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CONSTRUCTION AND EXPRESSION OF PROKARYOTIC EXPRESSION VECTOR OF TIA-ASSOCIATED ANTIGENS BY SEREX SCREENING

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Abstract: Objective: To construct the prokaryotic expressing vectors of cDNA insert fragments for TIA, and recombine GST-fusion proteins.Methods: A phage cDNA library of human aortic endothelial cell line was screened using sera of 19 patients with TIA, the cDNA insert of clones incorporated in pBluescript was cleaved by EcoRI/BamHI/SmalI and XhoI, and the insert fragments were isolated by 1% agarose gel electrophoresis. The expression plasmids of glutathione-S-transferase (GST)-fused proteins were constructed by recombining the cDNA sequence into pGEX-4T-1, 2, 3. The inserted DNA fragments were ligated in frame to pGEX-4T vectors by using Ligation or In-Fusion protocol. The reaction mixtures were used to transform ECOS™ competent E. coli BL-21 and appropriate recombinants were confirmed by DNA sequencing as well as protein expressions through SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The antigenic proteins succeeded in GST-tagged proteins recombinant could be candidate antigens. Results: 21 recombinant pGEX-4T expression vectors containing cDNA sequences of target genes were obtained from 70 SEREX antigens, and the expression of the GST-fusion proteins were shown on SDS-polyacrylamide gels stained with Coomassie staining, and thus, which could be as candidate antigens for further study. Conclusion: Recombination pGEX-4T expression plasmids could be constructed and expressed successfully by Ligation and In-Fusion methods, which may provide basis for the extraction and purification of these antigenic proteins aiming to identify serum antibodies in further study

Keywords: TIA; SEREX; genetic recombination; prokaryotic expression vector; GST tagged protein

INTRODUCTION

SEREX (serological identification of antigens by recombinant DNA expression cloning) was proposed and developed by Professor Sahin, a German serological immunologist[1], to screen disease antigen genes using autologous serum. In the past, it was mostly used to identify tumor-associated antigens, to date, more than 2,700 tumor-associated antigens have been discovered[2-7]. In recent years, the application of SEREX technology in autoimmune diseases has also been reported[8,9]. The suitability of the SEREX approach to define autoantigens associated with human autoimmune diseases had been verified[10].TIA (Transient ischemic attack) is a high-risk signal and a high-level early warning event of aCI (acute cerebral infarction). The detection of specific and sensitive TIA biomarkers can avoid the occurrence of aCI after TIA[11,12].

In the previous study, 70 recombinant cDNA clones pBluescript plasmids were screened, and the target gene was inserted into the prokaryotic expression vector plasmid pGEX-4T-1, 2, 3 by Ligation and In-Fusion gene recombination technology, and transformed into *E.coli* BL-21, the expressed product was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then identified by restriction enzyme digestion and DNA sequencing. Successfully recombined pGEX-4T vector inserts can be used as candidate target antigens for further investigation of serum antibodies.

1. MATERIALS AND METHODS

1.1 Materials

1.1.1 Serum and cDNA library

19 randomly selected TIA serum samples from Chiba Rosai Hospital. Human aortic endothelial cell λZAPII phage cDNA expression library was purchased from Stratagene (La Jolla, CA): Uni-ZAP XR Premade Library (1 ml, – 80 ℃), initial library size It is 2.0 × 109 pfu / ml (number of plaque-forming units / μl). The recombinant expression cDNA clone (cDNA expression library) was constructed on the Uni-ZAP XR phage vector (Uni-ZAP XR phage vector).

1.1.2 Enzymes and reagents

Escherichia coli (E. coli) XL1-Blue MRF 'strain was purchased from Stratagene (Bacterial Glycerol Stocks: – 80 ℃), Tetracyclin resistance; E. coli SOLR strain was purchased from Stratagene (Bacterial Glycerol Stocks: – 80 ℃), kanamycin (kanamycin) resistance. Prokaryotic expression vector pGEX-4T-1 (or 2, 3) was obtained from GE Healthcare Life Sciences. ECOS competent *E.coli* BL-21, Ligation-Convenience Kit and Blunting-Convenience Kit was purchased from Nippon Gene Company. In-Fusion HD Cloning Kit was purchased from Clontech Company. In-Fusion-pGEX-4T primer was ordered from Eurofins ProductService. Other common chemicals and consumables were bought from Sigma-Aldrich, Thermo, TaKaRa or Nippon Gene.

1.2 Methods

1.2.1 Immunoscreening of target genes

The serum of 19 TIA patients was subjected to three rounds of SEREX screening of the cDNA expression library of human aortic endothelial cells: 2 μL of E. coli XL1-Blue strain was extracted and added to 10 ml LB medium with a concentration of 12.5 μg / ml tetracycline at 30 ℃ , Centrifuge at 210 r / min, shake overnight in an incubator; take a human aortic endothelial cell cDNA expression library λZAPII phage dilution 10 μL and XL1-Blue 600 μL (A600 = 0.5) standby bacterial solution; mix the plate and culture until the Plaque formation; the plate was inverted and preheated at 37 ℃ for 1 h. The NC membrane was pretreated with 10 mmol / L IPTG solution for 30 min and then covered on the surface of the plate. It was incubated at 37 ℃ for 2.5 h and the plaque was transferred to the NC membrane. Top; gently peel off the NC membrane and immerse it in TBS-T [20 mmol / L Tris–HCl (pH 7.5), 0.15 mol / L NaCl, 0.05% Tween 20] buffer for 3 times; the NC membrane is 1% free of protease After BSA blocking, transfer to 1: 2000 diluted TIA patient serum (primary antibody) and shake slowly for 1 h; NC membrane was washed with TBS-T and soaked in 1: 5000 diluted HRP-labeled goat anti-human IgG (two Incubate for 1 h in the anti-); immerse the NC membrane in the NBT / BCIP developing solution in the dark to carry out the color reaction. To obtain purified positive monoclonals, refer to the first round of screening methods, and dilute the titer of the cDNA expression library for the second and third round of screening.

Prepare a mixture of 100 μL XL1-Blue MRF '(A600 = 1.0), 125 μL recombinant cDNA positive monoclonal and 0.5 μl ExAssist helper phage, add to 3 ml LB culture solution, and incubate at 30 ℃ with shaking for 2.5 ~ 3 h After heating and incubating in a dry constant temperature metal bath at 65-70 ℃ for 20 min, centrifuge at 1000 × g for 15 min; obtain pBluescript phagemid from the supernatant; transfect the strain E. coli SOLR, Plates were incubated at 37 ℃ and incubated overnight; the transformed positive monoclonal pBluescript plasmid was screened by Ampicillin.

1.2.2 Recombinant protein expression vector: Construction of expression vectors by Ligation

Using DNA Ligation gene recombination technology, the pBluescript SK (+) plasmid vector carrying SEREX antigen gene (insertion fragment of cDNA) was transformed into GST fusion protein expression vector pGEX-4T-1, 2, 3. Selection of suitable pGEX-4T vector based on sequence analysis of inserted fragment DNA sequencing results.

1.2.2.1 Midprep pGEX-4T vector plasmid

Extract pGEX-4T-1 10 µl, pGEX-4T-2 10 µl, pGEX-4T-3 5µl × 2 from glycerol bacteria stored at –80 ℃, add 4 sterile conical flasks containing 90 mL LB medium, which were shaken overnight at 37 ℃; 90 mL bacterial solution was divided into two 50 ml pointed bottom centrifuge tubes and centrifuge (8000 rpm, 3 min, 25 ℃), discard the supernatant, collect the precipitate, wash the cells with 15 ml 1 × PBS buffer, combine them into a 50 ml centrifuge tube, and centrifugate again (8000 rpm, 3 min, 25 ℃), discard the supernatant and retain the precipitate; re suspend the precipitate with 4 ml of cell suspension, add 4 ml of cell lysate and turn it for 6-8 times until the mixture becomes transparent and viscous, then add 4 ml of refrigerated Neutralization Solution and turn it for 4-6 times; add 3 ml of binding solution to the neutralized cell lysate, turn the centrifuge tube for 1-2 times manually, and then insert the GenElute HP Midprep Binding Column into the multi-head vacuumizing device, add 4 ml column preparation solution twice to wash the column and filter it, the Binding Columns are transferred into the new collection tube, 1 ml Elution Solution is added to the column, stand still for 1 min, then centrifuge (3000 rpm, 5 min, 25 ℃), harvest the plasmid DNA in the collection tube. Take 2 µ l plasmid DNA and measure the concentration of plasmid DNA by ultraviolet absorption method.

1.2.2.2 Double digestion pGEX-4T vector

Restriction enzymes (EcoR I / Xho I, BamH I / Xho I, or Sma I / Xho I) reaction system 40µl / tube, digested at 37 ℃ overnight and then agarose gel electrophoresis: add 8µl 6 × Gel Loading Dye to each tube and mix After that, 48µl of the mixed solution was equally distributed in two adjacent lanes of 1% agarose gel (DNA Marker 10ul; electrophoresis solution was 1 × TBE Buffer; voltage 100 v). Prepare Spin Columns: Insert Spin columns into 2 ml collection tubes, add 100µl of double-distilled water to centrifuge (15,000 rpm, 5-10sec, 25 ℃) and transfer to new collection tubes; cut the target band under the UV transilluminator and place it Prepare the Spin Columns, centrifuge (15,000 rpm, 10 min, 4 ℃), harvest the linear fragment of pGEX-4T vector in the collection tube.

1.2.2.3 Isolation and purification of target genes

According to the sequence analysis of the insert cDNA, select the appropriate digestion combination for separation and recovery to maximize the integrity of CDS. Restriction enzyme (EcoR I / Xho I, BamH I / Xho I, or Sma I / Xho I) reaction system 20µl / tube. After digestion at 37 ℃ for 2 h, perform agarose gel electrophoresis: add 4µl of 6 × Gel Loading Dye to each tube, mix well, and load in the lane of 1% agarose gel. For the rest, refer to pGEX-4T vector fragment recovery. Using the Gene Ladder Wide 2 DNA amount (ng / 5µl) correspondence table, using the EtBr fluorescence intensity method, the DNA of the inserted fragment was quantified by measuring the fluorescence intensity of the EtBr molecule.

1.2.2.4 Purified target fragment and pGEX-4T vector linear fragment Ligation

For the cDNA fragments isolated by EcoR I / Xho I, use Ligation-Convenience Kit; for the cDNA fragments isolated by BamH I / Xho I, use Ligation High ver. 2; for the cDNA fragments isolated by Sma I / Xho I, use Blunting-Convenience Kit. Ligation product ligation reaction system 6 µl / 0.2 ml PCR Tube (insert fragment cDNA 2µl, pGEX-4T vector 1µl, Ligation Kit 3µl). The mixed solution was placed in a PCR instrument, reaction conditions: 16 ℃, (Ligation-Convenience Kit:> 3 h; Ligation High ver. 2: = 30 min; Blunting-Convenience Kit: = 1 h). Melt the

competent *E.coli* BL-21 on ice, mix 6μl of the ligation product with 26μl BL-21, and let it stand on ice for 5-10 minutes. Then put it in a constant temperature metal bath at 42 ℃ for 1 min, transform competent cells under heat shock, take the transformed product and spread it on the LB agar plate, spread it on the LB agar plate, incubate at 37 ℃ overnight, select positive clones.

1.2.3 Recombinant protein expression vector: In-fusion

In-Fusion gene cloning technology recombinant pGEX-4T vector is suitable for insert CDS containing EcoR I / Xho I, BamH I / Xho I and Sma I / Xho I restriction sites and using the above restriction enzymes can not obtain the complete CDS target gene. According to the sequencing results of inserts, digestion analysis and pGEX-4T vector map, use the online support tool provided by clontech company (http://bioinfo.clontech.com/infusion) to design primers: Forward primer Forward primer-GTGGATCCCCGAATT ... AAAAGCTGGAGCTCC; Reverse primer -GATGCGGCCGCTCGA ... CGAATTGGGTACCGG. The pGEX-4T vector was digested with EcoRI / XhoI and subjected to agarose gel electrophoresis. The DNA amount of the vector fragment was estimated by EtBr fluorescence intensity method, and the linear vector fragment was recovered by excising the gel. Using the pBluescript plasmid carrying the SEREX antigen gene sequence as a template, the forward and reverse primers are added, and the inserted cDNA fragment is amplified under the action of hot start KOD-Plus. Total PCR reaction system 30 μl / tube, reaction parameters: pre-denaturation at 94 ℃ for 2 min, denaturation at 94 ℃ for 15 sec, annealing at 64 ℃ for 30 sec, extension at 68 ℃ for 3 min, amplification for 30 cycles, total reaction time: 2 h 13 min. Add 6 μl of 6 × Gel Loading Dye to each tube of PCR product (30 μl), mix it, and then load it into the lane of 1% Agarose large gel for agarose gel electrophoresis. The method of purification and recovery refers to the recovery of pGEX-4T vector fragments. Take 1μl of purified PCR product, dilute it to 10μl / tube with Milli-Q water, add 2μl of XL-DNA Ladder 100 plus and mix well, then add the sample to 1% agarose gel for agarose gel electrophoresis, according to XL -DNA Ladder 100 plus DNA amount (ng / 5μl) correspondence table to determine the DNA content of the sample. According to the ratio of the target gene to the carrier in the molar ratio of 2: 1, connect it in the 6μl reaction system at 16 ℃ for more than 3h. Take 6μl of the ligation product to transform competent cells under heat shock, spread the transformation product on a LB agar plate by spreading the plate method, incubate at 37℃ overnight, and obtain positive clones by screening.

1.2.4 Protein electrophoresis (SDS-polyacrylamide gel electrophoresis) to identify recombinant plasmids

After the amplification of the recombinant plasmid, 100 μl of each bacterial solution was divided into two 1.5 ml Reaction tubes (50 μl / tube), and then 450 μl of LB-Ampicillin Broth was added to each tube to 37 ℃, Shake the bacteria at 260 rpm for 1 hour. Then, 10μl of 5 mM IPTG (final concentration of 0.1 mM) was added to one tube of bacterial solution in each sample to induce GST protein expression, and the other tube of bacterial solution was used as a blank control, and the culture was continued for 2.5 to 3 h. Centrifuge the bacterial solution (8,000 rpm, 3 min, 4 ℃), discard the supernatant, collect the precipitate, wash the cells with 1 ml 1 × PBS Buffer, centrifuge again (8,000 rpm, 3 min, 4 ℃), completely remove the supernatant, Keep the precipitate; after fully resuspending the precipitate, place it in a 105 ℃ constant temperature metal bath for heat treatment for 3 min, and then immediately centrifuge at low temperature (4 ℃) for 1 ~ 2 sec. SDS-polyacrylamide gel electrophoresis analysis of expressed products: 11% separation gel; Protein Marker 8μl; 40 mA; 110 min / 2 gels. Take 10μl of each sample and load it in the stacking gel lane. SDS-PAG was placed in CBB staining solution (CBB staining solution) and slowly shaken for 1 h, then decolorized in decolorizing solution overnight. The next day, observe whether

there are bands of IPTG-induced GST-fusion proteins.

1.2.5 DNA sequencing to identify successfully expressed plasmids

Miniprep recombinant plasmid in the same way as pBluescript plasmid and agarose gel electrophoresis to obtain a recombinant plasmid with a larger molecular weight than pGEX-4T empty vector (pGEX-4T empty vector: 4,900bp). After identification, the plasmids with the corresponding size fragments were selected and sequenced. Recombinant plasmid DNA sequence detection: forward primer pGEX-F 22mer (100μM): CGACCATCCTCCAAAATCGGAT; reverse primer pGEX-R 22mer (100μM): CAGATCGTCAGTCAGTCACGAT, reaction system 14 ul (stored at 4 ℃), including Recombinant plasmid DNA 0.5 ug , 6.4pM Primer (4 ul of 1.6uM Primer), appropriate amount of DDW. The cDNA sequence homology search (Homology Search), coding region and reading frame are analyzed to identify whether the insertion direction and reading frame of the target gene are correct. At the same time, the sequencing results predict the molecular weight of the GST fusion protein according to the following formula.

Predicted size of the product (Total size) = product size + GST protein size

product size = Amino acid number × 110; GST protein size = 25,000 KDa;

Amino acid number = Base number/3; Base number = Length of CDS of insert fragment

2. RESULT

2.1 TIA sera screening of CDNA expression clones

Through three rounds of SEREX screening, 172 pBluescript SK (+) plasmids carrying SEREX antigen gene fragments (recombinant cDNA) were obtained. The phage cDNA library (plaque) was transferred to the NC membrane, and the expression of recombinant cDNA was induced by IPTG. The color reaction between the positive clone and the negative clone was significantly different from that of the negative clone: the negative clone appeared light Phage plaque, while the positive clones showed a clear purple color (Figure 1).

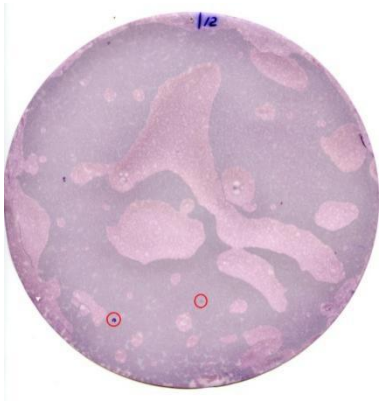

Figure 1a. Results of 1st Screening by SEREX (Plate size 150 mm).

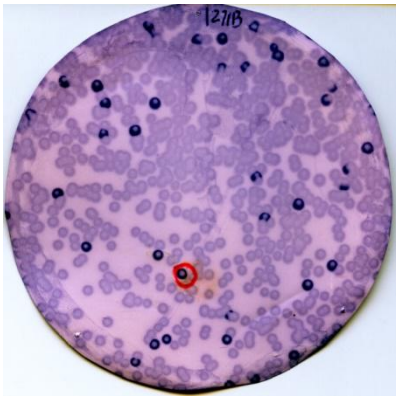

Figure 1b. Results of 2nd Screening (Plate size 100 mm).

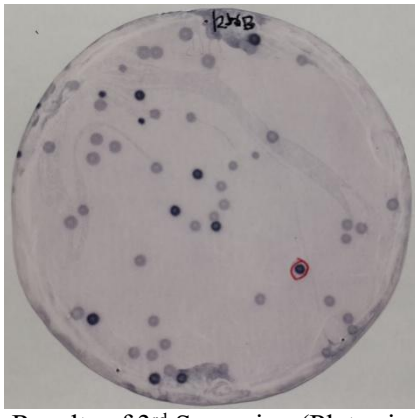

Figure 1c. Results of 3rd Screening (Plate size 100 mm).

Figure 1 Recombinant expression cloning proteins blotting onto nitrocellulose membranes

2.2 IPTG induces the expression of gst protein in recombinant vector of target gene

The expression vectors pGEX-4T-1, 2, 3 of GST protein of SEREX antigen gene (insert fragment of DNA) were constructed by DNA Ligation and In-Fusion techniques. The recombinant cloned plasmid was transformed into the competent cell *E.coli* BL-21, and the GST fusion protein was expressed under the induction of IPTG with the concentration of 0.1 mM. The bacterial precipitate containing the protein was directly taken for SDS-PAGE and stained with Coomassie Brilliant Blue, to verify whether the protein expression vector recombination was successful by obtaining IPTG-induced GST fusion protein bands or not (Figure 2).

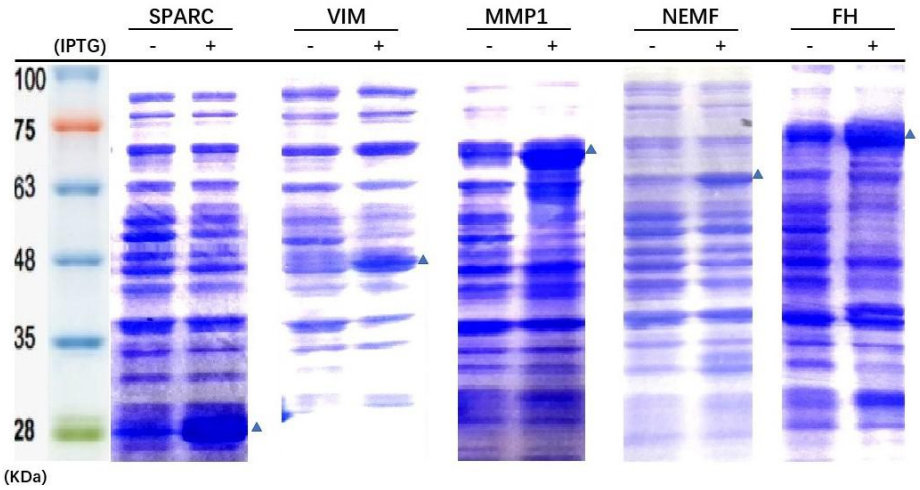

Figure 2 Expression of recombinant proteins by SDS-PAGE.

It was shown that *E.coli* BL21 containing pGEX-4T expression vector was treated with IPTG for 3 h, and cell lysates were subsequently separated by SDS-PAGE. The SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue (CBB). Triangles indicate the IPTG-induced GST fusion proteins which represent the GST fused-encoded cDNA product. Molecular weights are shown to the left.

2.3 SEREX antigen gene successfully expressed by GST fusion protein

The expression of recombinant GST fusion protein induced by IPTG was verified by SDS-PAGE. Twenty-one SEREX antigen genes successfully

expressed GST labeled protein were obtained(Table 1).

Table 1 SEREX antigen gene successfully expressing GST tag protein

Gene	Accession No.	Vector
MMP1	NM_002421	pGEX-4T-1
SERPINE1	NM_001193485	pGEX-4T-1
VIM	NM_003003	pGEX-4T-1
FH	NM_005561	pGEX-4T-2
KLC1	NM_021928	pGEX-4T-2
ECSCR	NM_001297776	pGEX-4T-2
ECI2	NM_002881	pGEX-4T-2
ALDOA	NM_018202	pGEX-4T-2
KIF20B	NM_019071	pGEX-4T-2
LAMP1	NM_001402	pGEX-4T-3
CBX1	NM_014733	pGEX-4T-3
BRAT1	NM_012145	pGEX-4T-3
SEC14L1	NM_002526	pGEX-4T-3
RNF41	NM_024911	pGEX-4T-3
CBX5	NM_001423	pGEX-4T-3
TPT1	NM_001136530	pGEX-4T-3
RIMKLB	NM_003118	pGEX-4T-3
CCT5	NM_003380	pGEX-4T-3
P3H4	NM_014832	pGEX-4T-3
WDR1	NM_001077693	pGEX-4T-3
NEMF	NM_012073	pGEX-4T-3

2.4 DNA sequencing of SEREX antigen gene recombinant plasmid

The above-mentioned SEREX antigen gene which successfully expressed the target protein in *E.coli* BL-21 showed that the target gene was completely consistent with the corresponding sequence in the RefSeq Database, and the reading frame was correct, the predicted size of the product of the recombinant GST fusion protein is basically consistent with the results of SDS-PAGE. This indicates that the SEREX antigen gene successfully reconstitutes the pGEX-4T vector with the GST-tagged protein and can be a candidate antigenic protein (Table 2).

Table 2 Candidate pGEX-4T-fusion antigenic gene.

Clone No.	Gene full name (abbr.)	Accession No.	Predicted size of the product
108-C-F	matrix metallopeptidase 1 (interstitial collagenase) (MMP1)	NM_002421	76.7 KDa
108-H-F	SEC14-like 1 (S. cerevisiae) (SEC14L1)	NM_002526	103.7 KDa
108J-T3-F	ribosomal modification protein rimK-like family member B (RIMKLB)	NM_003118	67.5 KDa
129-D-F	vimentin (VIM)	NM_003003	76.3 KDa
135-B-F	lysosomal-associated membrane protein 1 (LAMP1)	NM_001402	51.8 KDa
256-C-F	endothelial cell surface expressed chemotaxis and apoptosis regulator (ECSCR)	NM_001297776	42.7 Kda
256-J-F	chaperonin containing TCP1, subunit 5 (epsilon) (CCT5)	NM_003380	84.4 KDa

256-Q-F	aldolase A, fructose-bisphosphate (ALDOA)	NM_018202	65.2 KDa
265-C-F	fumarate hydratase (FH)	NM_005561	79.9 KDa
265-E-F	ring finger protein 41, E3 ubiquitin protein ligase (RNF41)	NM_024911	53.8 KDa
272-E-F	prolyl 3-hydroxylase family member 4 (P3H4)	NM_014832	56.6 KDa
278B-T3-F	kinesin family member 20B (KIF20B)	NM_019071	80.2 KDa
278-F-F	kinesin light chain 1 (KLC1)	NM_021928	86.7 KDa
297-G-F	chromobox homolog 5 (CBX5)	NM_001423	46.1 KDa
297-N-F	enoyl-CoA delta isomerase 2 (ECI2)	NM_002881	65.2 KDa
297-Q-F	WD repeat domain 1 (WDR1)	NM_001077693	59.0 KDa
297-S-F	chromobox homolog 1 (CBX1)	NM_014733	95.8 KDa
297-T-F	tumor protein, translationally-controlled 1 (TPT1)	NM_001136530	43.9 KDa
297-Z-F	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (SERPINE1)	NM_001193485	50.5 KDa
304-H-F	nuclear export mediator factor (NEMF)	NM_012073	51.2 KDa
322-A-F	BRCA1-associated ATM activator 1 (BRAT1)	NM_012145	115.4 KDa

3. DISCUSSION

TIA is a common neurovascular disease. Without special treatment, the recurrence rate of stroke is 5% in 48 hours and 10% in 3 months[13]. Early detection and treatment of TIA can effectively reduce the risk of stroke[14]. Moreover, acute specialized treatment of patients with TIA can significantly reduce (up to 80%) the risk of stroke and other related vascular events, such as myocardial infarction and vascular death[15,16]. Regrettably, however, there is currently no biomarker that has proven to be reliable enough to be diagnosed as a TIA[17]. In addition, TIA is closely related to atherosclerosis [18]，the unstable plaques on the surface of atherosclerotic vessels break up and fall off, thus forming microemboli to flow to the distal end, resulting in transient dysfunction of brain tissues in corresponding blood supply areas due to hypoxia and ischemia[19].Atherosclerosis is an inflammatory disease of the arteries. Vascular smooth muscle cells co-immunize with endothelial cells and immune cells in sclerotic plaques and resident cells in adventitia[20].Since TIA is recognized as an autoimmune disease, autoantigens identifcation is important.

With the maturity of molecular biology, the number of experimental methods that can be conceived and executed by cloning and copying of the gene has greatly increased. In our study, we used SEREX to screen autoantibodies IgG from TIA patients, because the stability of IgG globulin antibodies and the amplification of immune signals makes the concentration of antibodies much higher than that of target antigens. Therefore, autoantibodies are easier to detect than related antigens and can predict the disease earlier. Antibody markers are more sensitive and precise relative to serological detection of antigenic markers[21,22]. In view of this, the technical strategy of this study is to screen the relevant target antigens by serum antibodies of TIA patients, and then to identify autoantibodies with target antigens, aiming to adopt and draw on the research methods and techniques of immunology, genetics and molecular biology. Means to establish a new early serological screening technique for arteriosclerosis-related diseases. In serum SEREX screening of TIA patients, we successfully obtained the target antigens of 21 recombinant pGEX-4T vectors, which are essential for the induction, expression and subsequent detection of serological autoantibodies.

In this study, using Ligation and In-Fusion gene recombination technology, 70 SEREX antigen genes (target gene inserts) were vector-transformed to reconstruct the GST

protein expression vector plasmid pGEX-4T. GST as a kind of epitope tags can be added as markers for localization or for use in purification or two-hybrid assay systems[23].Among them, Ligation technology is preferred because Ligation technology is a classical strategy for gene transformation. For antigen genes containing restriction endonucleases (EcoR I/Xho I, BamH I/Xho I and Sma I/Xho I) in inserted CDS fragment, although it is not necessary to design a reaction primer and PCR technique to amplify a gene fragment, the time and energy required to transfer the cDNA to the vector using a conventional restriction-based and ligation-based subcloning method is huge. For the target gene of complete CDS fragment which can not be obtained by Ligation technology, in-Fusion technology must be used to recombine the vector. Compared with the traditional method, the major advantage to this system is that In-Fusion is convenient and efficient, unlike the restriction of restriction sites in the Ligation system. Based on the simple homologous recombination principle, In-Fusion system utilizes the recombination functional characteristics of vaccinia virus DNA polymerase with patent buffer. In addition, in-Fusion technology is not limited by vectors, the exposed complementary single-stranded ends of 15-bp homologous regions of the PCR fragments and linearized vector makes seamless cloning possible[24]. The cloning reaction of In-Fusion system takes only 15 minutes, and it takes only one step to construct even complex structures [25,26]. Because of its high efficiency and consistency, in-Fusion system can undoubtedly improve the success rate of gene cloning.

The technical route of this study is that the SEREX antigen gene plasmid was double-digested, and the insert was isolated and recovered. The target gene was inserted into the prokaryotic expression vector plasmid pGEX-4T by Ligation and In-Fusion technology, and transformed into *E.coli* BL21 competent cells. 21 recombinant pGEX-4T plasmids containing the SEREX antigen gene sequence successfully expressed IPTG-induced GST-fusion protein in *E.coli* BL21, and DNA sequencing showed the corresponding gene in the RefSeq Database. The sequence is completely consistent and the reading frame is correct. The predicted molecular weight of the recombinant GST fusion protein is basically the same as that of SDS-PAGE and GST fusion protein was successfully purified by Glutathione-sepharose column chromatography, indicating that the above 21 SEREX antigen genes successfully reconstruct the prokaryotic expression vector and pGEX-4T fused with GST-tagged protein. The technical strategy of TIA biomarkers is feasible, which provides a favorable means for the development of TIA serum markers. It lays a foundation for the extraction and purification of candidate antigenic protein and the recognition of TIA antibody markers. Through further screening and identification, we will strive to obtain molecular targets for diagnostic value of TIA early warning and immunotherapy, and provide decision-making information for individualized treatment of precision medicine.

4. CONCLUSION

In conclusion, recombination pGEX-4T expression plasmids could be constructed and expressed successfully by Ligation and In-Fusion methods, which may provide basis for the extraction and purification of these antigenic proteins aiming to identify serum antibodies in further study.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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REFERENCES

[1] U Sahin, O Türeci, H Schmitt, B Cochlovius, T Johannes, R Schmits, et al. Human neoplasms elicitmultiple specific immune responses in the autologous host. Proc Natl Acad Sci USA, 1995, 92(25): 11810-11813.

[2] Wen-Tao Yue, Zi-Tong Wang, Yue Wang, Lina Zhang. Construction of a T7 human lung cancer cDNA library. Chin J Lung Cancer, 2008, 11(5): 686-691.

[3] Sang-Yull Lee, Dooil Jeoung. The reverse proteomics for identification of tumor antigens. J Microbiol Biotechnol, 2007, 17(6): 879-890.

[4] Geng Li, Amanda Miles, Aija Line, Robert C Rees. Identification of tumor antigens by serological analysis of cDNA expression cloning. Cancer Immunol Immunother, 2004, 53(3): 139-143.

[5] James A Koziol, Jian-Ying Zhang, Carlos A Casiano, Xuan-Xian Peng, Fu-Dong Shi, Anne C Feng, et al. Recursive partitioning as an approach to selection of immune markers for tumor diagnosis. Clin Cancer Res, 2003, 9(14): 5120-5126.

[6] Sang-Yull Lee, Yuichi Obata, Masahiro Yoshida, Elisabeth Stockert, Barbara Williamson, Achim A Jungbluth, et al. Immunomic analysis of human sarcoma. Proc Natl Acad Sci USA, 2003, 100(5): 2651-2656.

[7] Michael Pfreundschuh. Exploitation of the B cell repertoire for the identification of human tumor antigens. Cancer Chemother Pharmacol, 2000, 46 (Supp l): S3-S7.

[8] Ken-Ichiro Goto, Takao Sugiyama, Ryutaro Matsumura, Xiao-Meng Zhang, Risa Kimura, Akiko Taira, et al. Identification of cerebral infarction-specific antibody markers from autoantibodies detected in patients with systemic lupus erythematosus. J Mol Biomark Diagnos, 2015, 6:2.

[9] Mayumi Muto, Masahiro Mori, Takaki Hiwasa, Masaki Takiguchi, Yasuo Iwadate, Akiyuki Uzawa, et al. Novel serum autoantibodies against talin1 in multiple sclerosis: Possible pathogen tic roles of the antibodies. J Neuroimmunol, 2015, 284:30-36.

[10] Philippe Krebs, Michael Kurrer, Ugur Sahin, Ozlem Türeci, Burkhard Ludewig. Autoimmunity seen through the SEREX-scope. Autoimmunity Reviews, 2003, 2(6):339-345.

[11] J Chris Bradberry, Susan C Fagan, David R Gray, Yong S K Moon. New perspectives on the pharmacotherapy of ischemic stroke. J Am Pharm Assoc, 2004, 44(2S l1): S46-S56.

[12] Peter M Rothwell, Charles P Warlow. Timing of TIAs preceding stroke: time window for prevention is very short. Neurology, 2005, 64(5): 817-820.

[13] Caren M Wu, Kevin McLaughlin, Diane L Lorenzetti, Michael D Hill, Braden J Manns, William A Ghali. Early Risk of Stroke After Transient Ischemic Attack: A Systematic Review and Meta-analysis. Archives of Internal Medicine, 2008, 167(22):2417-2422.

[14] Dominic Tse, Michael D. Hill, Shelagh B. Coutts. Early Secondary Prevention in Transient Ischemic Attack (TIA) and Minor Stroke. Current Neurology and Neuroscience Reports, 2019, 19(6).

[15] Peter M Rothwell, Matthew F Giles, Arvind Chandratheva, Lars Marquardt, Olivia Geraghty, Jessica N E Redgrave, et al. Effect of urgent treatment of transient ischaemic attack and minor stroke on early recurrent stroke (EXPRESS study): a prospective population-based sequential comparison. Lancet, 2007, 370(9596):1432.

[16] Philippa C Lavallée, Elena Meseguer, Halim Abboud, Lucie Cabrejo, Jean-Marc Olivot, Olivier Simon, et al. A transient ischaemic attack clinic with round-the-clock access (SOS-TIA): feasibility and effects. Lancet Neurology, 2007, 6(11):953-960.

[17] Glen C Jickling, Frank R Sharp. Biomarker Panels in Ischemic Stroke. Stroke, 2015, 46(3):915-920.

[18] Emmanuel Touzé, Olivier Varenne, David Calvet, Jean-Louis Mas. Coronary risk stratification in patients with ischemic stroke or transient ischemic stroke attack. International Journal of Stroke, 2010, 2(3):177-183.

[19] R Donnelly, A M Emslie-Smith, I D Gardner, A D Morris. ABC of arterial

and venous disease: Vascular complications of diabetes. BMJ, 2000, 320(7241):1062-1066.

[20] Desheng Hu, Changjun Yin, Shanshan Luo, Andreas J R Habenicht, Sarajo K. Mohanta. Vascular Smooth Muscle Cells Contribute to Atherosclerosis Immunity. Frontiers in immunology,2019,10:1101.

[21] Hideaki Shimada, Masaaki Ito, Akiko Kagaya, Tooru Shiratori, Mari Kuboshima, Masae Suzuki, et al. Elevated serum antibody levels against cyclin L2 in patients with esophageal squamous cell carcinoma. J Cancer Sci Ther, 2015, 7(2): 60-66.

[22] Hideaki Shimada, Satoshi Yajima, Yoko Oshima, Takaki Hiwasa, Masatoshi Tagawa, Kazuyuki Matsushita, Fumio Nomura. Impact of serum biomarkers on esophageal squamous cell carcinoma. Esophagus, 2012, 9: 131-140.

[23] Jaehong Park, Andrea L Throop, Joshua LaBaer. Site-Specific Recombinational Cloning Using Gateway and In-Fusion Cloning Schemes. Current protocols in molecular biology, 2015, 110:3.20.1-3.20.23.

[24] Chad R Irwin, Andrew Farmer, David O Willer, David H Evans. In-fusion® cloning with vaccinia virus DNA polymerase. Methods Mol Biol, 2012;890:23-35.

[25] Chao-Guang Chen, Louis J. Fabri, Michael J. Wilson, and Con Panousis. One-step zero-background IgG reformatting of phage-displayed antibody fragments enabling rapid and high-throughput lead identification. Nucleic Acids Res, 2014;42(4): e26.

[26] Roxane Lestini, Sergey P Laptenok, Joëlle Kühn, Mark A Hink, Marie-Claire Schanne-Klein, Ursula Liebl, Hannu Myllykallio. Intracellular dynamics of archaeal FANCM homologue Hef in response to halted DNA replication. Nucleic Acids Res, 2013;41(22):10358-10370.