·Basic Research·

Synergic effect of Na+-K+ ATPaseB1 and adriamycin on inhibition of cell proliferation and reversal of drug resistance in breast cancer MCF-7 cells

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[Abstract]   Background and Objective: Na+-K+ATPase (Na+-K+ pump) is an important cell energy conversion system which is probably associated with tumor metastasis. Expression of its B1 subunit gene-ATP1B1 is high in well differentiated and low in poorly differentiated tumor cells. This study was to investigate the synergic effect of Na+-K+ ATPaseB1 and adriamycin (ADM) on inhibition of cell proliferation and reversal of drug resistance in MCF-7 and MCF-7/ADM cells. Methods: Growth of MCF-7 and MCF-7/ADM cells transfected with PEGFP-ATP1B1 and shATP1B1 were measured by MTT. Intracellular fluorescence intensity of ADM was analyzed by inverted fluorescence microscopy and flow cytometry. ATP enzyme activity was measured by ultramicro-ATP enzyme, and mRNA expression of multi-drug resistance gene MDR1 was detected by RT-PCR and real-time PCR. The expression of P-glycoprotein (P-gp) was analyzed by western blot. Results: The sensitivity of MCF-7 and MCF-7/ADM cells transfected with pEGFP-ATP1B1 to ADM was higher in comparing to the negative control ADM-C3 (transfected with vector pEGFP-C3) and the control ADM-RPMI-1640 (cultured with RPMI-1640), and the differences between ADM-ATP1B1 and ADM-RPMI-1640 groups were statistically significant at different concentrations of adriamycin (P<0.05). After the B1 subunit was silenced, the sensitivity of cells to ADM in the ADM-shNC group was higher than that in the shATP and ADM-RPMI-1640 groups. The mean fluorescence intensity of ADM in the ADM-ATP1B1 group was higher than that in the ADM-C3 and ADM-RPMI-1640 groups (P<0.05). ATP enzyme activity was significantly higher in ADM-ATP1B1 group comparing to the ADM-RPMI-1640 group (P<0.05). mRNA expression of MDR1 gene and protein expression of P-gp were not significantly different among the ADM-ATP1B1 group and two control groups (P>0.05). Conclusion: Na+-K+ ATPase B1 can synergize with ADM and reverse drug resistance to ADM in the MCF-7/ADM cell line. This may be related to ATP enzyme activity, but not to influencing the expression of MDR1 gene.
Key words: Na+-K+ ATPase B1 subunit, adriamycin, breast cancer, MCF-7, reversal of drug resistance

Multi-drug resistance (MDR) is the main reason for the failure of chemotherapy. The reversal of drug resistance is the key point to improve the effect of chemotherapy and has significant values in the clinical management.[1] Resent researches revealed that the change in ATPase activity may be one of the basic factors of tissue dysplasia,

and the Na$^+$-K$^+$ ATPase may also relate to metastasis of cancer cells. Thus, Na$^+$-K$^+$ ATPase has been regarded as a target for breast cancer research.[2] Recent researches have been focusing on the A subunit of Na$^+$-K$^+$ ATPase, and the B subunit (ATP1B1) has been rarely studied. In this study, we investigate the synergic effect of Na$^+$-K$^+$ ATPaseB1 and adriamycin (ADM) on inhibition of cell proliferation and the reversal of drug resistance in MCF-7 and MCF-7/ADM cells.

Materials and Methods

Reagent. The eukaryotic expression plasmid pEGFP-ATP1B1 and pEGFP-C3, both of which contained the enhanced green fluorescent protein, pGPU6/GFP/Neo-shNC, pGPU6/GFP/Neo-shATP1B1 were constructed in our laboratory.[3] The following reagents were used: pGPU6/GFP/Neo-shNC, pGPU6/GFP/Neo-shATP1B1 (Jima Pharmaceutical Technology Co, Ltd, Shanghai), newborn calf serum (Wuhan Sanli Biotechnology Co, Ltd, China), RPMI1640 culture medium and typsin (Gibco, USA), MTT (Sigma, USA), DMSO (Kermel), Lipofectamine 2000 (Invitrogen, USA), cell total RNA rapid isolation kit (Huashun, Shanghai), RT-PCR Kit ReverTra Ace combined type (Boracker, Chengdu, China), RT-PCR, real-time PCR primers and real-time PCR kit (Takara, Dalian, China), DNA Marker 2000 (Tiangen, Beijing), ultramicro-ATPase detection kit (Jiancheng bioengineering institute, Najin), rabbit anti-human IgG P-gp (primary antibody) and HRP tagged goat anti-rabbit IgG (secondary antibody) (Boaosen biologica Co, Ltd, Beijing, China).

Cell culture. Breast cancer cell line MCF-7 and ADM-resistance cell line MCF-7/ADM (with high expression of P-gp) were stored in the Transplantation Immunity Laboratory of Sichuan University, West China Hospital, and Chengdu Diao Group. MCF-7 was cultured in RPMI1640 complete medium at 37℃, 5% CO2. MCF-7/ADM was also cultured in RPMI1640 complete medium containing ADM (1 μg/μL) to maintain its drug-resistant feature. The drug was withdrawn two weeks before the experiment.

Grouping and transfection. MCF-7 and MCF-7/ADM cells in logarithmic phase of growth were plated in cell culture plates. When the cell reached 80-90% confluence, transfection was performed using Lipofectamine 2000 according to the manufactures instruction. Twelve hours later, ADM was added to the culture. The groups are listed in Table 1.

Cell proliferation measured by MTT assay. The proliferation of cells transfected with ATP1B1.MCF-7 and MCF-7/ADM cells were seeded into a 96-well plate at a density of 5×10^3 cells/well. DNA (200 ng) and 0.3 μg liposome compound were transfected into cells of each group. ADM at gradient concentrations (0, 0.125, 0.25, 0.5, 1, 2, 4, 8 μg/mL) was added into each group, respectively. Each group had three duplicate wells. After 72 h culture, MTT was added to each group and incubated at 37℃, 5% CO_2. Then the supernatant was removed and 150 μL DMSO was added. The absorption (A) was measured at 570 nm. The experiment was repeated for three times and the average values were calculated. The inhibition ratio= (A_{570} value of blank group- A_{570} value of experimental group)/ A_{570} value of blank group $\times$ 100%.

Cell proliferation after shATP1B1 transfec-

Table 1   Group classification of experimental treatments

Group	Treatment	MCF-7	MCF-7/ADM	PEGFP-ATP1B1	pEGFP-C3
Treatment	ADM-ATP1B1-MCF-7	+	−	+	−
	ADM-ATP1B1-(MCF-7/ADM)	−	+	+	−
Negative control	ADM-C3-MCF-7	+	−	−	+
	ADM-C3-(MCF-7/ADM)	−	+	−	+
Blank control	ADM-RPMI-1640-MCF-7	+	−	−	−
	ADM-RPMI-1640-(MCF-7/ADM)	−	+	−	−

ADM：adriamycin

tion.   MCF-7 and MCF-7/ADM cells were seeded into six-well plates at a density of 5×10^5 cells/well.   Grouping was processed as described. shATP1B1   (2 μg)   and 8 μg liposome compound were transfected into cells of each group for 12 h,   and total RNA was extracted to determine the silencing effect on the B1 subunit by real-time PCR. shATP1B1 (0.3 μg) and 0.45μg liposome compound were transfected into cells of each group.   Then MTT assay as described in 1.4.1 was conducted to measure the cell suppression rate by shATP1B1.   The experiment was repeated for three times.

Inverted   microscopy.   MCF-7   and MCF-7/ADM cells were seeded into six-well plates at a density of 5×10^5 cells/well. Grouping and transfection were performed as discribed. DNA (1.25 μg) and 5 μg liposome compound were transfected into cells of each group for 12 h. ADM   (2.5μL)   of 1 μg/μL was add to each well and incubated for 24 h.   The green fluorescence emitted by EGFP and red fluorescence emitted by ADM were observed.

Flow cytometry.   The cell seeding and transfection were conducted as discribed.   ADM (2.5 μL) of 1 μg/μL was added to each group for 24h.   The cells were digested by 0.25% trypsin, washed by PBS twice and centrifuged for 5 min at $275 \times$ g. The precipitate was suspended in 300 μL PBS and the fluorescence intensity of ADM was determined by flow cytometry.   The experiment was repeated three times.

Activity of ATPase measured by ultramicro-ATP enzyme assay.   The medium was disposed after 12 h and the cells were harvested after digestion, centrifuged ($275 \times$ g, 5 min) and washing by normal saline twice.   The cells of the same amount in each group were suspended in normal saline of the same volume. The cell Lysis Solution was prepared by multigelation and centrifugation, the supernatant was collected to measure the protein concentration. The ATPase activity was measured by ultramicro-ATP enzyme assay in 120 μL sample. The A value at 630 nm was measure by the enzyme labeled instrument. ATPase activity= (A_{630} value of experimental group- A_{630} value of control group)   /   (A_{630} value of standard group- A_{630} value of blank group).   The experiment was repeated for three times.

The expression of MDR1 gene detected by PCR.   The total RNA of MCF-7/ADM cells of ADM-ATP1B1,   ADM-C3 and ADM-RPMI-1640 groups were extracted according to the manufactures instruction of the RNA quick kit. β-actin was served as the internal control,   and the expression of mdrl mRNA was measure by two-step RT-PCR.   The sense and antisense primers of MDR1 were 5-CCCATCATTG-CAATAGCAGG-3 and 5-GTTCAAACTTCT-GCTCCTGA-3, and the product length was 167 bp.   The sense and antisense primers of β-actin were 5-AGGGTGTGATGGTGGGAAT-3 and 5-CTCGGTGAGCAGCACAGG-3,   and the product length was 200bp.   PCR products were subjected to 1.5% agarose gel electrophoresis. The bands were observed by Bio-Rad Gel Imaging System and analyzed by Quantity One 4.5.0 Software.   The relative expression level of MDR1=density value of MDR1 band/ density value of β-actin band,   the experiment was repeated for three times.   Real-time PCR was performed to confirm the expression level of mdr1. Real-time PCR was performed using the SYBR green method[3] and the reaction system was prepared according to the instruction of the real-time PCR kit.   The expression level of MDRl gene was detected by the BIO-RAD iCycler IQ real-time RCR reactor.   β-actin was detected in another tube and each sample had three duplicate wells.   The reaction conditions were as follows: 95℃ for 2 min,   followed by 40 cycles of 94℃ for 10s, 60℃ for 10s, and 72℃ for 30s. The relative expression level of MDR mRNA was calculated by $2^{-\triangle\triangle Ct \pm S.}$ The $2^{-\triangle\triangle Ct \pm S}$ of ADM-RPMI-1640 group (blank group) was used as the reference standard. $\triangle\triangle Ct= (Ct_{MDR1}-Ct_{\beta\text{-actin}})$ experiment group- $(Ct_{MDR1}-Ct_{\beta\text{-actin}})$ control group.

Expression of P-gp by western blot.   The MCF-7/ADM cells of ADM-ATP1B1, ADM-C3 and ADM-RPMI-1640 groups were lysed to prepare the protein sample.   β-actin was served as the loading control. Lysate (40 μg) was subjected to SDS-PAGE electrophoresis.   After blocking for 1 h,   the membranes were washed with PBST for three times, and incubated with P-gp antibodies (1:300) overnight at 4℃. After being washed

again for three times,   the membranes were incubated with peroxidase-conjugated secondary antibodies (1:3000) for 2h at 37℃ .The bands were developed by ECL film to analyze the protein level.   The experiment was repeated for three times.

Statistical analysis.   All data are expressed as mean ± SD.   Variance analysis and paired comparison were performed using SPSS 13.0 software package. $p < 0.05$ was considered statistically significant.

Results

Transfection with ATP1B1 and shATP on the inhibitory effects of ADM on cell proliferation.   ADM-ATP1B1 and ADM-C3 inhibited proliferation of MCF-7 and MCF-7/ADM cells even without the treatment with ADM.   The drug exerted the inhibitory effect in a concentration-dependent manner. The saturation concentration of ADM was the lowest in the ADM-ATP1B1 group (~0.25 μg/μL). Cells in the MCF-7/ADM group were significantly resistant to ADM.   Each group did not reach saturation when the drug concentration was 8 μg/μL, meanwhile, the inhibition ratios of both ADM-C3 and ADM-RPMI-1640 groups were lower than that of the ADM-ATP1B1 group ($p < 0.05$) (Fig. 1, 2). Differences between ADM-ATP1B1 and ADM-RPMI-1640 groups were statistically significant at different concentrations of ADM ($p < 0.05$). pEGFP-ATP1B1 not only synergized with ADM to inhibit proliferation of MCF-7 and MCF-7/ADM cells,   but also reversed the drug resistance of MCF-7/ADM cells.

The silencing effect of shATP1B1 [(66.23± 0.13)%]   was significantly different from that of shNC [(11.56± 0.32)%] and RPMI-1640 (0) ($p < 0.05$).   The inhibition effect on cell proliferation of the group ADM-shNC was obviously higher than that of groups ADM-shATP1B1 and ADM-RPMI-1640 ($p < 0.05$)   (Fig 3,   4).   The non-specific effect of ADM-shNC was more outstanding;   yet the B1 subunit was silenced in the group ADM-shATP1B1,   leading to its relatively weak
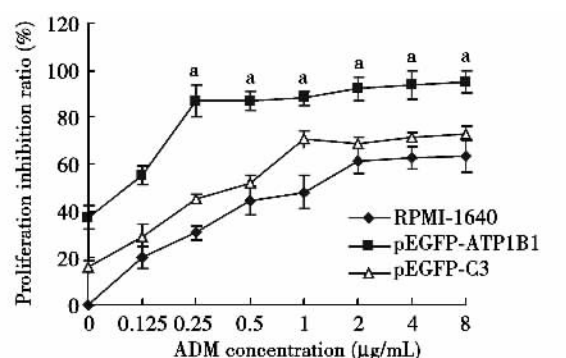

Figure 1   Influence of pEGFP-ATP1B1 on inhibition of MCF-7 cell growth by adriamycin（ADM）

[a]$P < 0.05$, vs. ADM-C3 and ADM-RPMI-1640. The results are presented as mean±SD of three independent experiments.
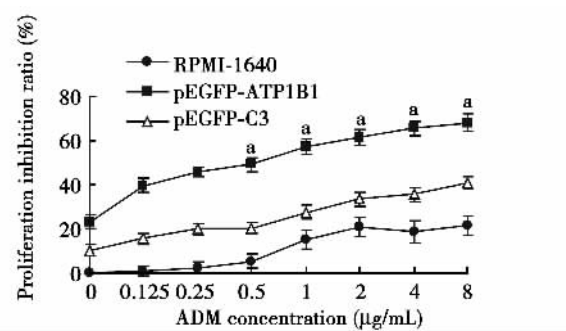

Figure 2   Influence of pEGFP-ATP1B1 on inhibition of MCF-7/ADM cell growth by adriamycin（ADM）

[a]$P < 0.05$, vs. ADM-C3 and ADM-RPMI-1640. The results are presented as mean±SD of three independent experiments.

non-specific effect to inhibit cell proliferation. This further confirmed that low expression of ATP1B1 reduced the inhibition ratio of cell proliferation,   which was consistent to the result of high expression of ATP1B1.

Intracellular distribution of ADM and cell morphology. As shown in Fig. 5, in MCF-7 and MCF-7/ADM cells,   the fluorescence density in groups ADM-RPMI-1640,   ADM-C3 and ADM-ATP1B1 was increased successively; significant morphological changes appeared in cells of ADM-ATP1B1 and ADM-C3 groups; the proliferation of cells was significantly inhibited;   cells became pyknotic and rounding without normal morphous;   a few vacuoles were observed in cytoplasm;   but cells in the group ADM-RPMI-1640 grew well,   with a long-polygon shape;   cells extended to join
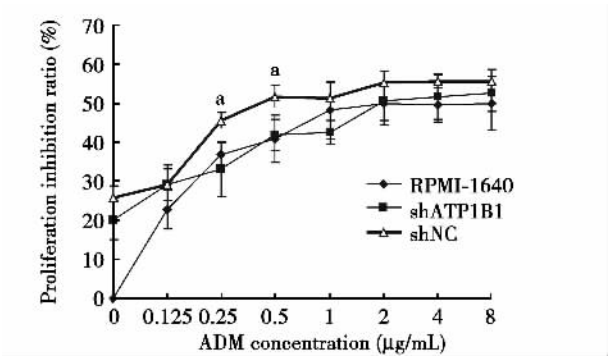
Figure 3　Influence of shATP1B1 on inhibition of MCF-7 cell
growth by adriamycin（ADM）

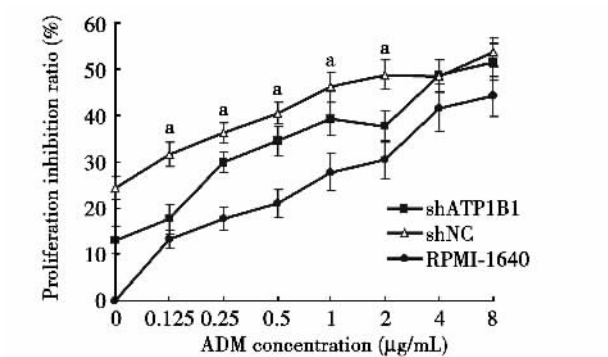[a]P<0.05, vs. ADM-shATP1B1 and ADM-RPMI-1640. The results are presented as mean±SD of three independent experiments.

Figure 4　Influence of shATP1B1 on inhibition of MCF-7/
ADM cell growth by adriamycin（ADM）

[a]P<0.05, vs. ADM-shATP1B1 and ADM-RPMI-1640. The results are presented as mean±SD of three independent experiments.

together. At the same time, the red fluorescence was significantly stronger in MCF-7 cells than in MCF-7/ADM cells.

Intracellular fluorescence intensity of ADM was analyzed by flow cytometry. As shown in Fig. 6, the mean intracellular fluorescence intensity of ADM was stronger in the ADM-ATP1B1 group than in ADM-C3 and ADM-RPMI-1640 groups, which were statistically significant (p<0.05). The fluorescence

intensity of MCF-7 cells was stronger than that in MCF-7/ADM cells. The green fluorescence intensities were not significantly different between the ADM-ATP1B1 and ADM-C3 groups (p>0.05). It confirmed that the synergic effect of pEGFP-ATP1B1 and ADM on the reversal of drug resistance.

Measure the ATP enzyme activity. As shown in Fig. 7, in MCF-7 cells, the activity of ATP enzyme of the ADM-ATP1B1 group ［
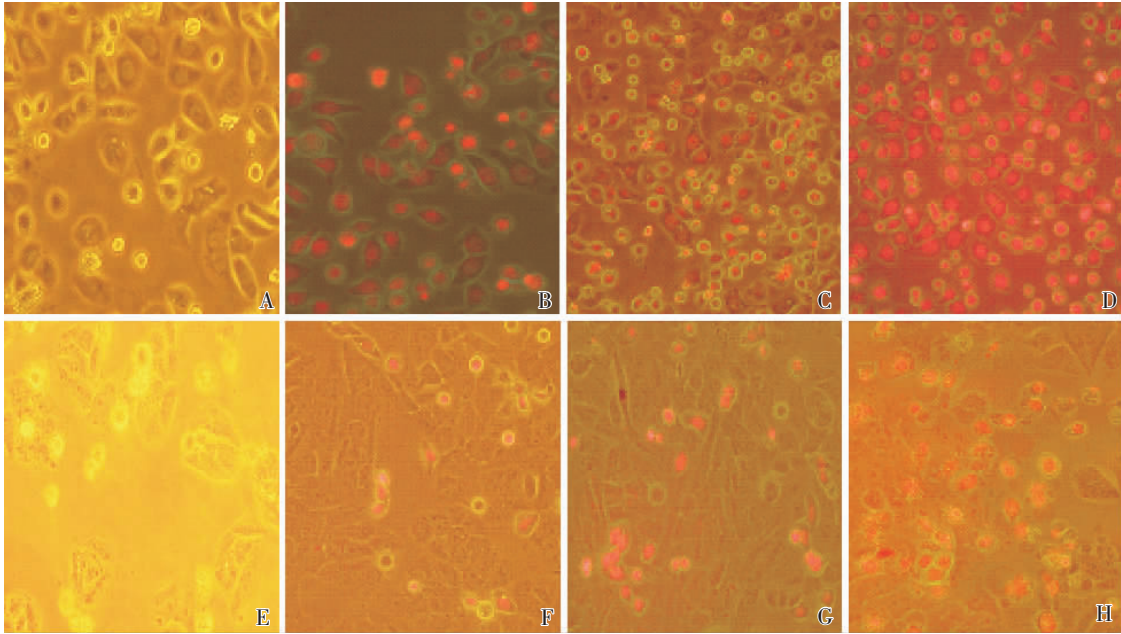

Figure 5　Fluorographic and morphological changes of MCF-7 and MCF-7/ADM cells after transfection with pEGFP-ATP1B1（×200）

Cell pyknosis and rounding with some vacuoles are observed.

A：MCF-7 cells；B：ADM-RPMI-1640-MCF-7；C：ADM-C3-MCF-7；D：ADM-ATP1B1-MCF-7 E：MCF-7/ADM cells；F：ADM-RPMI-1640-（MCF-7/ADM）；G：ADM-C3-（MCF-7/ADM）；H：ADM-ATP1B1-（MCF-7/ADM）.
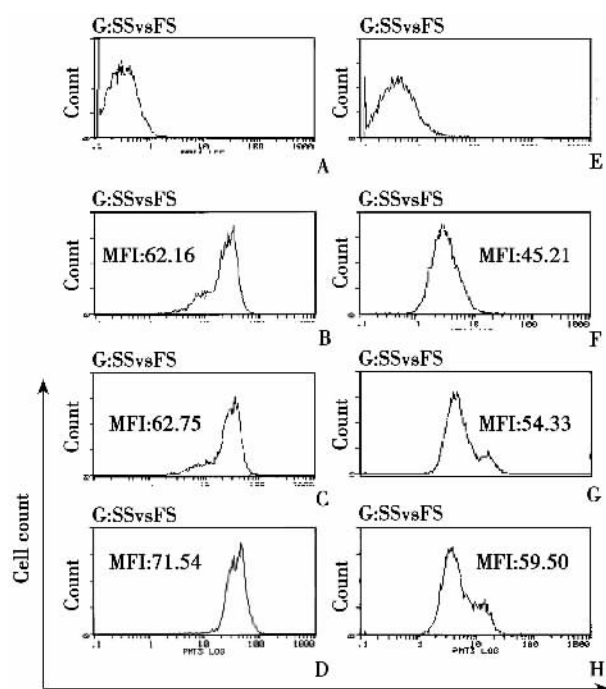
Figure 6　The mean fluorescence intensity of MCF-7 and
MCF-7/ADM cells transfected with pEGFP-ATP1B1
A：MCF-7 cells；B：ADM-RPMI1640-MCF-7；C：ADM-C3-MCF-7；
D：ADM-ATP1B1-MCF-7；E：MCF-7/ADM cells；F：ADM-RPMI1640-
（MCF-7/ADM）；G：ADM-C3-（MCF-7/ADM）；H：ADM-ATP1B1-
（MCF-7/ADM）

Figure 7　Relative expression of MDR1 mRNA in MCF-7/ADM
cells transfected with pEGFP-ATP1B1 detected by RT-PCR
Lane1：ADM-RPMI1640-（MCF-7/ADM）；Lane2：ADM-RPMI1640-
（MCF-7/ADM）；Lane3：ADM-C3-（MCF-7/ADM）；Lane4：ADM-
C3-（MCF-7/ADM）；Lane5：ADM-ATP1B1-（MCF-7/ADM）；Lane6：
ADM-ATP1B1-（MCF-7/ADM）；M：marker2000.

Figure 8　Expression of P-gp in MCF-7/ADM cells transfected
with pEGFP-ATP1B1 detected by western blot.
Lane 1：ADM-ATP1B1-（MCF-7/ADM）；Lane 2：ADM-C3-（MCF-7/
ADM）；Lane3：ADM-RPMI1640-（MCF-7/ADM).

$(2.89 \pm 0.26)\%$] was significantly higher than that of the ADM-RPMI-1640 group [$(1.02 \pm 0.11)\%$] ($p<0.05$), but that between the ADM-C3 [$(1.06 \pm 0.20)\%$] and the ADM-RPMI1640 group was no significantly different ($p>0.05$). In MCF-7/ADM cells, the activity of ATP enzyme of the ADM-ATP1B1 group [$(2.69 \pm 0.31)\%$] was significantly higher than that of the ADM-RPMI-1640 group [$(0.98 \pm 0.14)\%$] ($p<0.05$), but that was not significantly different between the ADM-C3 group [$(1.10 \pm 0.22)\%$] and the ADM-RPMI1640 group ($p>0.05$). pEGFP-ATP1B1 affected ATP enzyme activity through increasing the protein level of ATP1B1.

Detection the expression of MDR1 gene. As shown in Fig. 8, the relative mRNA expression of MDR1 gene was not statistically significant among the ADM-ATP1B1 ($1.03 \pm$
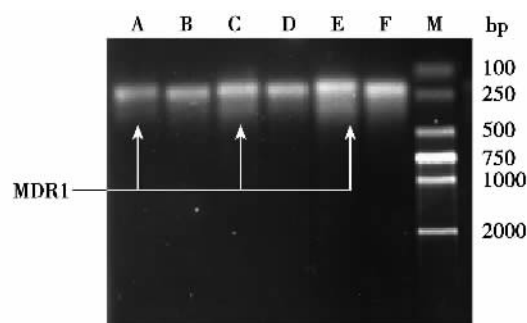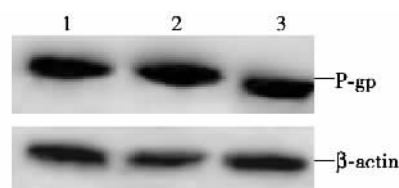
0.07), ADM-C3 (1.25 ± 0.06) and ADM-RPMI-1640 (1.10 ± 0.09) groups ($p>0.05$) measured by RT-PCR. There were not statistical significances in MDR1 mRNA expression among the DM-ATP1B1 (1.05 ± 0.12), ADM-C3 (1.15 ± 0.16) and ADM-RPMI-1640 (1.00 ± 0.15) groups detected by real-time PCR ($p>0.05$), which indicated that pEGFP-ATP1B1 did not affect mRNA expression of MDR1.

The expression of P-gp analyzed by western blot. As shown in Fig. 9, the protein levels of P-gp were not statistically significant among the ADM-ATP1B1, ADM-C3 and ADM-RPMI-1640 groups of MCF-7/ADM cells ($p>0.05$). There was no difference among the level of P-gp protein encoded by MDR1 mRNA, which was consistent to the mRNA results.

Discussion

Na$^+$-K$^+$ATPase (Na$^+$-K$^+$ pump) is mainly constituted by A and B subunits. B subunit, which is a regulatory subunit, with the structure and function as a typical adhesiove molecule, is the key factor in polarizing distribution of Na$^+$-K$^+$ATPase.[4] The B subunit contains three isoforms in the mammals as B1, B2 and B3. The most common one is B1, which lies on the cell membrane,[4,5] but the functions of each isoform is not very clear. The B subunit may play roles in the following three aspects: the maturity of the holoenzyme composition and function, the affinity of the regulatory enzyme to the cation and sending the A subunit to the membrane to exert the effect.[6,7] The main functions include holding cell osmotic pressure, maintaining the cell volume, keeping intracellular environment at a low level of Na$^+$ and a high level of K$^+$, retaining cell standing potential, and so on.[8] At present, some researches revealed that the Na$^+$-K$^+$ATPase not only adjusts the energy conversion but also acts as a key factor in determining cell growth, differentiation and life-span. They also discovered that the expression of the Na$^+$-K$^+$ATPase of many tumor tissue cells declines in varying degrees, which is mainly caused by the change in the expression of the B subunit. It has been confirmed that the expression of the B1 subunit is significantly decreased in the clear cell carcinoma of kidney, lung cancer, hepatocellular carcinoma and hormone-dependent prostate cancer.[9]

In this study, the MCF-7 and MCF-7/ADM cells were transfected with PEGFP-ATP1B1 to investigate the synergic effect of the B1 subunit and ADM on inhibition of cell proliferation of the breast cancer cells and the mechanism of the reversal of multi-drug resistance. After transfection of the MCF-7 and MCF-7/ADM cells with PEGFP-ATP1B1, PEGFP-ATP1B1 showed an evident synergic effect with ADM along with the increase in the concentration of ADM, and also displayed the reversal of drug resistance to MCF-7/ADM cells. Meanwhile, we designed the experiment with low expression of

ATP1B1 when the B1 subunit was silenced. The inhibition ratio was obviously decreased after silencing the B1 gene. To explain this conclusion more intuitively, we performed fluorometric analysis under microscopy and flow cytometry, whose results were consistent. We found out that cells transfected with PEGFP-ATP1B1 showed high ATPase activity, which further proves that PEGFP-ATP1B1 could increase the activity of ATPase through increasing the expression of ATP1B1 protein. The consistency between the results of RT-PCR, real-time PCR and western blot revealed that transfection with PEGFP-ATP1B1 did not changes the expression of MDR1 mRNA and P-gp protein.

Overexpression of MDR1 increases the synthesis of P-gp on the membrane, which leads to drug resistance.[10] Transfection of pEGFP-ATP1B1 into cells does not relate to the expression of MDR1, which implies that the synergic effect of ATP1B1 with ADM and its reversal effect of drug resistance may not through down-regulating the expression of MDR1 mRNA or decreasing the synthesis of P-gp. Our study indicated that after the induction of exogenous ATP1B1 into breast cancer cells, the gene expression of ATP1B1 was increased, and an increase in translated ATP1B1 protein was led. Subsequently, the activity of ATPase was increased through a series of regulation, probably accompanied with the increase in the Na$^+$-K$^+$ exchange, which induced the imbalance of the ion concentration inside and outside of the cells; changes in the osmotic pressure might result in the confusion of the intracellular environment, leading to a serial changes in cells.[11,12] Therefore, we suggest that ATP1B1 gene coding the B1 subunit of the Na$^+$-K$^+$ ATPase is through a new path- the "ion pump", which transfers the light concentration drugs outside the cells to the inside of the cells; or, ATP1B1 might transfer the drugs into the cells acting as a plasmid vector to synergize with ADM.[13] However, the underlying mechanism remains unclear.

So far, we have not found any literatures regarding the function of ATP1B1 in multi-drug resistance of tumor cells. In this study, we reported the synergic effect of ATP1B1 with

ADM and its effect in the reversal of drug resistance. This study may offer some information to overcome drugs resistance of anti-cancer drugs in the future.

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