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Cloning expression, monoclonal antibody preparation and serologic study of mammaglobin in breast cancer

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Mammaglobin may be a potential serum biomarker for the differential diagnosis of breast cancer. 260 serum samples were collected from 127 untreated breast cancer patients and 133 healthy volunteers to analyze the sera expression of mammaglobin and its implications for both. The expression vector of pGEX-4T-2-Mammaglobin and pBVIL1-Mammaglobin were constructed and transformed into E.coli.HB101 for expression. The mice were immunized with the purified recombinant protein to prepare monoclonal antibody and to detect by ELISA the serum of normal people and breast cancer patients. Recombinant mammaglobin antigen was effectively expressed in *E.coli*. Two hybridoma cell lines were obtained after the mice were immunized by pGEX-4T-2-mammaglobin. 133 cases of normal serum and 127 cases of breast cancer serum were analyzed by ELISA. The sera expression level of mammaglobin in breast cancer group (average OD value 0.645±0.223) was significantly ($p<0.001$) higher than that in the normal control group (0.255±0.109). Mammaglobin has a higher expression in the serum of breast cancer patients than normal control. The primary results indicate that mammaglobin may be a potential serum biomarker for the differential diagnosis of breast cancer.

Key words: mammaglobin; cloning expression; monoclonal antibody; serologic study; breast cancer

Mammaglobin (MGA) is also called human mammaglobin (hMAM), a 93 amino acid tissue-specific protein connected with the growth of mammary gland epithelium, which was cloned by Watson and Flemingin 1996 [1]. It has been reported that the mRNA of mammaglobin can be detected by RT-PCR in peripheral blood [2,3,4] Fanger et al. confirmed that mammaglobin in the serum produced by breast cancer cells could be detected by ELISA [5]. So far, only a few studies of the serologic detection of mammaglobin were reported. Therefore, its efficiency in the diagnosis to breast cancer needs to be further evaluated.

In this study, we received mammaglobin recombinated antigen by cloning expression and prepared the monoantibody of mammaglobin. Finally, A new method of ELISA to detect mammaglobin in the serum of breast cancer patients and normal people was established, which may be beneficial for the serological diagnosis of breast cancer.

Materials and methods

Materials.Serum sample preparation. This study was approved by the ethics committee of our hospital. 260 serum samples were collected and stored at -80°C immediately after centrifugation of 3ml blood from 127 patients of untreated breast cancer and 133 healthy volunteers. Specimens were provided by Department of General Surgery, 307 Hospital, Beijing, China.

Clone and expression. Prokaryotic fusion expression vector pBVIL was constructed in Institute of Basic Medical Science, Academy of Military Medical Sciences, China. The vector Pgex-4T-2 and E.coli HB101 were stored in the laboratory. The main reagents were used in this study including T4 DNA ligase (Promega), T4 DNA polymerase, DNA restriction enzymes, Tryptone, yeast extract, and PCR Product Purification Kit (Sanbo Bioengineering Ltd, Beijing, China).

Monoclonal antibody preparation. Recombination antigen vector pBVIL-mammaglobin and Pgex-4T-2-mammaglobin were prepared in the section of clone and expression, and the mouse myeloma cell series SP2/0 was provided by Institute of Basic Medical Science, Academy of Military Medical Sciences, China. Female BALB/c mice (8 weeks) was supplied by the Center of Experimental Animal, Academy of Military Medical Sciences, China.

ELISA reagent. Sulfo-NHS-LC-Biotin (Pierce Ltd), strepavidin (Promega), HRP, BSA, TMB (Sigma) were used in this study.

Methods Selection of mammaglobin epitope. It has been reported that mammaglobin is constructed with 93 amino acid [5,6] and 8-93 is the active sequence of the protein through the analysis of BioSun [7].

Synthesis of the gene of mammaglobin epitope. The sequence coding epitope was decided based on the sequence of the amino acid of the selected epitope and the optimized codon used in E. coli. The sequence was synthesized by PCR and the primers are as follows.

F 1 TGGTTGTCCACTGCTGGAAAACGTGATCTC-CAAGACCATC;
R 1 CTCCGTCTTCGACACTTGTGGATTGAT-GGTCTTGGAGATC
F 2 GAGCCAGCATTGCTATGCTGGCTCTGGTT-GTCCACTGCTG
R 2 GAACTCCTGCAGGAGTTCCTTGTACTC-CGTCTTCGACACT
F 3 GC CTCGAGATGTTAGCTGCCCTGAGCCAG-CATTGCTAT
R 3 GGTAGTTGCGTTGTCATCGATGAACTCCT-GCAGGAGT
F (1) TGAAGGAATGCTTCCTGAACCAGACAGATGA-GACGCTGAG
R (1) AACTGCATGAACACCTCGACGTTGCT-CAGCGTCTCATCTG
F (2) CAACTACCAACGCGATCGACGAGCTGAAG-GAATGCTTCCT
R (2) TCGCACAGGGAGCTGTCATAGATTAACT-GCATGAACACCT
F (3) TTCATCGATGACAACGCAACTAC-CAACGCGAT
R (3) GC TCTAGATTAGAACAGGTCGCACAG-GGAGCTGT

All the primers were synthesized by Sanbo bioengineering Ltd and sequenced by Lijiafucheng Ltd.

Expression vector construction and target protein expression . All the steps refer to «Experiment Guide of Molecular Cloning». The condition of PCR is as follows: denaturing at 95°C for 1min, annealing at 58 °C for 1min, then extension at 72 °C for 1min, and then performing 30 cycles of denaturing-annealing-extension, finally extension at 72°C for 5mins.

Antigen purification. After expression in the engineered bacteria, mammaglobin in the inclusion body was released by ultrasound and then dissolved in 8mol/L Urea. The negative laminar analysis was performed using Sepharose FF anion exchange column (Balance solution: PH8.0 0.20mmo/L TE including 6mol/L urea, 0.1% beta-ME). Then, the solution of nonbeing peak was collected. Q-Sepharose FF cationic exchange column was used for purification and NaCl solutions of gradient concentration (prepared with the balance solution) were used for elution. The solution of the eluting peak was collected and tested by SDS-PAGE, and then desalted by Sephardex G-50 solvent resistant column. Finally, the first peak was recorded.

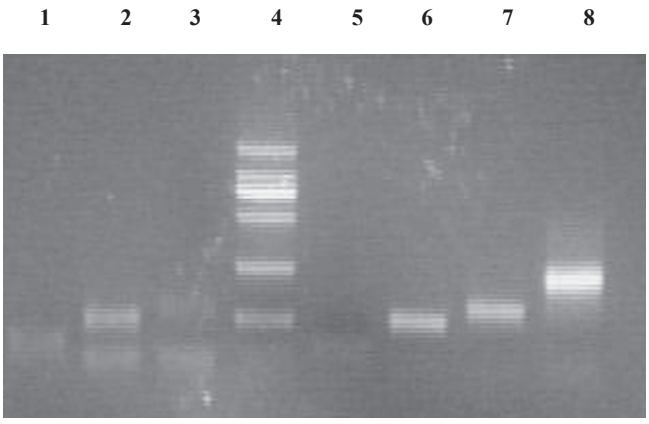

Figure 1. The result of PCR for mammaglobin gene amplification (2%agarose) 1, F1/R1; 2, F2/R2; 3, F3/R3; 4, DL2000; 5, F(1)/R(1); 6, F(2)/R(2); 7, F(3)/R(3); 8, F1/R(3) Full length of target gene

Preparation of monoclonal antibody. Mammaglobin recombination antigen (50μg per mouse) was administrated by the back and peritoneal injection. The 2nd and 3rd administration was given at 4 and 8 weeks, respectively. The myeloma cells and spleen cells were fused at a ratio of 1:10. The masculine clone was selected using indirect ELISA and then the clone was stored. The hydrogaster was used to prepare mouse monoclonal antibody.

Stable ELISA method. We coated the two monoclonal antibodies with different concentrations, marked the two Abs with biotin, performed cross combination, and then selected the best group. The coated antibody was diluted with carbonate to different concentrations of 20, 10, 5, 2.5 (μg/ml). The biotin labeled antibody was diluted 500, 1000, 2000, 4000, 8000 times. Then, the ODs values of the antibody were measured by using UV spectrophotometer.

Statistical analysis. The data of ODs were analyzed by rank sun test, and the data in the subsets were analyzed by t-test.

Results

Target gene. The amino acid sequence of the Mammaglobin was analyzed with BioSun and the result showed that the peptide of 8-93 amino acid was the epitope .The target gene was too long to obtaine by one PCR. Thus, we used Fusion PCR to get the complete gene with enzyme cutting site of XhoI and XbaI. (Fig.1)

The construction expression and purification of the vector of Mammaglobin. We chose vector PGEX-4T-2 and pBVIL1 to link the target gene. To avoid cross-reaction we constructed vector pGEX-4T-2/ Mammaglobin and pBVIL1/Mammaglobin.The target gene was respectively inserted into the vector PGEX-4T-2 and the vector pBVIL1.Both were effectively expressed in *E.coli*. The protein of pGEX-4T-2-Mammaglobin

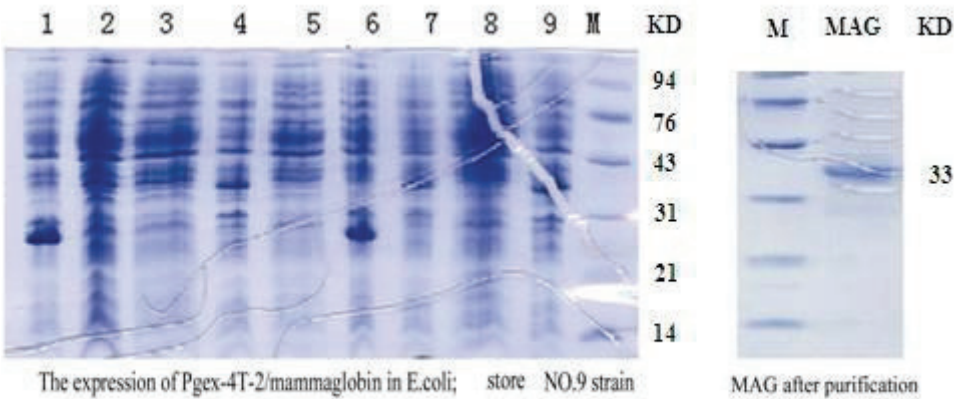

Figure 2. SDS-PAGE: The expression and purification of pGEX-4T-2-Mammaglobin
(1-9: colony; M: protein marker; MAG: the protein of pGEX-4T-2-Mammaglobin from NO.9)

was 33KD. The protein of pBVIL1-Mammaglobin was 26KD. The results of sequencing showed that the insert was correct. (Fig.2, 3)

Preparation of mouse antibody to mammaglobin After the laminar analysis, the two proteins which expressed by pGEX-4T-2/ Mammaglobin and pBVIL1/Mammaglobin were electrophoretically pure, and their purity exceeded 95%of the total protein. We used the two proteins to immunize the mouse respectively and utilize ELISA to get the positive clone after cell fusion: The two proteins were coating to plate respectively to select the fusion cell. The cells from the mouse immunized by pBVIL1/ Mammaglobin were low activity .From the other group we obtained two high activity cells, named 1E12 and 4B11.The hypotype verification showed that the two stable cells all secreted IgG3. (Fig.4)

Serological analysis. The monoclonal antibody , secreted by 4B11 and 1E12 coating the plate and labeling biotin respectively , was used to detect the serum of normal people and breast cancer patients by ELISA.

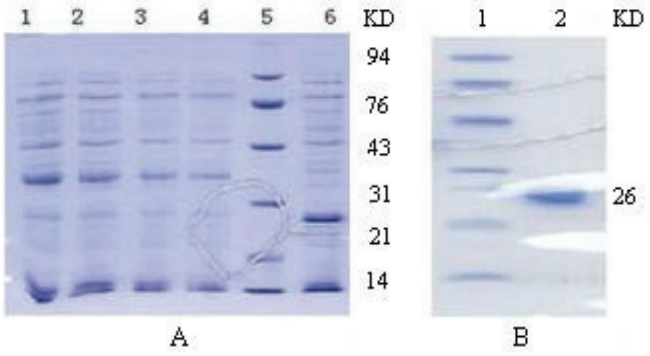

Figure 3. SDS-PAGE: the expression and purification of pBVIL1–Mammaglobin
(A: 1-4 and 6, colony; 5, Marker; B: After purification, 1,marker; 2, the protein of pBVIL1-Mammaglobin from colony 6)

The level of mammaglobin expressed in the serum of normal people and breast cancer patients. The result showed that the level of mammaglobin expressed in the serum of breast cancer patients group (OD average: 0.645±0.223) was significant higher than in normal people group (OD average:0.255 ±0.109), P=0.0001. (Fig.5)

Relationship of mammaglobin expression level in the serum with clinical parameter. The subset result showed that there were not the statistical significance between mammaglobin expression level in the serum of breast cancer patients and the age of patient, the size of the tumor, clinical stage, the metastasis of axillary lymph nodes and the ER status.(Tab1).

Discussion

The tissue-specificity of mammaglobin was first utilized in the study of axillary node micrometastasis. The mRNA detec-

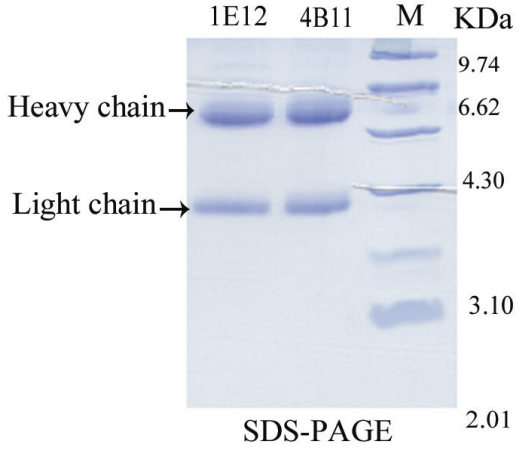

Fig 4, The result of the monoclonal antibody secreted by 1E12, 4B11 (SDS-PAGE)

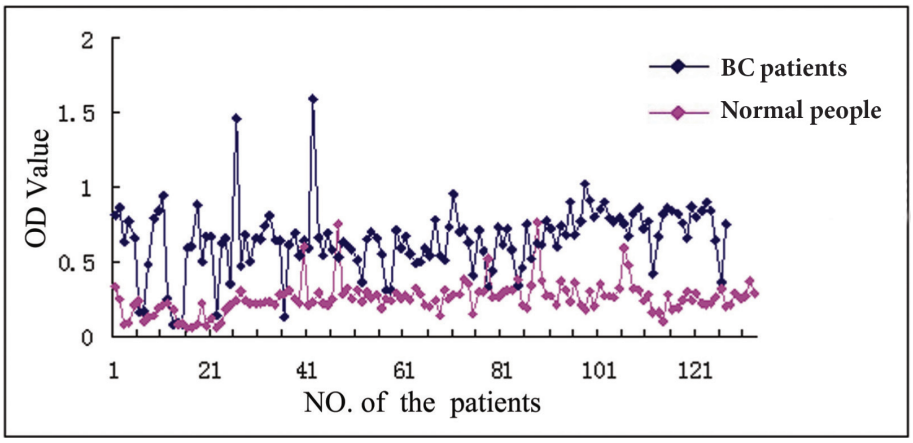

Fig 5. The mammaglobin expressed in the serum of breast cancer patients and normal people

Table 1. Relationship of mammaglobin expression level in serum with the age of patient, the size of the tumor, clinical stage, the metastasis of axillary lymph nodes and the status of the ER

group		NO.	average	S.D	maximal value	minimum value	median	p value
age	≤50	59	0.652	0.230	1.456	0.081	0.667	0.765
	>50	68	0.639	0.219	1.587	0.091	0.644	
Tumor size	≤ 2cm	52	0.681	0.180	1.456	0.308	0.675	0.126
	>2cm	74	0.620	0.247	1.587	0.081	0.646	
clinical stage	I – II stage	88	0.642	0.218	1.456	0.081	0.664	0.528
	III-IV stage	38	0.664	0.234	1.587	0.142	0.670	
metastasis of axllary nodes	positive	56	0.684	0.225	1.587	0.165	0.669	0.0798
	negative	70	0.614	0.218	0.949	0.081	0.648	
ER	positive	78	0.648	0.216	1.456	0.081	0.659	0.909
	negative	41	0.643	0.249	1.587	0.082	0.672	

tion of mammaglobin by RT-PCR can elevate the percentage of the diagnosis for micrometastasis of axillary or sentinel lymph node [8,9,10]. Using RT-PCR to test the mRNA of mammaglobin and CK-19 have been validated by FDA for biopsy of sentinel lymph node in operation [11,12] Many other studies also showed that RT-PCR can test mRNA of mammaglobin in breast cancer patients' blood, which had great sensitivity to micrometastasis of breast cancer cell in blood vessel [3,4,5,6].

An ideal marker in the blood for detection of breast cancer should have the sensitivity and also be simple and fast. To detect mammaglobin, the ELISA may be the good way for the diagnosis of breast cancer. Fanger et al. proved that using the method of ELISA could detect the mammaglobin protein[5]. They detected the mammaglobin in the fluid of two mammary cancer cells using the western blot and then ELISA to test the mammaglobin in the serum of breast cancer patient and normal people. Their result showed that the concentration of mammaglobin in the serum of breast cancer patients is 0.07-9.6ng/ml,which is significant higher than the concentration of mammaglobin(0-0.07ng/ml) in the serum of normal people.

We got the gene of mammaglobin by PCR and constructed two prokaryotic vectors to avoid cross-reaction and got high yield of protein in E.coli. Getting monoclonal antibody by using the protein expressed of the two vectors, we established the method of ELISA to detect mammaglobin protein in the serum. We detected 260 cases, 127 breast cancer patients and 133 normal people, the result showed that the level of mammaglobin expressed in the serum of breast cancer patients group(OD average: 0.645±0.223) is significant higher than normal people group(OD average:0.255 ±0.109), P=0.0001. The results showed that the expression lever of mammaglobin in serum may be useful for the differential diagnose of breast cancer. However,we failed to find the serum expression of mammaglobin has the relation with the prognosis of breast cancer.

Fanger GR et al's research showed that the mammaglobin is scarcely expressed in the serum of normal people[5]; the concentration was 0-0.07ng/ml. In our study, the mammaglobin level was low in healthy people, although the OD value of 5 cases of 133 healthy people were close to the average OD value of breast cancer patients. Bernstein JL et al. [6] also found that the ELISA was highly sensitive and specific for detection of mammaglobin protein in tissue culture fluids of breast cancer

cells and sera of breast cancer patients. Zehentner BK et al. [13] had similar report, they found that Circulating mammaglobin protein was detected in 68% of the breast cancer sera, and was increased in 38% in comparison with a mixed control population. The RT-PCR assay and the ELISA for mammaglobin produced a combined sensitivity of 84% and specificity of 97%. In some healthy people a high level of mammaglobin is detected, which may lead to false suspicion of breast cancer. The reason is still unknown. As so far, there were few reports of serum mammaglobin detection as a breast cancer marker by ELISA. More researches are needed to determine the stability of the antibody and the sensitivity of the method.

In this study, we established a method to detect mammaglobin protein in the serum by ELISA. Using this method, the expression of mammaglobin in the serum of breast cancer patients and normal people were analyzed. The primary results indicate that this biomarker may be useful for the identification of breast cancer.

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