Screening a Novel Human Breast Cancer-Associated Antigen from a cDNA Expression Library of Breast Cancer

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OBJECTIVE The aim of this research was to clone and express the antigen of the previously prepared monoclonal antibody named M4G3.
METHODS Western blots were used to screen a breast cancer cell line that overexpresses the M4G3-associated antigen. A λ zap cDNA expression library of breast cancer cells was constructed and screened using M4G3 as a probe to clone the antigen. The positive clones were subcloned and identified by homologous comparison using BLAST.
RESULTS The λ zap cDNA expression library had 1.0×10^6 independent clones. Fifteen positive clones were isolated following 3 rounds of immunoscreening and identified as being from *Mycoplasma pulmonis*.
CONCLUSION The specific antigen that matched the monoclonal M4G3 antibody is an unknown protein of *M. pulmonis*. This work is helpful for the further study of the association of *M. pulmonis* infection with breast cancer.

KEYWORDS: breast cancer, antibodies monoclonal, antigens, tumor-associated, mycoplasma.

Breast cancer-associated tumor antigens provide an important role in diagnosis and therapy of mammary carcinoma.[1-3] They provide information for early detection of primary tumors, prediction of the extent of the disease and prognosis, early detection of a recurrent disease, evaluation of a therapeutic response, monitoring patient outcome, and differential diagnosis of metastatic tumors of unknown origin.[4] Recently, advances in the use of tumor-specific immunotherapies, such as the anti-ErbB2 monoclonal antibody, Herceptin (or TrastuzuMab), have shown clinical efficacy for the treatment of metastatic breast cancer with ErbB2 overexpression.[5-7] Because only 25%~30% of human breast cancers overexpress ErbB2,[8-10] there is a great need for the identification of more breast tumor-specific immunotherapy targets. Therefore, it has become the focus of breast cancer-specific immunotherapies to identify new breast cancer associated-antigens with a high specificity that can be targeted.

In our previous work, we immunized mice with an extract of human breast cancer tissue, and then made a specific mouse anti-breast cancer monoclonal antibody named M4G3 using hybridoma technology, which was then shown to be highly specific.[11] In addition, through repeated identification using immunohistochemical staining, we found that the antigen-matched McAb was different from the breast tumor markers previously reported, such as c-erbB-2, ER and c-myc. The M4G3 has been used for diagnosis and localization in breast cancer clinical trials, which has had ideal effect.[12,13] The use of M4G3 in-

spired our interest to explore the nuclear production of the antigen. Thus, it became the focus of this research to clone and express the antigen for further investigation of its function.

MATERIALS AND METHODS

Cell culture and regents

Human breast cancer cell lines T47D, MDA-MB-231, MDA-MB-435 and MCF-7, and the M4G3 McAb were all maintained in our laboratory. Rabbit-anti-mouse IgG-HRP, goat-anti-mouse IgG-biotin and avidin-HRP were purchased from DOKO Co. Trizol was from Invitrogene Co. A λ ZAP kit and E. coli strains were purchased from Strantegen Co. X-gal, isopropylthiogalactoside (IPTG), DEPC, and a λ DNA package kit were from Promega. M-MLV reverse transcriptase, a cDNA synthesis kit and T4 DNA ligase were from Takara. DAB was from Sigma. A S-P immunohistochemical kit was purchased from Sangon Crop (Shanghai China). PVDH membranes were acquired from PALL. NC membranes were obtained from Millipore.

Western blot of breast cancer cells

Cells were screened, which strained for overexpression of the antigen that matches M4G3 McAb. Human breast cancer cell lines T47D, MDA-MB-231, MDA-MB-435 and MCF-7 were cultured at 37℃ in RPMI 1640 containing 100 ml/L FCS in 5% CO_2.[14,15] The cells were kept in logarithmic growth phase by trypsin digestion and reinoculation every 2~4 days.[16,17]

A total of 10^7 cells of each cell strain was lysised with 500 μl lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 1 mM PMSF), then centrifuged at 14,000×*g* for 15 min at 4℃ and the supernatants collected. The protein concentration of each sample was assessed using the Bradford method, and all samples adjusted to an equal protein content before analysis. Samples (100 μg of total protein) were separated on a 12% denaturing polyacrylamide gel. Separated proteins were transferred to a PVDH membrane (37 mA, 90 min; transfer buffer 48 mmol/L Tris, 39 mmol/L glycine, 20% methanol, 0.037% sodium dodecyl sulfate) using electrophoretic buffer (25 mmol/L Tris, 192 mmol/L glycine, 1% sodium dodecyl sulfate). The membrane was placed into blocking buffer (0.5% non-fat milk in TNT (20 mmol/L Tris/HCl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) for 1 h at 37 ℃. Blocking buffer was decanted and the membrane was incubated with the M4G3 McAb (1:500 diluted in blocking buffer) at 37 ℃ for 2 h with gentle shaking. After being washed with TNT, the membrane was incubated with rabbit-anti mouse IgG-HRP (1:2000 diluted in blocking buffer; 1 h; 37 ℃; gentle shaking). Following washing, the membrane was restained with DAB buffer.

RNA extraction

Trizol was used to isolate total RNA from 10^8 breast cancer cells that overexpressed the antigen. The total RNA was electrophoresed on 1% agarose gel to evaluate its size range and quality.

cDNA library construction

The first-strand was synthesized with 2 μl oligo d(T) primer in a mixture containing 100 μg total RNA which was denatured at 65℃, 1 μl M-MLV, 4 μl 5× buffer, 1 μl dNTP, 1 μl RNase inhibitor and RNase-free H_2O in a final volume of 20 μl at 42℃ for 1 h. Then, second-strand synthesis was carried out using 20 μl of first-strand reaction volume, 4 μl of second-strand enzyme cocktail, 3 μl dNTP, 30 μl 5× buffer, 89 μl of RNase-free H_2O for 2 h at 16 ℃, followed by heating at 70℃ for 10 min. The polymeric synthetic reaction was performed at 37℃ for 10 min after adding 4 μl of T4 DNA polymerase into the above reaction volume. The second-strand synthesis was terminated by adding 15 μl of 0.25M EDTA and 15 μl of 10% SDS. After a 2-fold phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) extraction and ethanol precipitation of the second- stranded cDNAs with 3 M NaAc (pH 7.0), the pellet was dissolved in 4 μl of sterile H_2O. Then the precipitate was washed in 75 % ethanol and the residual ethanol evaporated.

The 18 μl of ds cDNA was ligated with 5 μl EcoR I adaptor (10 pmol/μl) by 1 μl T4 DNA ligase (350 U/μl) in a final volume of 10 μl at 16℃ for 1 h. The action was terminated by heating at 70 ℃ for 10 min. Then the cDNAs with adaptor were mixed with 4 μl 10×PNK buffer, 0.7 μl 75 mM ATP, 1 μl T4 polynucleotide kinase (10 U/μl) and 4.3 μl sterile H_2O at 37 ℃ for 30 min. Adding 5 μl of 0.5M EDTA mix terminated the action. The resulting cDNAs were extracted, precipitated, and resuspended in 30 μl TEN Buffer (100 mmol/L NaCl, 10 mmol/L Tris/HCl, 1 mmol/L EDTA). Upon passing the mixture of cDNAs through Sephacryl-S400 spin column, less than 400 bp cDNA fragments and adaptors were removed. The remaining

cDNAs were ligated into the λ ZAP phage expression vector DNA according to the user's manual. The recombinants were packaged in vitro using a λ DNA package kit. A small portion of the packaged phage was used to infect *E.coli* XL1-Blue-MRF' for titration. The plaques were counted and the capacity of the cDNA library was calculated.

Examination of recombinant efficiency and cDNA fragment size

Ten μl of different titer-packaged λ phage was added to 100 μl XL1-Blue-MRF' bacteria, then incubated at 37 ℃ for 20 min. After adding 3 μl NZCY tetracycline-agar (containing 1 mmol/L IPTG and 0.25 g/L X-galand, preheating at 48℃), the mixture was added into Luria-Bertan agar plates (diameter 6 cm), and then incubated at 37 ℃ overnight. We counted the numbers of white plaques (recombinant) and blue plaques (nonrecombinant), and calculated the recombinant efficiency.

Several white plaques were picked out and boiled for 5 min in 80 μl sterile H_2O, and following centrifugation at 4, 000 rpm for 10 min, the supernatant was collected. Two μl of supernatant was used as a template for PCR amplification with the T7 and T3 as forward and reverse primer. The PCR program consisted of 30 cycles at 94 ℃ for 1 min, at 55 ℃ for 1 min, at 72℃ for 1 min, and at 72℃ for 7 min for the final extension. The PCR products were electrophoresed on 1% agarose gel to identify the size of the cDNA inserts.

Screening the cDNA library with M4G3 McAb

The M4G3 McAb was diluted 1:10 and preabsorbed with lysate from *E.coli* strain XL1-Blue-MRF' to remove antibodies reacting with *E.coli* components. After 100 μl of XL1-Blue-MRF' bacteria was infected with 103 recombinants (1 μl cDNA library) for 20 min at 37℃, 3 ml of melted NZCY top agarose preheated at 48℃ was added. The mixture was inverted once and spread onto a prewarmed, dry Luria-Bertan agar plates. The plates were inverted and incubated at 42℃ for 4 h to induce protein synthesis in *E.coli*. A nitrocellulose membrane treated for 15 min with 10 mM IPTG was placed onto the NZCY soft top agarose. The plate was incubated at 37 ℃ overnight to transfer the expressed polypeptide onto the nitrocellulose membrane. The filter was marked with 3 asymmetric locations, and peeled off carefully. After being washed with TNT, the filter was blocked with blocking buffer for 1 h at 37 ℃ with gentle shaking, then incubated with M4G3 McAb (1:500 diluted in blocking buffer, 37℃, 2 h, gentle shaking), washed with TNT, incubated with goat-anti-mouse IgG-biotin (secondary Ab; 1: 3000 diluted in blocking buffer; 37 ℃ ; 1 h; gentle shaking), washed with TNT, and incubated with avidin-HRP (tertiary Ab; 1:5000 diluted in blocking buffer; 37℃; 1 h; gentle shaking). After being washed, the reaction clones were restained with DAB buffer. The above process was repeated until the whole library was screened. Only clones which appeared nut-brown were considered positive. The positive clones were picked out and rescreened 2 times to obtain monoclonality.

Identified and subcloned positive clones were converted to the pBK-CMV phagemid form by using Ex-Assist helper phage with XLOLR strain, the phagemids were purified and digested by EcoR I to determine the size of the cDNA fragments. BioAsia Crop was used to identify the sequences of the cDNA insert. DNA and the predicted amino acid sequences were compared with sequences in the GenBank and other public databases by using the BLAST program.

RT-PCR

Total RNA was isolated from breast cancer cell T47D by using Trizol according to the manufacturer instructions. First strand cDNA was reversibly transcribed from total RNA with M-MLV reverse transcriptase. The mixture was amplified with the oligonucleotides as forward and reverse primer respectively that were designed and synthesized by Sangon Co according to the sequence of positive clones. PCR was performed by using the following protocol: initial denaturing at 94℃ for 3 min followed by 33 cycles of reaction including denaturing at 94 ℃ for 1 min, annealing at 55℃ for 30 s and extension at 72 ℃ for 1 min, and a final bonus extension for 7 min.

RESULTS

Screening of breast cancer cells

The total protein from every cell line was analyzed by Western blots with M4G3 McAb. The results showed that the antigen for M4G3-McAb staining was very abundant in the T47D cells, and only weakly in the MCF-7 cells. In contrast, the MDA-MB-231 and MDA-MB-435 cells did not act with M4G3 McAb. Besides, a special 48, 000 dalton band could be found in extracts of the T47D cells (Fig.1). So, we showed that the breast cancer T47D cells overexpressed McAb M4G3-associated antigen.

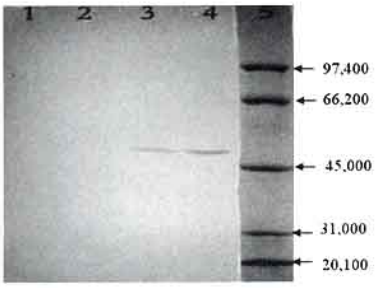

Fig.1. Western blot analysis of the protein extracted from 4 kinds breast cancer cells with M4G3 McAb. Lane 1: MDA-MB-435, Lane 2: MDA-MB-231, Lane 3: MCF-7, Lane 4: T47D, Lane 5: Markers.

The analysis of RNA integrity

The A260/A280 ratio of RNA from T47D cells was 1.9~2.0, showing that the extracted RNA was highly purified. Good total RNA quality was confirmed by finding 28 S/18 S ≥ 1.5 in agarose gel electrophoresis. These results showed that RNA was not destroyed by ribonucleases (Fig.2). Size range of the reverse transcription cDNA products was represented in a smear from 0.5~2kb.

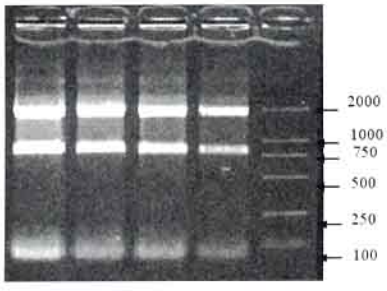

Fig.2. Quality identification of total RNA extracted from the T47D cell line in 4 runs.

Identification of cDNA library

A cDNA library was generated from the RNA as described in Materials and Methods. The result of titration is shown in Fig.3. The plaques were counted and the cDNA library was calculated as follows: pfu = number of plaques×dilution×volume of library (μl)/ volume of phage plated (μl). The results showed that the cDNA library consisted of 1.0×10^6 pfu recombinants.

Color selection was used to assess recombination efficiency. On the agar plates with 100 μl of transformants, 93 white plaques and 3 blue plaques were obtained, thus providing a ratio of white plaques (recombinant) to total plaques [recombinant and blue (nonrecombinant)] of 96.9 % (Fig.4).

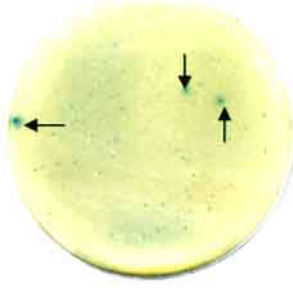

Fig.4. Blue/white screening for target clones. Ninety-three white plaques(96.9%) and three blue plaques(arrows) were seen on the agar plate.

PCR products of white clones amplified with T3 and T7 primers on a larger scale showed that each recombinant included one inserted fragment ranging from 500bp~2,500bp (Fig.5).

These data suggest that the cDNA library was successfully established.

Fig.3. Titration of the cDNA library. (A) More than one thousand clones were seen on the agar plant of 1:10 dilution. (B) There were one hundred and eighty-nine clones on the 1:100 dilution agar plant. (C) Only nineteen clones were seen on the agar plant of 1:1000 dilution.

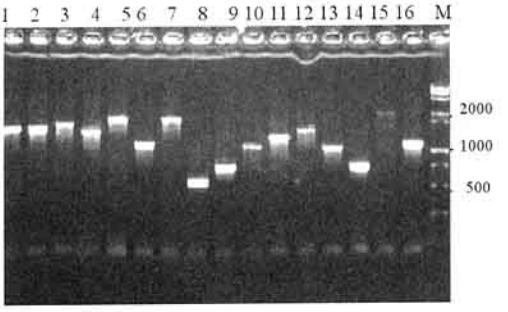

Fig.5. Different lengths of cDNA fragments from white clones amplified by PCR, Lanes 1-16: Randomly-selected white clones, Lane M: DNA mass markers.

Screening for the cDNA library

The cDNA library was screened with M4G3 McAb (Fig.6). After rescreening and eliminating false-positive clones, 15 positive clones were obtained. Identified and subcloned positive clones were converted to the pBK-CMV phagemid forms by using ExAssist helper phage with the XLOLR strain, the phagemids were purified and the sequences of cDNA inserts identified by BioAsia Crop (Fig.7). The results showed the length of the cDNA inserts were 1.7~3.3 kb. A BLAST homology search revealed that all of the sequences were homologous with *M.pulmonis* and coded 2 unknown proteins of *M.pulmonis* in the GenBank (Table 1, Fig.8).

RT-PCR

We designed two pairs of primers A1 (5'-ATT TAT AGT TTG GCT ACC ACC-3')/A2(5'-CAG TTG CCG ATA CTG CT-3'), B1 (5'-CTT TTA ATA AAA TCT TCA CGG-3')/B2 (5'-AAA AGC GGT AGA ATA AGT AGA-3') according to the sequence of positive clones using the oligo 6.0 software. The procedure of RT-PCR for *M.pulmonis* was carried out in breast cancer cell T47D with the 2 pairs of primers and took the primers of beta actin (5'-ATC ATG TTT GAG ACC TTC AAC A-3') /(5'-CAT CTC TTG CTC GAA GTC CA-3') as control. The results showed there was a *M. pulmonis* sequence in the total RNA of the T47D cells. The length was approximately 1,700 kb that was consentaneous with the length of a cDNA fragment (Fig. 9).

DISCUSSION

Immunotherapy is a promising new approach for breast cancer treatment, complementing surgery, chemotherapy, radiation and antihormonal therapy. [18] Recent advances have demonstrated the clinical utility of specific monoclonal antibodies that recognize tumor-associated antigens on the surface of tumor cells. These antibodies are useful for treating human breast cancer and B cell lymphoma. [19-21] For example, Trastuzumab antibody against the HER2/neu protein has opened new prospects in the treatment of breast cancer. Tumor markers may be helpful for the early di-

Table 1. The sequence of positive clones.

Clones	T3-end sequence	T7-end sequence
A	Mycoplasma pulmonis(AL445565) (128030~128891)	11 chromosome
B	Mycoplasma pulmonis(AL445565)(128030~128891)	Heat shock 90Kd prol
C	Mycoplasma pulmonis(AL445563)(294321~295152)	Mycoplasma pulmonis(AL445563)(293040~293735)
D	Mycoplasma pulmonis(AL445563)(294521~295163)	Y-like protein
E	Mycoplasma pulmonis(AL445565)(128034~128781)	Homosapians ribosomal protein L41
G	Mycoplasma pulmonis(AL445563)(294616~295173)	Mycoplasma pulmonis(AL445563)(293043~293435)
J_2	Mycoplasma pulmonis(AL445563)(294592~295153)	Mycoplasma pulmonis(AL445563)(293044~293509)
K	Mycoplasma pulmonis(AL445565)(130508~131057)	Mycoplasma pulmonis(AL445563)(128030~128242)
L	Mycoplasma pulmonis(AL445563)(294609~295173)	Mycoplasma pulmonis(AL445563)(293043~293450)
S	Mycoplasma pulmonis(AL445563)(294041~295180)	Mycoplasma pulmonis(AL445563)(293036~293816)
Q	Mycoplasma pulmonis(AL445563)(294043~295179)	Mycoplasma pulmonis(AL445563)(293043~293999)
R_2	Mycoplasma pulmonis(AL445565)(129249~130308)	Mycoplasma pulmonis(AL445565)(130022~131057)
N	Mycoplasma pulmonis(AL445563)(294529~295147)	Mycoplasma pulmonis(AL445563)(293045~293473)
O_1	Mycoplasma pulmonis(AL445563)(294561~295179)	Mycoplasma pulmonis(AL445563)(293045~293487)
R_1	Mycoplasma pulmonis(AL445565)(129249~130215)	Mycoplasma pulmonis(AL445565)(130102~131057)

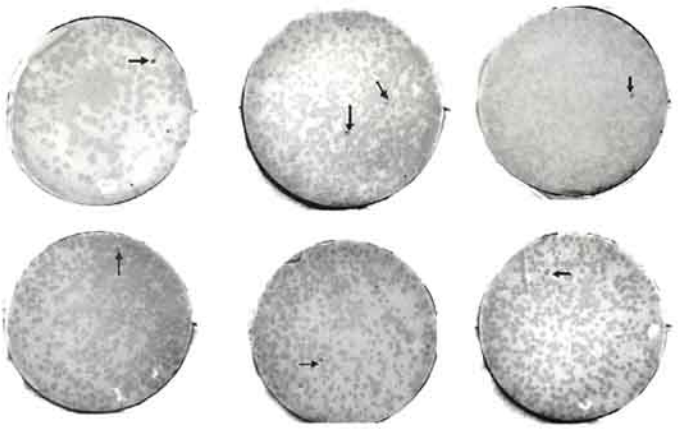

Fig.6. The randomly-selected positive clones (arrows) were obtained by immunoscreening the cDNA library with M4G3 McAb.

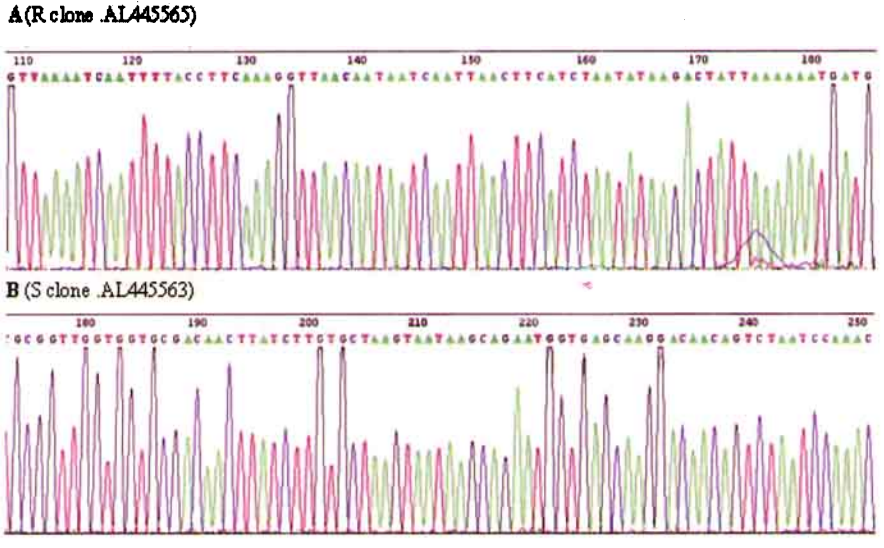

Fig.7. The sequence display of randomly-selected positive clones. A: R-clone: AL445565 B: S-clone: AL445563

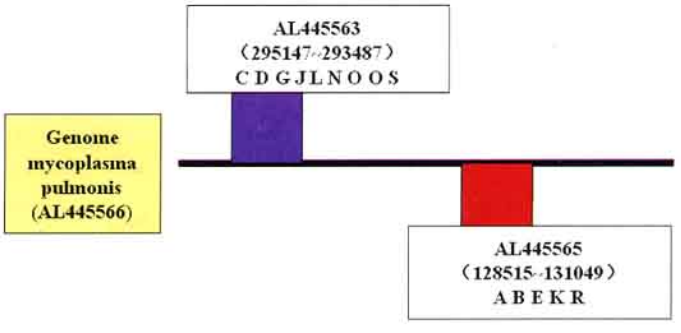

Fig.8. The positive simulation of the positive clone sequence in the sequence of the *M.pulmonis* genome.

agnosis of breast cancer and for the initial assessment of the extent of disease, as well as in monitoring tumor growth or volume reduction, and cancer recurrence. Therefore, finding novel tumor-associated antigens has become the focus of breast cancer-specific immunotherapies.

Fig.9. RT-PCR products of *M.pulmonis* were carried out in breast cancer cells T47D, MDA-MB-231 and MDA-MB-435. Lane 1:T47D primer beta actin, Lane 2, 3: T47D primer A1A2 and B1B2, Lane 4: MDA-MB-231 primer beta actin, Lane 5, 6: MDA-MB-231 primer A1A2 and B1B2, Lane 7: MDA-MB-435 primer beta actin, Lane 8, 9: MDA-MB-435 primer A1A2 and B1B2, Lane 10: DNA Markers.

Research on the application of tumor cell cDNA libraries is very extensive, especially the studies of unknown proteins that specifically react with tumor tissue.[22] At present, SEREX, the serological analysis of a recombinant cDNA expression library, is the prevalant method.[23, 24] The most efficient method to clone a novel protein is the following process: constructing a cDNA expression library, screening and isolating the objective cDNA fragment using matched antibody, then subcloning and identifying the nucleotide sequence, searching the function of the aimed protein for gene diagnosis and gene therapy. The aim of our research was to find the tumor-associated antigen for M4G3 McAb from breast cancer cells, whose concentration was unknown. Therefore we identified the expression of the antigen in tissue and cells by Western blot, and selected the overexpressed cells to construct a library, in order to increase the probability of screening positive clones.

We adapted the classical method of constructing a library with little modification. In the classical method, before constructing a library, the total RNA is purified to generate mRNA. But after several trials, we found this method had some deficiencies: ① The purification of mRNA is a complicated process, involving coupling, washing, eluting, etc, which can increase the possibility of contamination by exterior RNase; ②We detected excess oligo d (T) pillar after washing, so the process might have lost the potential template; ③Because we took the oligo d (T) as primer for reverse transfer, it is unnecessary to purify mRNA. Therefore, we extracted high quality total RNA, and reverse transfered directly using primer to gain the whole template.[25]

After screening the library using M4G3 as probe and eliminating 8 false positive clones, 15 positive clones were obtained and identified coding 2 unknown proteins of *M. pulmonis* Through identification and BLAST homology search, the result revealed that all of the sequences were homologous with *M. pulmonis* and coded 2 unknown proteins of *M. pulmonis* in the GenBank.

It is very interesting that the sequences of T7-end and T3-end were not same in A, B, C and E clones. We drew the conclusion that different cDNA fragments connected then inserted into the carrier. So upon cutting the PCR products of A, B, C and E clones with EcoR I, the result indeed showed there were 2 bands. The T3-end contained the down stream of the promoter, and translation should start from T3-end, so the insert of T3-end represented the positive clone.

Mycoplasmas as a heterogeneous group are the smallest recognized free-living bacteria with a size of 0.1 μm~0.8 μm, and genomes of approximately 500~800 kb. In addition to their small size, these bacteria lack cell walls and hence can be deformed.[26] They can cause a wide variety of diseases in animals, and some mycoplasmas have been identified to cause respiratory or urogenital diseases in humans.[27,28] Association of mycoplasma infection with tumorigenesis is not clear, but there have been some relevant reports concerning this relationship.

Mycoplasma infections can be detected in tumors from many tissues. In the 1960s, Dmochowski et al.[29] used electron microcopic analysis to show that mycoplasmata could be found in the blood of leukemic patients. Henceforth, a number of studies among leukemic patients were soon published that served to raise the possibility of an association between mycoplasmata and leukemia. Among these citations, several species of mycoplasmata were isolated from bone marrow of leukemic patients. Some isolates were assessed in animal models and were found to cause a leukemoid reaction after systemic infusion.[30]

Recently, with the development of molecular biological technology, mycoplasmata were also found in tissue cultures of other malignancies. In 1995, Sasaki et al.[31] reported that in Southern-blot analysis using

mycoplasma 16S ribosomal DNA (rDNA) as a probe, positive signals was detected in DNA samples from surgical specimens of gastric cancers. In addition, these organisms were detected in paraffin-embedded malignant ovarian cancer tissue and cervical condyloma tissues. These were categorized as cervical intraepithelial neoplasia (CIN) using combined PCR-ELISA with the consensus primers targeted for 15 species of mycoplasmata.[32,33] In 2001, the Beijing Cancer Institute reported the association of *Mycoplasma hyorhirnis* infection with tumorigenesis.[34] But others suggested that suppression of the immune system was induced by the malignant tumors leading to mycoplasma infection. Consequently the identification of mycoplasmata from the tissues might be due to contamination and thus they questioned the importance of the findings.[35]

There have been many reports concerning continuous infection of mycoplasma causing chromosomal changes and leading to malignant transformation of mammalian cells in vitro through gradual progressive chromosomal loss and translocations.[36] However, investigating the possible association between mycoplasma infection and carcinogenesis did not become a subject of active research until Tsai et al.[37,38] reported that continuous infection with *M. penetrans* or *M. fermentans* could lead to multiple stage malignant transformation of murine embryonic C3H cells, accompanied by abnormal karyotypes and some oncogene upregulation. In addition, some studies have indicated that infection by mycoplasmata could change some biological characteristics of tumor cells, facilitate their metastasis and enhance their invasive properties, however these changes could be suppressed by relevant monoclonal antibodies.[39-41] These reports, taken together, suggest association of mycoplasma infection with tumorigenesis.

It is well known that mycoplasma contamination is very common in routine cell cultures devised for preparing monoclonal antibodies using hybridoma technology. When mice are immunized with mycoplasma-infected tumor cells, mycoplasma may be more prone to stimulate an immune response, thereby obtaining a false-positive result. However, the M4G3 McAb used in this research was prepared through immunizing mice with an extraction of breast cancer tissue not tumor cells, which excludes the possibility of a false-positive result. Our study indicated that the antigen matching M4G3 McAb was a protein from *M. pulmonis*. Undoubtedly, the characteristics and function of the antigen, and the association between *M. pulmonis* infection and tumorigenesis needs to be intensively investigated using methods, such as Fluorescence In Situ Hybridization(FISH) and epidemiological surveys.

Our research has raised many interesting questions. Does M. pulmonis integrate into the chromosomes of breast cancer cells and become expressed? Since the normal host of *M. pulmonis* is rodents, how does it infect humans? Work to answer these questions is now in progress.

REFERENCES

1 Liu M, Acres B, Balloul JM, et al. Gene-based vaccines and immunotherapeutics. Proc Natl Acad Sci USA. 2004; 101:14567-14571.

2 Jager E, Jager D, Knuth A. Strategies for the development of vaccines to treat breast cancer. : Recent Results Cancer Res. 1998;152:94-102.

3 Ditzel HJ. Human antibodies in cancer and autoimmune disease. Immunol Res. 2000;21:185-193.

4 Kurebayashi J. Tumor markers in breast cancer. Gan To Kagaku Ryoho. 2004;31:2077-2081.

5 Bange J, Zwick E, Ullrich A. Molecular targets for breast cancer therapy and prevention. Nat Med. 2001;7:548-552.

6 Shak S. Overview of the trastuzumab (Herceptin) anti-HER2 monoclonal antibody clinical program in HER2-overexpressing metastatic breast cancer. Semin Oncol. 1999;26:71-77.

7 Choudhury A, Kiessling R. Her-2/neu as a paradigm of a tumor-specific target for therapy. Breast Dis. 2004;20:25-31.

8 Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987;235:177-182.

9 Pegram MD, Lopez A, Konecny G, et al. Trastuzumab and chemotherapeutics: drug interactions and synergies. Semin Oncol. 2000;27:21-25.

10 Pero SC, Shukla GS, Armstrong AL, et al. Identification of a small peptide that inhibits the phosphorylation of ErbB2 and proliferation of ErbB2 overexpressing breast cancer cells. Int J Cancer. 2004;111:951-960.

11 Huang JY. Preparation and immunopathology study of anti-human breast cancer specific glycoprotein McAb. Chin Oncol.1993;3:280-286.

12 Niu Y, Fu XL, Niu RF. Significance of the monoclonal antibody M4G3 against human breast cancer in the diagnosis of the occult breast cancer. Chin J Exp Surg. 2003; 20:654-658.

13 Niu RF, Chang KL, Niu DQ. Radioimmunoimaging in human breast cancer xenografted nude mice with single chain antibody against breast cancer. Chin J Clin Oncol. 2001;28:729-732.

14 Nadeau I, Sabatie J, Koehl M, et al. Human 293 cell metabolism in low glutamine-supplied culture: interpretation of metabolic changes through metabolic flux analysis. Metab Eng. 2000;2:277-292.

15 Liu SJ, Hu GF, Liu YJ, et al. The effect of human osteo-

pontin on the proliferation, transmigration and expression of matrix metallproteinase-2 and 9 of osteosarcoma cells in vitro. Chin Med J. 2004;117:235-240.
16 Christoffers AB, Kreisler M, Willershausen B. Effects of esradiol and progesterone on the proliferation of human gingival fibroblasts. Eur J Med Res. 2003;8:535-542.
17 Song J, Rolfe BE, Hayward IP, et al. Effects of collagen gel configuration on behavior of vascular smooth muscle cells in vitro: association with vascular morphogenesis. In vitro Cell Dev Biol Anim. 2000;36:600-610.
18 Untch M, Ditsch N, Hermelink K. Immunotherapy: new options in breast cancer treatment. Expert Rev Anticancer Ther. 2003;3:403-408.
19 Kennedy RC, Shearer MH. A role for antibodies in tumor immunity. Int Rev Immunol. 2003;22:141-172.
20 Barriuso Feijoo J, Sundlov A, Gonzalez Baron M. Antibodies against cancer. Rev Clin Esp. 2004;204:649-654.
21 Vlad AM, Finn OJ. Glycoprotein tumor antigens for immunotherapy of breast cancer. Breast Dis. 2004;20:73-79.
22 Li Y, Yang L, Cui JT, et al. Construction of cDNA representational difference analysis based on two cDNA libraries and identification of garlic inducible expression genes in human gastric cancer cells. World J Gastroenterol. 2002;8:208-212.
23 Krackhardt AM, Witzens M. Identification of tumor-associated antigens in chronic lymphocytic leukemia by SEREX. Blood. 2002;100:2123-2131.
24 Hartmann TB. SEREX identification of new tumor antigens linked to melanoma-associated retinopathy. Int J Cancer. 2005;114:88-93.
25 Kuo J, Inman J. Evaluation of vector-primed cDNA library production from microgram quantities of total RNA. Nucleic Acids Res. 2004;32:e183.
26 Waites KB, Talkington DF. Mycoplasma pneumoniae and its role as a human pathogen. Clin Microbiol Rev. 2004; 17:697-728.
27 Wisniewska-Ligier M, Wozniakowska-Gesicka T, Sobanska A, et al. Extrapulmonary complications of Mycoplasma pneumoniae infections. Przegl Lek. 2003;60:832-835.
28 Jensen JS. Mycoplasma genitalium: the aetiological agent of urethritis and other sexually transmitted diseases. J Eur Acad Dermatol Venereol. 2004;18:1-11.
29 Dmochowski L, Taylor HG, Grey CE, et al. Viruses and mycoplasmas (PPLO) and human leukemia and lymphoma. Cancer. 1965;18:1345-1368.
30 Plata EJ, Abell MR, Murphy WH. Induction of leukemoid disease in mice by Mycoplasma fermentans. J Infect Dis. 1973;128:588-597.
31 Sasaki H, Igaki H, Ishizuka T, et al. Presence of Streptococcus DNA sequence in surgical specimens of gastric cancer. Jpn J Cancer Res. 1995;86:791-794.
32 Chan PJ, Seraj IM, Kalugdan TH, et al. Prevalence of mycoplasma conserved DNA in malignant ovarian cancer detected using sensitive PCR-ELISA. Gynecol Oncol. 1996; 63:258-260.
33 Kidder M, Chan PJ, Seraj IM, et al. Assessment of archived paraffin-embedded cervical condyloma tissues for mycoplasma-conserved DNA using sensitive PCR-ELISA. Gynecol Oncol. 1998;71:254-257.
34 Huang S, Shou CC, Wu J, et al. Mycoplasma infection in human gastriointestinal carcinoma tissues. National Medical J Chin. 2001;8181:601-604.
35 Cimolai N. Do mycoplasmas cause human cancer? Can J Microbiol. 2001; 47:691-697.
36 Kotani H, Phillips D, McGarrity GJ. Malignant transformation of NIH-3T3 and CV-1 cells by a helical mycoplasma, Spiroplasma mirum, strain SMCA. In Vitro Cell Dev Biol. 1986;22:756-762.
37 Tsai S, Wear DJ, Shih JW, et al. Mycoplasmas and oncogenesis: persistent infection and multistage malignant transformation. Proc Natl Acad Sci USA. 1995;92:10197-10201.
38 Zhang B, Shih JW, Wear DJ, et al. High-level expression of H-ras and c-myc oncogenes in mycoplasma-mediated malignant cell transformation. Proc Soc Exp Biol Med. 1997;214:359-366.
39 Oredipe OA, Barth RF, Rotaru JH, et al. Alterations in monoclonal antibody affinity and antigenic receptor site expression on mycoplasma-infected human colorectal cancer cells. Proc Soc Exp Biol Med. 1990;194:301-307.
40 Ushio S, Iwaki K, Taniai M, et al. Metastasis-promoting activity of a novel molecule, Ag 243-5, derived from mycoplasma, and the complete nucleotide sequence. Microbiol Immunol. 1995;39:393-400.
41 Yang J, Hooper WC, Phillips DJ, et al. Interleukin-1beta responses to Mycoplasma pneumoniae infection are cell-type specific. Microb Pathog. 2003;34:17-25.