ABCG2 gene amplification and expression in esophageal cancer cells with acquired adriamycin resistance

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Abstract. Resistance to chemotherapeutic agents is the main reason for treatment failure in patients with cancer. The primary mechanism of multidrug resistance (MDR) is the overexpression of drug efflux transporters, including ATP-binding cassette transporter G2 (*ABCG2*). To the best of our knowledge, the MDR mechanisms of esophageal cancer have not been described. An adriamycin (ADM)-resistant subline, Eca109/ADM, was generated from the Eca109 esophageal cancer cell line by a stepwise selection in ADM from 0.002 to 0.02 ng/μl. The resulting subline, designated Eca109/ADM, revealed a 3.29-fold resistance against ADM compared with the Eca109 cell line. The *ABCG2* gene expression in the Eca109/ADM cells was increased compared with that of the Eca109 cells. The cellular properties of the Eca109/ADM cells were detected by reverse transcription polymerase chain reaction (RT-PCR), flow cytometry and western blotting. The ABCG2 expression levels were detected by RT-PCR and flow cytometry, and the drug efflux effect was detected by flow cytometry. The present study detected the correlation between ABCG2 and the multidrug resistance of esophageal cancer. *ABCG2* gene expression and the drug efflux effect of the Eca109/ADM cells were increased compared with those of the Eca109 cells. Collectively, the results of this study indicated that the overexpression of ABCG2 in the Eca109/ADM cells resulted in drug efflux, which may be responsible for the development of esophageal cancer MDR.

Introduction

Cancer therapy is often based on surgery, chemotherapy and radiation therapy. Chemotherapy is one of the main therapies for the majority of cancers, particularly for those with a proneness to invade adjacent tissues and to metastasize to other organs. The effectiveness of chemotherapy is often limited by toxicity to other normal tissues in the body and multidrug resistance (MDR). Malignant tumors exhibit MDR and toxicity, which are major therapy-limiting factors that lead to a poor patient prognosis. As a consequence, the requirement to understand these resistance mechanisms on a molecular level is urgent. The development of resistance to chemotherapeutic agents is a serious problem in the treatment of numerous human cancers. A tolerance to one agent is often accompanied by cross-resistance to a variety of other compounds. This MDR is caused by several mechanisms, including increased drug efflux (1-8), enhanced drug detoxification, alterations of targets, and modification of DNA damage repair systems (9-11) and apoptosis pathways (12). The drug efflux effect is mediated by the enhanced expression of one or more ATP-binding cassette (ABC) transporters (2,13-15). One of these ABC transporters is breast cancer resistant protein (BCRP), which is encoded by the *ABCG2* gene (16). A number of chemotherapeutic agents, including adriamycin (ADM), mitoxantrone (MIT) and daunorubicin (DNR), are substrates for the ABCG2 drug efflux pump (2). The exposure of the Eca109 cell line, an esophageal squamous cell carcinoma cell line, to increasing concentrations of ADM leads to the development of the MDR phenotype, the resulting subline being designated Eca109/ADM. The objective of the present study was to investigate the correlation between ABCG2 expression and the MDR of esophageal cancer.

Materials and methods

Cell line and cell culture. The Eca109 esophageal squamous cell carcinoma cell line was obtained from the Tumor Institute of The Fourth Hospital of Hebei Medical University (Shijiazhuang, China). The Eca109 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 5% penicillin (100 U/ml) and streptomycin (100 mg/ml) in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. The medium was changed three times a week.

Establishment of the ADM-resistant cell line. An ADM-resistant cell line was established from the Eca109 cells by continuous exposure to increasing concentrations of ADM, from 0.002 ng/μl to 0.02 ng/μl, for eight months. One of the surviving clones was isolated and designated as Eca109/ADM.

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Cytotoxicity assay. The sensitivity of the Eca109 and Eca109/ADM cells to the anticancer drugs ADM, DNR and MIT was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the capacity of viable cells to metabolize (via mitochondrial succinate dehydrogenase) a yellow tetrazolium salt MTT into purple formazan crystals dissolved in acidified propan-2-ol. The resulting purple solution is spectrophotometrically measured at 490 nm. An increase or decrease in cell number results in a concomitant change in the quantity of formazan formed, indicating the degree of cytotoxicity caused by the test material. The absorbance at 490 nm was read using a microplate reader (BioTek, Winooski, VT, USA). The cells were seeded into 96-well culture plates at a density of $5x10^4$ cells/ml. A serial concentration of ADM, DNR or MIT was added in a final volume of 200 μl per well. Following drug treatment for 24 h, the medium was replaced with an equal volume of fresh medium containing 0.5 mg/ml MTT (Sigma, St. Loius, MO, USA) and incubated for 4 h. The medium was then removed and 180 μl dimethylsulfoxide was added and incubated for 10 min at room temperature. The cytotoxic effects of the drugs were determined according to the optical density values using a microplate reader at an absorption wavelength of 490 nm. Cell viability was expressed as the relative formazan formation in the treated samples compared with the control cells [(A490-treated cells / A490 control cells) x 100]. The IC_{50} (i.e., the drug concentration causing 50% growth inhibition), was determined using the MTT assay. The resistance index was determined as the IC_{50} of the resistant cells/the IC_{50} of the parental cells.

ABCG2 mRNA levels determined by reverse transcription polymerase chain reaction (RT-PCR). The total RNA was isolated from the Eca109 and Eca109/ADM cells using TRI reagent (Sigma) according to the manufacturer's instructions, and then treated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) to form cDNA. The cDNA was amplified by PCR using Taq DNA polymerase (Promega), which was performed by denaturation at 94˚C for 30 sec, annealing at 57˚C for 30 sec and extension at 72˚C for 30 sec. This was repeated for 35 cycles. The PCR-amplified products were then run on a 1.5% agarose gel and visualized by ethidium bromide staining. The expression intensities of the optimized bands were quantified with Quantity One software (Bio-Rad, Mississauga, ON, Canada), and expressed as a ratio (*ABCG2* versus *GAPDH*). The PCR primers were as follows: Forward: 5'-GGT CAG AGT GTG GTT TCT GTA GCA-3' and reverse: 5'-GTG AGA GAT CGA TGC CCT GCT TTA-3' for *ABCG2* (product of 280 bp); and forward: 5'-ACC ACA GTC CAT GCC ATC AC-3' and reverse: 5'-TCC ACC ACC CTG TTG CTG TA-3' for *GAPDH* (product of 452 bp).

ABCG2 protein levels determined by flow cytometry. The flow cytometric analysis was performed based on the standard staining procedure. The Eca109 and Eca109/ADM cells were grown to 80% confluence in cell culture bottles, gently dislodged with pancreatic enzymes solution (Gibco, Carlsbad, CA, USA), centrifuged for 5 min at 1,200 x g (Bai Yang, Beijing, China) and resuspended in phosphate-buffered saline (PBS). Samples of unfixed Eca109 and Eca109/ADM cells (10^6 cells/100 μl) in flow cytometry tubes were incubated subsequently with mouse anti-ABCG2 labeled with fluorescein isothiocyanate (BioLegend, San Diego, CA, USA) for 30 min in the dark. Fluorescence was measured on a Epics-XL II flow cytometer (Beckman Coulter, Miami, FL, USA).

ABCG2 protein level detection by western blotting. The ABCG2 protein expression level was detected by western blotting. Each cell line was grown to 80% confluence, trypsinized, transferred to eppendorf tubes and rinsed with ice-cold PBS. The contents of each tube were suspended in 200 μl lysis buffer in the presence of protease inhibitors (Sigma). The cell suspension was incubated for 20 min (4˚C) and centrifuged (10 min, 32,000 x g) to provide a clear supernatant. The protein concentration was measured by the Bio-Rad protein assay, with bovine serum albumin used as a standard (Bio-Rad Laboratories, Mississauga, ON, USA). For the western blotting analysis, the protein extract was analyzed by SDS gel electrophoresis on appropriate polyacrylamide gels (5%), with 50 μg protein loaded on each lane. The proteins on the gels were then transferred to polyvinylidene difluoride membranes by a semi-dry transfer method. Following blocking with Tris-buffered saline containing 5% skimmed milk and 0.1% Tween-20, the membranes were probed with a primary antibody against ABCG2 (mouse monoclonal antibody, clone no sc-18841; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The antibody was used at a dilution of 1:1,000. The membrane blots were then reacted with secondary antibody (horseradish peroxidase anti-mouse IgG), washed extensively with Tris-buffered saline (0.1% Tween-20) and submerged in DAB, then developed to visualize the antibody-antigen complexes.

ADM accumulation and efflux by flow cytometry. The cellular accumulation and efflux of ADM were analyzed by flow cytometry. In total, $4x10^5$ cells (Eca109 and Eca109/ADM) were incubated with 0.02 μg/ml ADM at 37˚C for 2 h and then washed twice with ice-cold PBS. The cells were resuspended in ADM-free RPMI-1640 for 1 h. The cells were washed with ice-cold PBS and the ADM retained in the cells was detected by flow cytometry. The ADM generated red fluorescence (fluorescent peak at 620 nm) after being stimulated by a 488-nm laser (Beckman Coulter).

Apoptosis rate of cells. The apoptosis rate of the Eca109 and Eca109/ADM cells following treatment with 0.02 μg/ml ADM for 24 h was analyzed by flow cytometry. The cells were harvested with trypsin-EDTA (1:20), washed with PBS and then centrifuged (5 min, 1,200 x g). The cells were dyed in 1 ml flourescence liquor containing 50 μg/ml propidium iodide following incubation for 30 min in the dark at 4˚C, then kept at 4˚C until used. The cells were analyzed by an Epics-XL II type cytometer (Beckman Coulter) equipped with a 488 nm argon ion laser. For each sample, 10,000 events selected in the living cell gate were measured. Forward scatter and side scatter data were used to establish a gate excluding dead cells and debris.

Statistical analysis. The statistical analysis was performed with SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA). The data are displayed as the mean ± standard deviation, and three individual experiments were conducted in triplicate. Student's

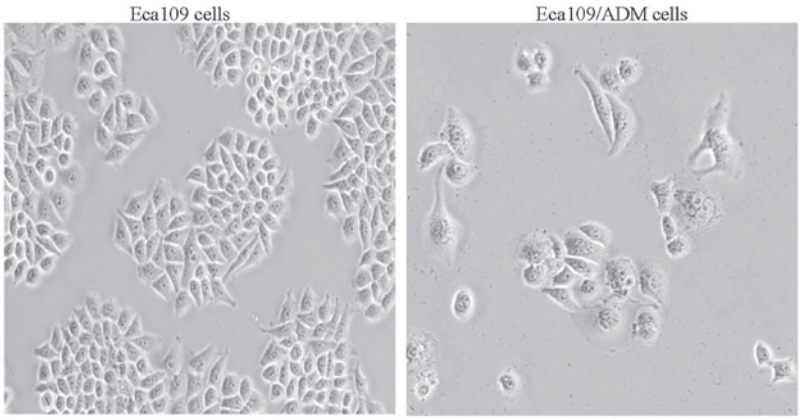

Figure 1. Morphology of Eca109/ADM cells and Eca109 cells detected by optical microscopy (magnification, x200). The Eca109/ADM cell line was established from Eca109 cells by continuous exposure to increasing concentrations of ADM for eight months. The cell morphology of the Eca109/ADM cells was irregular compared with that of the Eca109 cells. ADM, adriamycin.

t-test was used to compare data. P<0.05 was considered to indicate a statistically significant difference.

Results

Cell morphology of Eca109/ADM. The Eca109/ADM cell line was successfully established from the Eca109 cells by continuous exposure to increasing concentrations of ADM, from 0.002 ng/μl to 0.02 ng/μl, for eight months. One of the surviving clones was isolated and designated as Eca109/ADM. The cell morphology of the Eca109/ADM cells was more irregular compared with that of the Eca109 cells (Fig. 1).

Drug-resistant phenotype. An ADM-resistant subline derived from the parental sensitive cell line, Eca109, was established by a stepwise selection in ADM. The subline was designated as Eca109/ADM. The IC_{50} value of ADM, DNR and MIT in the Eca109/ADM cells was 15.45±1.15, 7.27±0.30 and 3.91±0.53 μg/ml, respectively, compared with 4.69±0.88, 1.94±0.21 and 1.24±0.30 μg/ml, respectively, in the Eca109 cells. The Eca109/ADM cells exhibited 3.29-, 3.75- and 3.15-fold resistance, respectively (Fig. 2).

ABCG2 gene expression level. The *ABCG2* gene expression level was detected by RT-PCR and compared with that of the parental cell line, Eca109. The *ABCG2* mRNA expression level of the Eca109/ADM cells was significantly higher than that of the Eca109 cells (P<0.05; Fig. 3).

ABCG2 protein expression level. The ABCG2 protein expression level was examined by flow cytometry and western blotting. The result of the flow cytometry revealed that the ABCG2 protein expression level of the Eca109/ADM cells was significantly higher than that of the parental cell line, Eca109 (P<0.05; Fig. 4). The western blotting result was consistent with the flow cytometry result (Fig. 5).

Drug efflux effect of Eca109/ADM cells. To investigate the resistance of the Eca109/ADM cells to the anticancer agent, ADM, the ADM efflux effect was detected using flow cytometry (Fig. 6). When the cells were incubated at 37˚C with

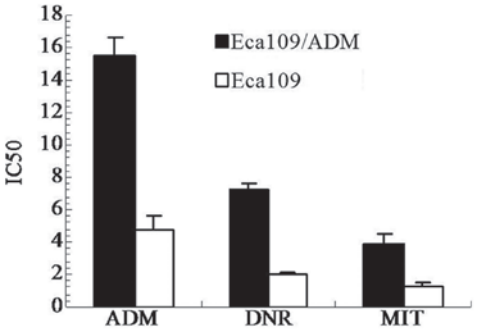

Figure 2. IC_{50} value of cells against ADM, DNR and MIT detected by MTT. The IC_{50} value of Eca109/ADM against ADM, DNR and MIT was 15.45±1.15, 7.27±0.30 and 3.91±0.53 μg/ml, respectively, compared with 4.69±0.88, 1.94±0.21 and 1.24±0.30 μg/ml, respectively, for Eca109. The Eca109/ADM cells exhibited 3.29-, 3.75- and 3.15-fold resistance. ADM, adriamycin; DNR, daunorubicin; MIT, mitoxantrone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

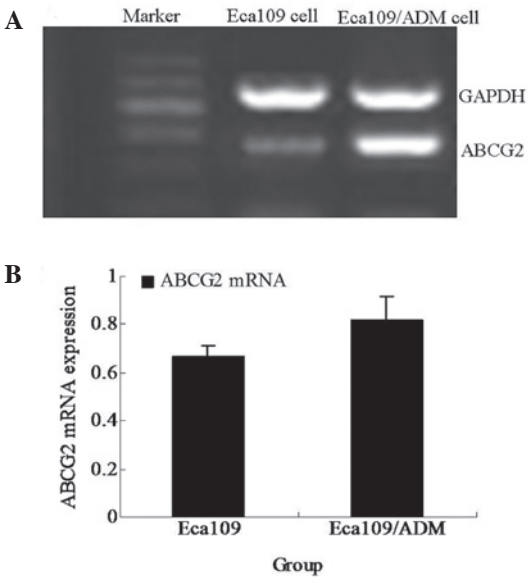

Figure 3. ABCG2 mRNA exprssion levels in Eca109 and Eca109/ADM cells. (A) ABCG2 mRNA expression in cells detected by RT-PCR. (B) ABCG2 mRNA expression levels in the Eca109/ADM cells (0.82±0.10) were higher than those of the Eca109 cells (0.67±0.04; P<0.05). ABCG2, ATP-binding cassette transporter G2; ADM, adriamycin.

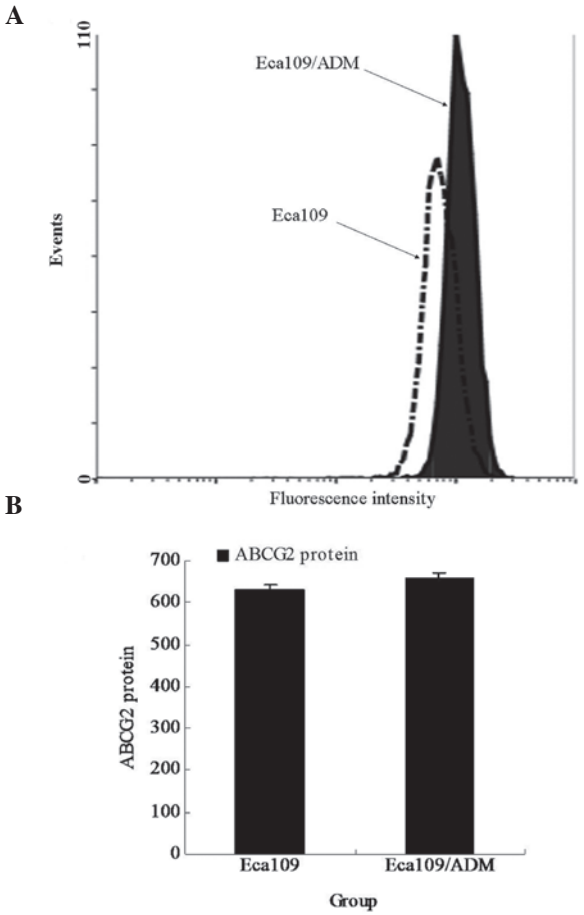

Figure 4. ABCG2 protein expression levels of Eca109 and Eca109/ADM cells. (A) ABCG2 protein expression in cells detected by flow cytometry. (B) The ABCG2 protein expression level of the Eca109/ADM cells (659.88±10.60) was significantly higher than that of the Eca109 cells (628.25±12.49) (P<0.05). ABCG2, ATP-binding cassette transporter G2; ADM, adriamycin.

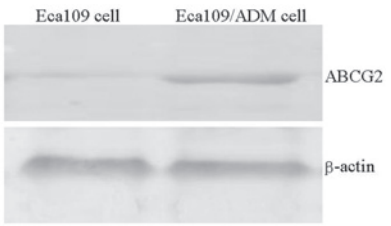

Figure 5. ABCG2 protein expression level of Eca109 and Eca109/ADM cells detected by western blotting. The ABCG2 protein expression level of the Eca109/ADM cells was significantly higher than that of the Eca109 cells. The result was consistent with the flow cytometry result. ABCG2, ATP-binding cassette transporter G2; ADM, adriamycin.

0.02 μg/ml ADM for 2 h and then without ADM for 1 h, the level of ADM in the Eca109/ADM cells was decreased more than that in the Eca109 cells. The ADM efflux effect of the Eca109/ADM cells was more than that of the Eca109 cells.

Apoptosis rate of cells. The apoptosis rate of the Eca109/ADM cells following the treatment with 0.02 μg/ml ADM for 24 h was significantly lower than that of the Eca109 cells (P<0.01; Fig. 7).

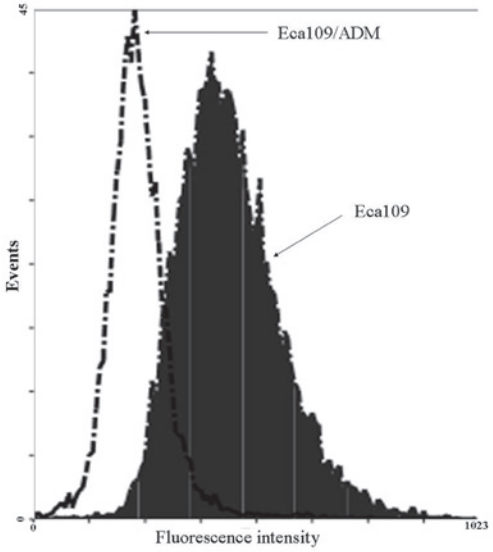

Figure 6. Fluorescence intensity of ADM in Eca109 and Eca109/ADM cells as assayed by flow cytometry. The cells were incubated at 37˚C with 0.02 μg/ml ADM for 2 h and then without ADM for 1 h. The level of ADM in the Eca109/ADM cells was decreased more than that in the Eca109 cells. The ADM efflux effect of the Eca109/ADM cells was more than that of the Eca109 cells. ADM, adriamycin.

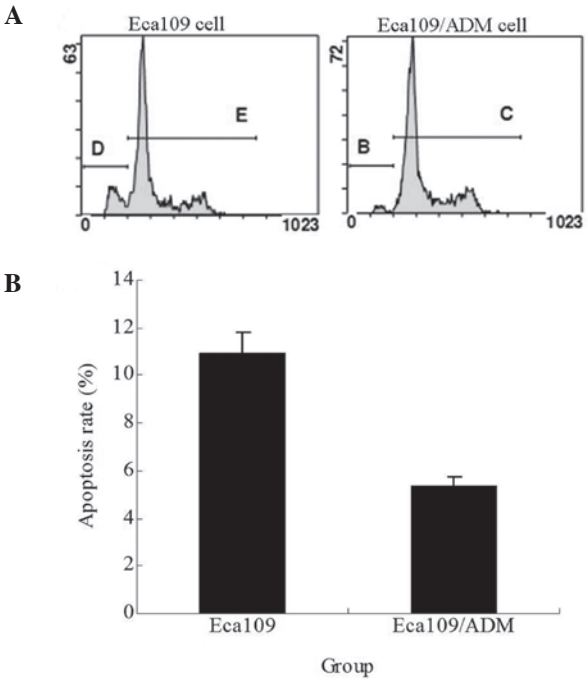

Figure 7. Apoptosis rate of Eca109 and Eca109/ADM cells (A) Apoptosis rate of Eca109 and Eca109/ADM cells, as detected by flow cytometry, following treatment with 0.2 μg/ml ADM for 24 h. (B) The apoptosis rate of the Eca109/ADM cells was significantly lower compared with that of the Eca109 cells (P<0.01). ADM, adriamycin.

Discussion

At present, the high mortality rate of cancer is a serious threat to an individual's life and health. Chemotherapy is a major form of treatment for various cancers. However, cancer cells often become resistant to chemotherapy via MDR, thereby resulting in chemotherapy failure. This resistance may be due to the

drug extrusion activity of MDR ABC transporters, particularly ABCG2. MDR, by which cells resist numerous structurally and functionally unrelated drugs, is a major obstacle for the effective chemotherapy of cancer. As for the mechanisms of MDR, the most significant is the decreased accumulation of drug within cells by an increased drug efflux, including overexpression of the cell membrane transporters, the majority of which are ABC transporters. ABC transporters are transmembrane proteins capable of expelling a large variety of structurally and mechanistically diverse anticancer drugs, which consequently reduces the concentration of anticancer drugs in the cells. ABCG2 is also known as BCRP and is a member of the ABC protein family. A large number of hematological malignancies and solid tumors have been detected as exhibiting BCRP (17). This indicates that this transporter may be significant in the clinical drug resistance of cancers, however there have been few studies in esophageal cancer.

The multidrug transporters (ABC transporters) are present in a number of normal tissues with barrier functions (18-23), and may modulate the absorption of orally administered cytotoxic compounds. For example, ABCG2 is expressed mainly in the placenta, and it is indicated that ABCG2 may control the penetration of drugs from the maternal plasma into the fetus, thus protecting the fetus against the potential toxicity of the drugs (24).

Based on the ability of ABCG2 to extrude drug agents (25,26), it is possible that multidrug transporters affect the absorption, distribution, cellular levels and effectiveness of these agents (27), resulting in drug resistance. There is a significant correlation between ABCG2 expression and the drug resistance of tumors.

In order to investigate the correlation between ABCG2 expression level and the drug resistance of esophageal cancer, the ADM-resistant subline, Eca109/ADM, was generated from the Eca109 esophageal cancer cell line by continuous exposure to ADM, an anticancer drug agent. The Eca109/ADM cells exhibited 3.29-, 3.75-and 3.15-fold resistance against ADM, DNR and MIT, respectively, and the MDR phenotype against various anticancer drugs. Analysis of the gene and protein expression profile using RT-PCR, flow cytometry and western-blotting demonstrated the highly elevated expression level of ABCG2 in the Eca109/ADM cells, compared with its parental cell line, Eca109. The MDR phenotype of the Eca109/ADM cells may be associated with the level of ABCG2 overexpression.

ABCG2, a member of one of the ABC family, may extrude the drug agents from cells. For further research in the present study, the drug efflux effect of the Eca109/ADM cells was detected using flow cytometry. The flow cytometry results demonstrated that the drug efflux effect of the Eca109/ADM cells was stronger than that of its parental cell line, Eca109.

The MTT and cell apoptosis rates as detected by flow cytometry were compared with the Eca109 and Eca109/ADM cells sensitivity to ADM. The MTT results demonstrated that the IC_{50} of the Eca109/ADM cells following the treatment of various concentrations of ADM for 24h was significantly higher than that of the Eca109 cells. The Eca109/ADM cells exhibited 3.29-fold resistance against ADM compared with its parental cell line, Eca109. The apoptosis rate results demonstrated that the apoptosis rate of the Eca109/ADM cells following the treatment of 0.02 μg/ml ADM for 24 h was significantly lower than that of the Eca109 cells. These results indicated that the Eca109/ADM cell line was resistant to the ADM drug compared with its parental Eca109 cell line.

Overall, the Eca109/ADM cell line was a multidrug cell line with an ABCG2 MDR phenotype. High expression levels of ABCG2 in Eca109/ADM cells may extrude ADM from cells, which decreases the ADM concentration in the cells and results in MDR. Therefore, ABCG2 amplification and expression in esophageal cancer cells may acquire resistance.

Acknowledgements

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