillation and elution, the elution liquid was finally collected. The E. coli with not completely pyrolyis is broken by ultrasonic. According to the instructions manual, it was coupled with the 4B material of agarose gel which was activated by potassium bromide after the quantitative analysis of protein through ultraviolet spectrophotometry. After the serum of 96 of ovarian cancer patients and the serum of 96 of normal controls, which were waiting for the inspection, they were diluted with dihydroxy methyl amino (1:10) which contained 1% BsA, and mixing the cracking liquid column of E. coli. The oscillation at 4°C was maintained overnight. Then the serum was collected after the elution.

(2) The serological detection: the reaction between the related antigens of ovarian cancer which was screened (including positive phage liquid of related antigen gene of ovarian cancer) and the serum of waiting for detection showed that it was positive when the spot color with the positive of phage liquid was stronger than the spot (no-load phage liquid) with the negative control of phage liquid. It reacted positive between the related antigen and corresponding of autoantibody response of serum, namely ovarian cancer antigen and corresponding autoantibodies in serum (IgG and IgM with the markers of alkaline phosphatase which were used to distinguish them).

Statistical methods

Data processing and analysis were performed utilizing MATLAB7.0 software. The comparison of count data were analyzed by the Chi-square test ($\chi^{2)}$. Logistic regression analyis was used to assses the value of conjoint analysis of the related antigen in the diagnosis of ovarian cancer.

Results

The construction and amplification of cDNA library

The original capacity of cDNA library was 1.8 x 106 pfu/ml. After the amplification, the capacity was 8.0 x 109 pfu/ml, namely, the cDNA library of phage owned the high performance.

cDNA library screened by SEREX technology

In the first round of serological screening, 261 of bacteriophage plaques were achieved. Then, the second round of subcloning was conducted. After two rounds of screening, 245 of positive monoclonal phages were obtained and they accepted the internal shear immediately. They all get the single colony with pBluescript phage. The recombinant plasmid DNA was extracted after expanding each colony. The 245 of positive plasmid clones, which were appraised by enzyme digestion, includes the exogenous inserted fragment. The size of fragment was 0.5~2.0 kb.

Spot hybridization and the analysis of nucleotide sequence

The 245 plasmid DNA and the SSH probe of marking, which were screening by two rounds of serological, was detected by spot hybridization. The results showed that 59 plasmids had significantly positivity in the cut hybridization probes and had negative reaction in the reverse cut hybridization. It was preliminarily confirmed that these 59 plasmids stand for the candidate antigen genes that were related to ovarian cancer.

The nucleotide sequencing results of 59 plasmids were sent for the similarity comparisons and bioinformatics analyses. It was found that the antigen gene fragments of 59 candidates of ovarian cancer represented 50 genes, which were divided into six categories: (1) the homologous genes of the known related gene of ovarian cancer; (2) the homologous genes of related gene of other tumor; (3) the genes were expressed in some special groups; (4) the homologous genes of some protein gene with special function; (5) the homologous genes of the source of embryonic genes; (6) the unknown genes with no homologous sequence and comparison in the gene pool of GeneBank.

Table 1. — *The recombinant antigen of ovarian cancer and positive reaction of autoantibody of IgG corresponding in serum.*

Gene	1 serum carcinoembryonic (n=105)		0 normal serum (n=105)		χ^2	*p* value
	Case	Positive rate (%)	Case	Positive rate (%)		
TM4SF1	30	28.57	10	9.52	14.251	0.008
C1D	23	21.90	7	6.67	7.719	0.004
BARD1	23	21.90	5	4.76	10.455	0.006
FXR1	24	22.86	8	7.62	8.743	0.002
OV-189	33	31.43	14	13.33	9.874	0.001

Table 2. — *The recombinant antigen of ovarian cancer antigen gene and the positive rate of corresponding IgG autoantibody response in serum.*

Gene	Serum carcinoembryonic (n=105)		Normal serum (n=105)		χ^2	*p* value
	Case	Positive rate (%)	Case	Positive rate (%)		
TIZ	27	25.71	9	8.57	10.975	0.012
FXR1	29	27.62	12	11.43	9.399	0.019
OV-189	19	18.09	8	7.62	4.562	0.034

Serology detection of the related antigen of ovarian cancer

SMARTA method was used to detect the reaction of corresponding autoantibody in serum, with which 50 cases of related antigen of ovarian cancer was compared with the serum of 105 cases of ovarian cancer patients, and the serum of 105 cases of normal control women, respectively. The positive rate, which the recombinant and fusion antigen of phage of TM4SF1, C1D, BARD1 gene, FXR1, OV – 189 genes were compared with the corresponding IgG autoantibody response in the cancer serum and the normal serum, respectively, showed a statistical significance ($p < 0.01$). When the recombinant antigens of gene TIZ, FER1 together with OV-189 and the related positive rate of IgM autoantibody reaction in the tumor serum were compared with the normal serum, respectively, the difference showed statistical significance ($p < 0.05$), as reported in Tables 1 and 2.

The reaction conditions of recombinant antigen of ovarian cancer antigen gene and corresponding autoantibody in serum of ovarian cancer patients in different clinical pathological characteristics: the recombinant antigen of FXR1, OV - 189 gene and the positive rate of corresponding IgG autoantibody response of Stages I–II of ovarian cancer patients in serum was higher than Stages III–IV. They were compared respectively with each other and showed statistically significant differences (p values were 0.042, 0.025).

The recombinant antigen of OV - 189 gene and the positive rate of corresponding IgG autoantibody response of ovarian cancer patients with well differentiated in serum was higher

Table 3. — *The corresponding IgG autoantibody in serum of ovarian cancer patients and the positive rate of recombinant antigen of ovarian cancer antigen gene in different clinical pathological characteristics.*

The clinicopathological features	Total cases	TM4SF1		C1D		BARD1		FXR1		OV-189	
		case	Positive rate (%)	case	Positive rate (%)	case	Positive rate (%)	case	Positive rate (%)	case	Positive rate (%)
Clinicopathological features											
Epithelial	85	25	29.41	19	22.35	19	22.35	14	16.47	25	29.41
Non-epithelial	20	6	30	4	20	5	25	8	40	7	35
Clinical stage											
I-II period	35	11	31.43	6	17.14	8	22.86	13	37.14 [a]	17	48.57[a]
III-IV period	70	16	22.86	15	21.43	15	21.43	9	12.86	13	18.57
Pathological differentiation											
Well differentiated	24	3	12.5	5	20.83	7	29.17	7	29.17	17	70.83[b]
Middle and low differentiated	63	25	39.68	20	31.75	17	26.98	19	30.16	14	22.22

Note: "a" signifies the comparison with III-IV period, $p < 0.05$ "b" signifies the comparison with middle low differentiation $p < 0.05$

Table 4. — *The corresponding IgM autoantibody in serum of ovarian cancer patients and the positive rate of recombinant antigen of ovarian cancer antigen gene in different clinical pathological characteristics.*

The clinicopathological features	Total cases	TIZ		FXR1		OV-189	
		Case	Positive rate (%)	Case	Positive rate (%)	Case	Positive rate (%)
Clinicopathological features							
Epithelial	85	22	25.88	22	25.88	15	17.65
Non-epithelial	20	5	25	8	40	4	20
Clinical stage							
I-II period	35	16	45.71 [a]	18	51.43 [a]	9	25.71
III-IV period	70	10	14.29	12	17.14	11	15.71
Pathological differentiation							
Well differentiated	24	10	41.67	10	41.67	5	20.83
Middle and low differentiated	63	19	30.16	21	33.33	15	23.81

Note: "a" signifies the comparison with III-IV period, $p < 0.05$

than middle low differentiation. The comparative difference was statistically significant ($p = 0.001$), as shown in Table 3. The recombinant antigen of TIZ, FXR1 gene and the positive rate of corresponding IgG autoantibody response of Stages I–II of ovarian cancer patients in serum was higher than Stages III–IV. They showed statistically significant differences (p values were 0.021 and 0.021), as shown in Table 4.

The value of the corresponding autoantibody, which was in correlation with antigen that related to ovarian cancer, was analyzed and applied in the diagnosis of ovarian cancer. The results were as follows: combining the clinical value of IgG and IgM autoantibody in the diagnosis of ovarian cancer (which were produced by the recombinant antigen of this six gene: M4SF1,C1D, TIZ, BARD1, FXR1, and OV-189) with Logistic regression analysis, the diagnostic value of autoantibody repertoire combined with CA125 was analyzed. As a result, when conjoint analysis of relevant IgG autoantibody, which was in correlation with the recombinant antigen of gene TM4SF1, C1D, TIZ, and FXR1 was conducted, and more than three of them were predicted as positive autoantibody repertoire, the diagnostic results of the sensitivity, specificity, and accuracy of ovarian cancer were 68%, 81%, and 75%, respectively, in the diagnosis of ovarian cancer. Sensitivity, specificity, and accuracy were 85%, 80%, 81%, respectively, and sensitivity and accuracy were obviously improved combining CA125 with the spectrum of autoantibodies.

Discussion

Ovarian cancer is one of the three largest tumors in gynaecology and has the worst prognosis, because its onset is concealed by a lack of specific signs and symptoms. Patients that are first visited are already in an advanced stage [7]. Currently the diagnosis of ovarian cancer mainly relies on conventional physical and pathological examination, due to the lack of more effective means [8, 9]. Therefore searching for the specific antigen of ovarian tumor, or related antigen for the early diagnosis of ovarian cancer has important theoretical and practical significance.

Since 1991, when Boon [10] used the T cell clone technology to find and identify the first melanoma antigen, there are a variety of ways to screen the tumor antigen. Sahin *et al.*, [11] in 1995, founded the technology of Serological Analysis of Recombinant cDNA Expression Libraries (SEREX).

The basic steps of this technology include the establishment of cDNA expression library; screening the cDNA expression library with the serum of patient; positive cloning

sequencing; testing the expression of positive clone in normal tissues and various kinds of tumors. The immunogenicity of tumor antigen is weak in patients with tumor. It cannot activate the effective anti-tumor immune response in the patient's body, so the antibody level in serum of tumor patients is relatively low. The key point of SEREX is to obtain the display of all cells protein antibody in serum and antibody serum with high titer. The tumor antigen, which has been screened, belongs to the antigen which can be identified by the B cell antigen. Most antigen molecules are many full-length cDNA, both can induce the humoral and cells immune.

This study used SEREX technology and a variety of pathological ascites tumor cells to construct a cDNA library, and combining with the SSH method to screen the related antigen gene of ovarian cancer to ensure the diversity of antigen gene and improve the sensitivity. After screening analysis, 59 candidate antigen gene fragments of ovarian cancer represented 50 genes. These proteins of genes encode includes protein involved in cancer gene, the zinc finger protein and the protein on the surface of cell membrane, and some functions of unknown protein. Such as BARD1 is a protein, with a certain ring structure domain, which is related to the tumor suppressor genes BRCA1. The gene mutations of BARD1 can destroy the structure of the compound of BRCA1/BARD1 and lead to the tumor [12]. The recombinant antigen of TM4SF1 gene is a transmembrane protein [13]. The recombinant antigen of TIZ gene is a zinc finger protein which can restrain the related factor 6 (TRAF6) of tumor apoptosis and participate the regulation of TRAF6 for osteoclast differentiation [14]; OV - 189 is a protein of unknown function. The role of these proteins in the development and mechanism of ovarian cancer need further research.

SMARTA owns many features, such as high flux, good repeatability advantages, and so on. This experiment detected the serum of related antigens of 50 ovarian cancers by using SMARTA. The results show that the comparison is remarkable between the recombinant and fusion antigen of phages of TM4SF1, C1D, BARD1, FXR1, OV - 189 gene and the positive rate of corresponding IgG autoantibody response in serum of cancer and normal serum. The comparison was also remarkable between the recombinant antigen of phages of TIZ, FXR1, OV - 189 gene and the positive rate of corresponding IgM autoantibody response in serum of cancer and normal serum. These show that the occurrence and development of ovarian cancer in each stage has its related antigens with specific open expressions; searching for their autoantibodies can provide the important serum markers for diagnosing and screening. In addition, the value of a single of tumor markers is limited for cancer diagnosis, combining multiple markers that can improve the sensitivity and accuracy of the diagnosis [15]. The related antigen of ovarian cancer by joint analysis and the positive results of corresponding IgG, IgM antibody can greatly improve the screening of ovarian cancer and accuracy of diagnosis. In conclusion, this study provides a solid foundation for exploring the effect of related antigen of ovarian cancer in diagnosis, immune treatment, and the application of disease detection.

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