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


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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 Fleming in 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 monoclonal antibody 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.

Key words: mammaglobin; cloning expression; monoclonal antibody; serologic study; 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), streptavidin (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  TGTTGTCCACGCTGGAAAAAGTGATCTC-CAAGACACATC;
R 1  CTCCGTCTCCTGGACACTTGTGGATG-TGTCTTTGAGATCG
F 2  GAGCCAGCTTTGCTGCTGTCGTTGTCGTTGTCGTTGTCCGACAG
R 2  GAACCTCGAGGAGATCTTCTTGATCTC-CTGTCCTGACACT
F 3  GCCTCTTGCTGTGCTGAGCTGACGAGCAGCAG
R 3  GGTAGTTGCTGGTCACTGATGAACCTCCT-GCAAGGATCG
F (1) TGAAGGAATGCTTCCTGAACCAGACAGATGA-GAGCCTGAG
R (1) AACTGCATGAACACCTGACGCTTGCT-CAAGCCTCTCATTCT
F (2) CAACTACAACACGCGATCGACGAGCAGCTGAAG-GAAGTCTCTCCT
R (2) TCGCAGAGCAGGAGCTGCTCATGATGAACCTC-CTGACGAGGATCG
F (3) TTTACTGATAGATGACACAGCGAACCAGCAGCA
R (3) GCCTCTTGCTGTGCTGAGCTGACGAGCAGGAGGAG

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 0.20mmol/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.

Preparation of monoclonal antibody. Mammaglobin recombinant 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 obtain 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
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 significantly 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-
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 level 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.

| 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 axillary 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  |        |

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

![Graph showing the expression of mammaglobin in serum of breast cancer patients and normal people](image-url)
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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References


