Establishment of recombinant lentiviral vector with ABCG2 overexpression and effects of the recombinant on human breast cancer MCF-7 cells' biological characteristics

Yuanming Fan, Chengyi Wu

Department of Endocrine Breast Surgery, The First Affiliated Hospital, Chongqing Medical University, Chongqing, China

Aim of the study: ATP-binding cassette super family G2 (ABCG2) is recognized as the key point of the cancer cells' resistance to chemotherapy drugs. But there have been rare reports about the relationship between ABCG2 and the invasion, migration and animal tumor formation abilities of cancer cells. We want to establish a recombinant lentiviral vector with ABCG2 overexpression, and study the vector's effect on human breast cancer MCF-7 cells' biological abilities above to improve our understanding about ABCG2.

Material and methods: The recombinant lentiviral vector with ABCG2 overexpression was transfected into human breast cancer MCF-7 cells. The cells' abilities of migration and invasion were tested by wound healing assay, and transwell invasive assay. The MCF-7 cells infected were injected in the left back of the nude mice. In the meantime the MCF-7 cells without anything were injected in the right back of same nude mice as the control group. Western blotting was used to detect the expression of ABCG2 in MCF-7 cells infected, the transplantation tumor tissue and the control group MCF-7 cells.

Results: The recombinant lentiviral vector with ABCG2 overexpression infected human breast cancer MCF-7 cells successfully, and the transfection efficiency was 95.4 ±2.8%. The wound line of MCF-7 cells infected healed after 48 hours, but the line of control group MCF-7 cells still existed. The number of the cells going through the membrane in infected MCF-7 cells was 78.34 ±0.25, and the number in control group MCF-7 cells was 15.28 ±0.12, $p < 0.05$. A much bigger transplantation tumor appeared in the MCF-7 cell infected nude mice. The expression of ABCG2 in infected MCF-7 cells and the transplantation tumor tissue was significantly higher than that in control group MCF-7 cells, $p < 0.05$.

Conclusions: We obtained ABCG2 overexpression in human breast cancer MCF-7 cells which showed the increasing migration, invasion and animal tumor formation abilities. Therefore, the results revealed that there might be a relationship between overexpression of ABCG2 and MCF-7 cells with increasing invasion, migration and animal tumor abilities.

Key words: breast neoplasm, ATP-binding cassette super family G2, gene transfection.

Contemp Oncol (Pozn) 2013; 17 (5): 435–439
DOI: 10.5114/wo.2013.35290

Introduction

Breast cancer has become an important threat to women's health and lives at the present. There are many cases of failure in clinical treatment. And as we know, the cancer cells' invasion, migration and infinite proliferation are the key reason for the failure.

ATP-binding cassette super family G2 (ABCG2) located in the cell membrane is a key enzyme which is responsible for cell transport of toxins out of the cell. ATP-binding cassette super family G2 is also thought to play an important role in maintaining cell structure and multidrug resistance to chemotherapy.

We want to establish a recombinant lentiviral vector with ABCG2 overexpression, and test the vector's reliability. Meanwhile the vector will be used for infecting human breast cancer MCF-7 cells and the effects of the cells also will be observed and recorded.

Material and methods

The ABCG2 sequence obtained through PCR

The sequence of ABCG2 is shown below.

F: 5'-GAGGATCCCCGGGTACCGGTCGCCACCATGTCTTCCAGTAATGTCGAAG-3'

R: 3'-TCACCATGGTGGCGACCGGAGAATATTTTTTAAGAAATAACA-5'

The conditions of amplification: 94°C, 5 min → 4°C, 30 s → 55°C, 30 s → 72°C 2 min 10 s, cyclic amplification of 30 times above, 72°C extension 10 min.

Construction and identification of the recombinant lentiviral vector

The pGC-FU-EGFP-3FLAG vector and ABCG2 gene PCR fragment were digested by Age I enzyme. 20 µl connect system was prepared as the formula of the enzyme recovery linearization vector DNA 5 µl, purified PCR product 2 µl, In-Fusion exchange enzyme buffer 2 µl, In-Fusion exchange enzyme 0.5 µl, plus ddH_2O 2.5 µl. The system reacted for 30 minutes at 25°C, and then reacted at 42°C for 15 minutes. The ratio of the linear vector DNA and purified PCR product must be 1 : 3–1 : 9. We used calcium chloride to prepare the fresh *Escherichia coli* competent cells. 200 µl of competent cells and 10 µl of connected liquid were transferred to the sterile micro-centrifuge tubes. Each tube was placed in ice for 30 minutes. The tubes were placed in the pre-heated to 42°C circulating water bath for 90 seconds, and must not be shaken. Then the tubes were quickly cooled in an ice bath. Each system with 800 µl of LB medium was put in the 37°C table for 45 minutes to make the bacteria recovery. 150 µl of competent cells prepared were transferred to AMP resistance (100 µg/ml) LB agar medium and were placed at room temperature until the liquid had been absorbed. Then the flat dish was inverted at 37°C to make the bacteria grow naturally. A follow-up PCR identified the new clones after 16 hours. The reac-

tion system contained ddH2O 12.4 μl, Primer (+) (ABCG2-SEQF, 10 μM) 0.4 μl, Primer (–) (EGFP-N-R, 10 μM) 0.4 μl, DNTPs (2.5 mM) 1.6 μl, Taq polymerase 0.2 μl, 5 × Taq buffer solution. Finally the volume was added to 20 μl by ddH_2O. The reaction conditions were as shown below. 94°C hot start for 30 seconds → 94°C degeneration for 30 seconds → 60°C recover for 30 seconds → 72°C extension 45 seconds. The procedure cycled 30 times, and the reaction system was placed at 72°C for 6 minutes for polymerization. Finally the system inoculated with positive transformants was kept at 37°C, and the end products were sent to be sequenced.

Western blotting test of the recombinant lentiviral vector

393T cells after cultured for pre-cooling 24 hours were added with 2 × Lysis Buffer protein lysate cracking. Every protein sample was adjusted to 2 μg/μl terminal. We put the protein samples on the agarose gel prepared in accordance with the target protein molecular weight size, and the electrophoresis lasted for 2 hours at 30 mA. The samples were divided into 4 groups: positive group (No. 1, standard goods), control group (No. 2), and 293T cells infected by the recombinant lentiviral vector (No. 3, 4). The target proteins were transferred to the PDVF membrane by the transfer electrophoresis apparatus in the conditions of 4°C and 400 mA constant current. The TBST containing 5.

5% skim milk was used to close the antigens. The primary antibody incubation lasted for the night at 4°C, and the secondary antibody lasted for 2 h at room temperature. Finally we used the ECL + plusTM WB kit (Amersham company) for color, and developed the photographs under X-ray. The experiment was repeated 3 times.

Packing of the recombinant lentiviral vector

Lipofectamine 2000 co-infected the 293T cells, and the culture mediums were exchanged after 8 hours. The high purity lentiviral concentrated liquid was packed after being cultured for 48 hours from the cell supernatant.

The recombinant lentiviral vector with ABCG2 overexpression infected the human breast cancer MCF-7 cells

The virus titer was 2×10^8 TU/ml, and the infection index was 20 : 1. We observed the fluorescence intensity of the cells' GFP by fluorescence microscopy after being cultured in a 37°C 5% CO_2 incubator for 48 hours and 96 hours. The infection efficiency was calculated by the following formula after being cultured for 5 days:

number of green fluorescent cells / number of total cells × × 100%).

Wound healing assay

At first a 13 ml cell suspension was prepared containing about 3×10^5 MCF-7 cells infected in a good growth state and some culture solution. The cell suspension was placed in a 24-well plate. The plate was kept in a 37°C 5% CO_2 incubator overnight. We used a pipette to scratch the plate along the middle line of it after observing the cell adherent cell. After 24 and 48 hours of culture the plate was photographed and recorded. At the same time the MCF-7 cells without the recombinant lentiviral vector were chosen as a control.

Transwell invasive assay

200 μl of MCF-7 cell suspension with the recombinant lentiviral vector were piped into each hole of the upper Transwell chamber, and 500 μl culture solution was piped into the lower Transwell chamber. The upper Transwell chamber was fixed with 95% alcohol after being cultured for 48 hours. Then we observed the cell dyed by the crystal violet and choose the MCF-7 cells without the recombinant lentiviral vector as control.

Animal experiment

The MCF-7 cells infected by the recombinant lentiviral vector were diluted to 2×10^3/ml suspension with the culture medium, and then it was injected subcutaneously to the left back of the nude mouse. Each mouse accepted 0.1 ml suspension. At the same time the right back of the mouse accepted the MCF-7 suspension without virus as the control group. We observed the transplantation tumors of the nude mice every 7 days. The mice were killed after 1 month, and the tumors were removed for the next processes.

HE staining and immuno-histochemistry

This part of the experiments was done as described in the kit instructions. The membrane had appeared brown was judged for the positive results.

Statistical method

The data are shown as mean ± SD and were managed by using statistical analysis software SPSS 15.0. The statistical difference of each treatment was compared by Student's *t*-test. A p value less than 0.05 was considered as statistically significant.

Results

Cloning of ABCG2 gene PCR and agarose gel electrophoresis results

We obtained target fragments which were 2011 bp long after amplification (Fig. 1).

Enzyme agarose gel electrophoresis results

Homogeneous electrophoresis bands appeared when the plasmid accepted enzyme digestion (Fig. 2).

Identification of positive clones for the recombinant lentiviral vector with ABCG2 overexpression

The size of the recombinant lentiviral vector was 668 bp, and was consistent with human ABCG2 sequence (Fig. 3).

Western blotting results

ABCG2-EGFP-3FLAG proteins co-expressed at the same time, and the molecular weight was 100 kDa (Fig. 4).

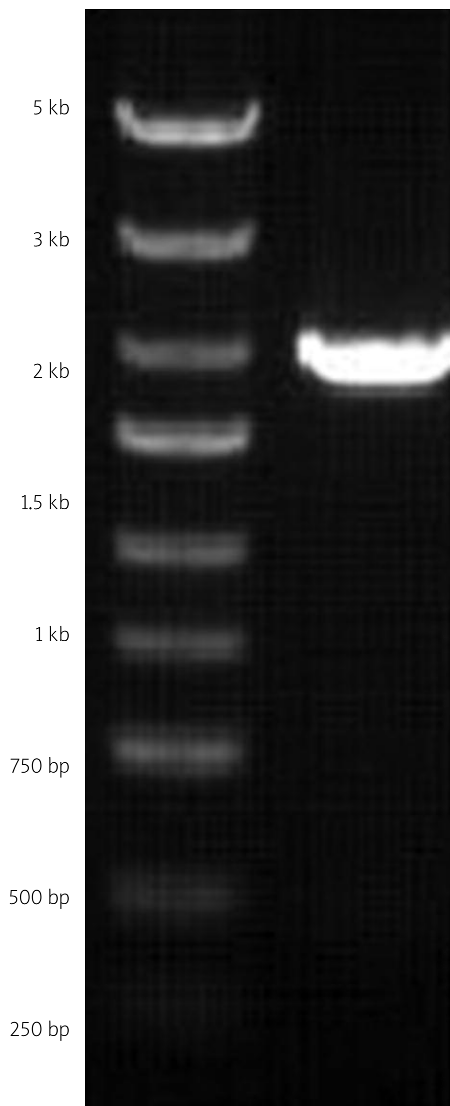

Fig. 1. PCR agarose gel electrophoresis

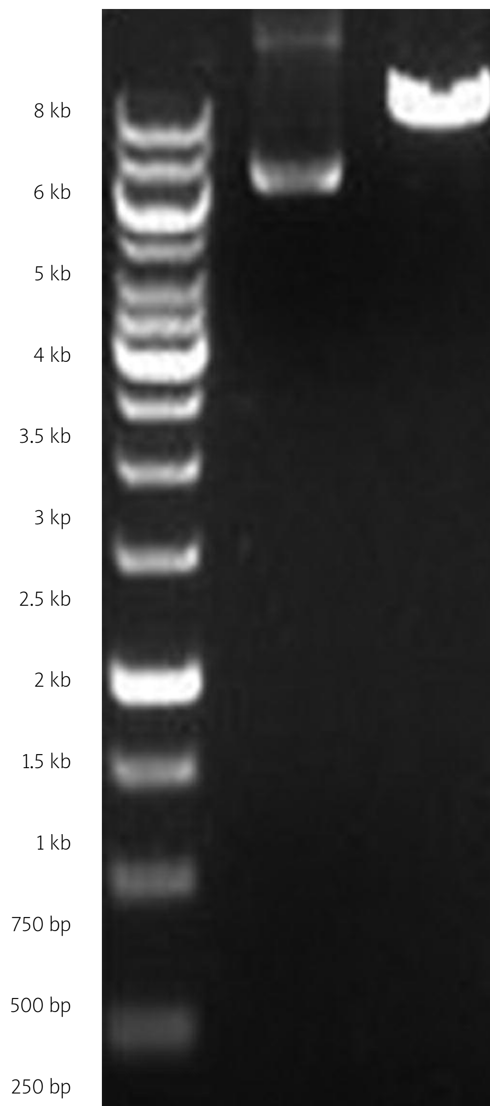

Fig. 2. Vector digested by agarose gel electrophoresis

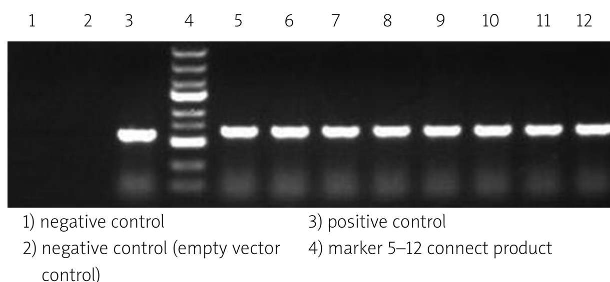

Fig. 3. Identification of positive clones for the recombinant lentiviral vector with ABCG2 over expression

The recombinant lentiviral vector with ABCG2 overexpression infected the MCF-7 cells

In the inverted fluorescence microscope the MCF-7 cells infected successfully displayed green. The infection efficiency was 92.4 ±1.8% (Fig. 5).

Wound healing assay

After 24 hours some cells appeared in the wound tape. The wound of the infected MCF-7 could heal after 48 hours and was full of the cells. The control group wound could not heal after 48 hours (Fig. 6).

Transwell invasive assay

We chose 10 visions randomly under the microscope, and calculated the average number of one vision cells. In the infected MCF-7 cell group 38.24 ±0.35 cells appeared, but only 12.21 ±0.25 cells appeared in the control group ($p < 0.05$) (Fig. 7).

Animal tumor formation

The transplanted tumor appeared in the left back of the nude mouse after 14 days. The size of the transplantation tumor increased gradually and stopped increasing after 34 days. The transplanted tumor was observed in the right back of the nude mouse until 20 days, and its size increased slowly. After 29 days the increase of size disappeared (Fig. 8).

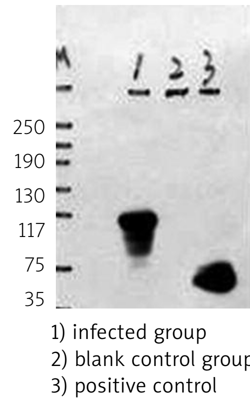

Fig. 4. The western blotting results

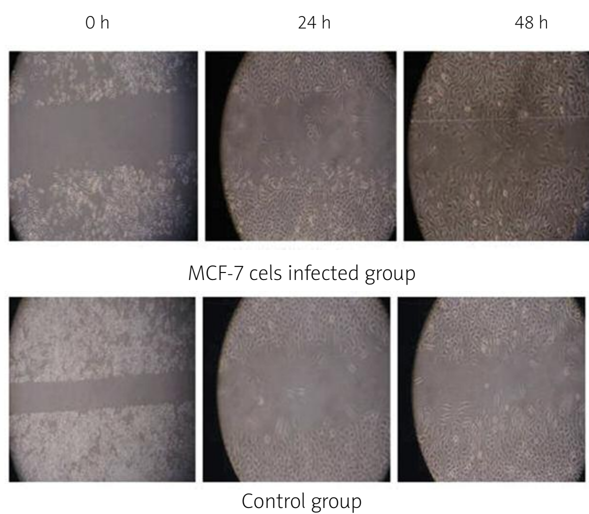

Fig. 6. Wound healing assay

Fig. 8. Transplanted tumor of the nude mouse

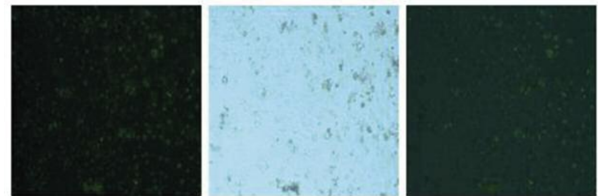

Fig. 5. MCF-7 cells infected by the recombinant lentiviral vector with ABCG2 over expression

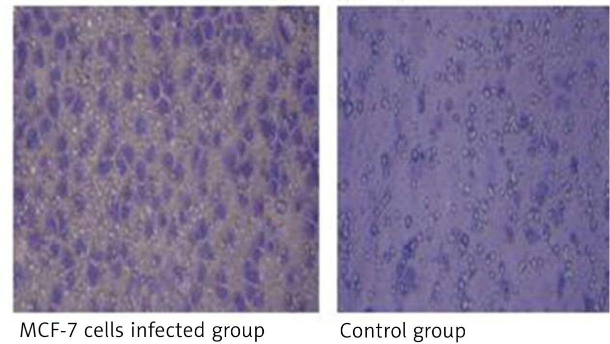

Fig. 7. Transwell invasive assay

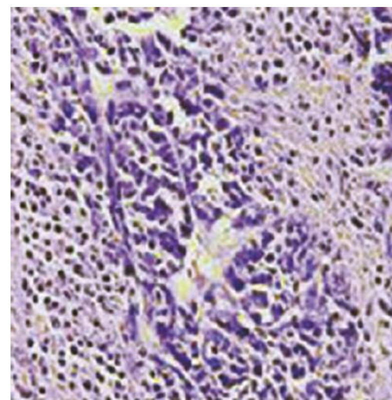

Fig. 9. HE staining result

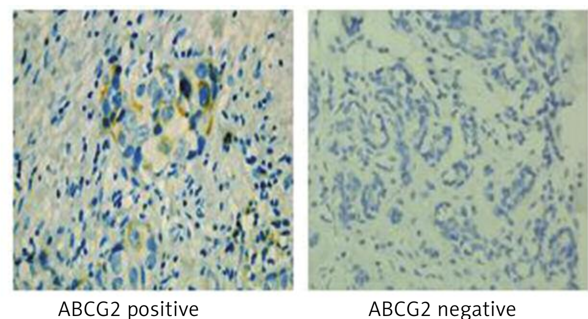

Fig. 10. Transplanted tumor immunohistochemistry results

HE staining and immuno-histochemistry results

We found the cancer cells in the transplanted tumor by HE staining. The immuno-histochemistry test showed positive results in transplanted tumor of MCF-7 cells infected, but negative results in transplanted tumor of the control group (Figs. 9, 10).

Discussion

Gene transfection refers to the biological function of nucleic acid is transported into the cells, and the nucleic acids in

the cell can maintain their biological functions. Nucleic acid comprises DNA (plasmid and linear double-stranded DNA), antisense oligonucleotides and RNAi (RNA interference). At present gene transfection technology has been widely applied in regulation of gene expression, gene function, signal transduction and drug screening studies and gene therapy research. Usually the plasmid vectors and the virus vectors are used widely as a carrier in gene transfer studies. In order to make the target gene maintain the activity stably and consistently the lentiviral vector is used widely. The lentivirus could infect the aperiodic and post-mitotic cells effectively, and integrate the exogenous gene into the host chromosome. In this study we established a lentiviral vector carrying ABCG2 overexpression (Figs. 1–4), and demonstrated that the vector could infect MCF-7 cells successfully and stably (Fig. 5), which was consistent with Han's studies [4]. So we think that the gene transduction technology is a reliable and effective method used in breast cancer research.

ABCG2 located in the membrane of cells is a member of the ATP binding assembly protein G subfamily, and is considered as the key enzyme for the cell transporting toxins out [1–3]. ABCG2 joins the process of maintaining the cell's structure and the formation of the cancer's chemotherapy drug resistance [5, 6]. The migration and invasion of the cancer cells are the key reasons why cancer recurrence and metastasis occur. We promoted the expression of ABCG2 in the human breast cancer MCF-7 cells by gene transduction technology, and the cells infected showed stronger migration and invasion abilities (Figs. 6, 7). The results seemed to be related to the MEK/ERK-ABCG2 pathway [7]. We speculated that the reason might be that ABCG2 increases tumor cell structure stability, and thus the tumor cell gains a strong survival and proliferation ability.

The infinite animal tumor formation is considered one of the cancer stem cell's characteristics. We obtained one type of MCF-7 cells by using lentiviral vector infecting them which had a strong animal tumor formation ability (Fig. 8). So we thought the MCF-7 cells infected by the lentiviral vector seemed some cancer stem cell's characteristic. Semenza confirmed that HIF (hypoxia inducible factor) and ABCG2 are highly expressed in breast cancer cases with lung metastasis, and thought the HIF family member had led to the cancer cells' infinite proliferation by promoting the expression of Ang (angiopoietin) [8]. We speculated that the overexpression of ABCG2 promoted the growth of the vessels around the transplanted tumor, and thus made the animal obtain a greater volume tumor.

The reliability of the recombinant lentiviral vector with ABCG2 overexpression has been verified by some experimental methods. And the MCF-7 cells after being infected showed the changes expected. All of these helped us promote the understanding about the relationships between ABCG2 and breast cancer cells, whereby ABCG2 did not only promote the resistance of the chemotherapy drug, but also promoted the migration and invasion abilities of cancer cells. However, we must admit that the detailed mechanism between ABCG2 and cancer cells is not clear. In the next step we will carry out further studies about all above to reveal the reason for these changes caused by ABCG2.

The authors declare no conflict of interests.

References

1. Li FG, Chu Y, Meng DM, Tong YW. Association of ABCG2 gene C421A polymorphism and susceptibility of primary gout in Han Chinese males. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 2011; 28: 683-5.
2. Geng F, Jiao Z, Dao YJ, Qiu XY, Ding JJ, Shi XJ, Li ZD, Zhong MK. The association of the UGT1A8, SLCO1B3 and ABCC2/ABCG2 genetic polymorphisms with the pharmacokinetics of mycophenolic acid and its phenolic glucuronide metabolite in Chinese individuals. Clin Chim Acta 2012; 413: 683-90.
3. Takara K, Matsubara M, Yamamoto K, Minegaki T, Takegami S, Takahashi M, Yokoyama T, Okumura K. Differential effects of calcium antagonists on ABCG2/BCRP-mediated drug resistance and transport in SN-38-resistant HeLa cells. Mol Med Rep 2012; 5: 603-9.
4. Han M, Liu M, Wang Y, et al. Re-expression of miR-21 contributes to migration and invasion by inducing epithelial-mesenchymal transition consistent with cancer stem cell characteristics in MCF-7 cells. Mol Cell Biochem 2012; 363: 427-36.
5. Sakata S, Fujiwara M, Ohtsuka K, Kamma H, Nagane M, Sakamoto A, Fujioka Y. ATP-binding cassette transporters in primary central nervous system lymphoma: decreased expression of MDR1 P-glycoprotein and breast cancer resistance protein in tumor capillary endothelial cells. Oncol Rep 2011; 25: 333-9.
6. Meyer zu Schwabedissen HE, Kroemer HK. In vitro and in vivo evidence for the importance of breast cancer resistance protein transporters (BCRP/MXR/ABCP/ABCG2). Handb Exp Pharmacol 2011; 201: 325-71.
7. Liu W, Liu H, Gao D, Ge G, Zhang P, Sun S, Wang H, Liu S. ABCG2 protects kidney side population cells from hypoxia/reoxygenation injury through activation of the MEK/ERK pathway. Cell Transplant 2012 [Epub ahead of print].
8. Semenza GL. Molecular mechanisms mediating metastasis of hypoxic breast cancer cells. Trends Mol Med 2012; 18: 423-7.

Address for correspondence

Prof. Chengui Wu, PhD
Department of Endocrine Breast Surgery
The First Affiliated Hospital
Chongqing Medical University
Chongqing, China
e-mail: wuchengyi1192@163.com

Submitted: 10.01.2013
Accepted: 25.02.2013