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Original Article

Reversing the ABCG2-mediated multidrug resistance by AG1478 and small interfering RNA in laryngeal squamous cell carcinoma

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Abstract: Background: ABCG2 is a member of ATP-binding cassette transmembrane proteins that play an important role in conferring multidrug resistance (MDR) to carcinoma cells. The aim of this study is to evaluate the regulation of ABCG2 by epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) -AG1478 and small interfering RNA (siRNA) with ABCG2 in laryngeal squamous cell carcinoma (LSCC). Methodology and Principal Findings: LSCC MDR cells lines Hep-2T and it parental cell line Hep-2 were used in vitro. MTT assay showed that AG1478 and siRNA-ABCG2 inhabit cell proliferative activity at both cell lines, and enhance the sensitive of chemotherapeutic drug, such as Mitoxantrone, 5-Fu and Doxorubicin. Using flow cytometry, we found that introduction of AG1478 and siRNA also enhanced the intracellular accumulation of Mitoxantrone and Doxorubicin. Conclusion and Significance: Our data show that siRNA and AG1478 could modulate MDR in vitro and may present a new approach to overcome ABCG2-mediated drug resistance in LSCC.

Keyword: ABCG2, Multidrug resistance, AG1478, small interfering RNA, Laryngeal squamous cell carcinoma

Introduction

Laryngeal carcinoma, 90% to 95% of which is squamous cell carcinoma, is the second-most-common malignancy in the head and neck region and the eleventh-most-common cancer among men worldwide [1]. Because of the unsatisfied results of single treatment in cancer, comprehensive treatment is playing more and more important roles in cancer. ABCG2 is a member of ATP-binding cassette (ABC) transporter cloned from atypical multidrug-resistance MCF-7 human breast cancer cells selected with intracellular accumulation of anthracyclines despite the absence of overexpression of ABCB1 or ABCC1 [2].

In our early research, we have found that ABCG2 was not only over-expression in Laryngeal squamous cell carcinoma (LSCC), but also reduced the overall survival rate of LSCC patients [3]. In vitro, we also demonstrated that ABCG2 was expression and conferred the function of efflux pump to LSCC cell lines Hep-2 and Hep-2T [4].

To date, several methods were used to circumvent ABCG2-mediated multidrug resistance (MDR), such as small interfering RNA (siRNA), a specific gene knockdown of ABCG2 [5], and AG1478, a potent and specific Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) [6].

In our study, we explored the effects of siRNA and AG1478 on MDR-linked ABCG2 in LSCC cell lines. We demonstrated that siRNA and AG1478 significantly inhibit ABCG2-mediated MDR by direct effect of gene knockdown and drug efflux.

Materials and methods

Chemicals

Mitoxantrone was purchased from Sigma-Aldrich (USA). Monoclonal antibodies anti-ABCG2 BXP-21 was purchased from Abcam (USA). PE anti-human CD338 (ABCG2) was purchased from Biolegend (USA). AG1478 was purchased from Cell Signaling Technology (USA). Lipofectamine 2000 was from Invitrogen (USA).

Exscript RT reagent kit was from Toyobo (Japan). β-Actin antibody which was used to detect the internal control in Western blotting test was from Santa Cruz (USA). MTT was purchased from Sigma (USA). Realtime PCR MasterMix kit was from Roche (Germany). Cisplatin was from Qilu Pharmaceutical co., LTD. (China). 5-Fu was from Xudong help Pharmaceutical co., LTD (China). Doxorubicin was from BenVenueLaboratores, Inc (USA). Taxol was from Bristol-Myers Squibb Company (USA).

Cell lines and culture

Hep-2 cell lines from humanlaryngeal cancer, was obtained from the American Type Culture Collection (ATCC). Besides, a Toxal-resistant cell line (Hep-2T) was developed by continuous exposure of Hep-2 cells to stepwise escalating concentrations of taxol for 12 months [7]. All the cell lines were cultured in Dulbecco modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere composed of 5% carbon dioxide.

Design and application of anti-ABCG2 siRNA

Three different small interfering RNA (siRNA) sequences targeting human ABCG2 gene were designed to be homologous to the ABCG2-encoding mRNA consensus sequence (GenBank accession no. HM_004827). The siRNA duplexes were all synthesized by Shanghai GenePharma Co., Ltd. The sense and antisense sequences of siRNA-ABCG2 duplex were as follows: 483: 5'-CGACCUGCCAAUUUCAAAUTT-3' (sense) and 5'-AUUUGAAAUUGGCAGGUCGTT-3' (antisense); 580: 5'-GGCAUUUACUGAAGGAUCUTT-3' (sense) and 5'-AGCUCCUUCAGUAAAUGCCTT-3' (antisense); 741: 5'-GGAGGCAAAUCUUCGUUAUTT-3' (sense) and 5'-AUAACGAAGAUUUGCCUCCTT-3' (antisense). A nonspecific control siRNA was designed: 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense). The sequence analysis of the nonspecific control siRNA by basic alignment search tool (BLAST) search (National Center for Biotechnology Information database) showed no homology to any human transcripts in records. Hep-2 and Hep-2T cells were cultured in antibiotics-free medium for 24 h before transfection, and then transfected with siRNA (100 nM) using Lipofectamine 2000. Sliencing was examined 48 h after transfction.

Western blot analysis of ABCG2

Exponentially growing cells were washed with ice-cold PBS for 2 times, and lysed in RIPA buffer with protease inhibitor PMSF for 30 minutes with occasional rocking followed by centrifugation at 12,000 g at 4°C for 15 minutes. Protein concentration of the lysates was quantified by the BCA protein assay. Equal amounts of protein (40 μg) were electrophoresed in 8% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were blocked for 1 hour with 5% skim milk in PBS, and incubated with anti-ABCG2 mouse monoclonal antibody BXP-21 diluted 1:20 in skim milk at 4°C overnight. After washing, membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Pierce) diluted 1:20000 in skim milk. The immunocomplexes were visualized with an enhanced chemiluminescence detection kit according to the manufacturer's instructions (Pierce).

Real-time quantitative RT-PCR

Total cellular RNA was isolated from Hep-2 and Hep-2T cells using a Trizol reagent (invitrogen). Single-stranded oligo (dT)-primed cDNA was generated form 2 μg of total RNA in a 20 μl reaction mixture. Pirmers used for the analysis of ABCG2 was sense primer 5'-CTCAGTTTATCCGTGGTG-3' and anti-sense primer 5'-AGATGATTGTTCGTCCCT-3', 180 bp) and β-actin was used as internal standard (sense primer 5'-ATCATGTTTGAGACCTTCAA-3' and anti-sense primer 5'-CATCTCTTGCTCGAAGTCCA-3' 318 bp). The amplification reaction was carried out with 2 μl of cDNA product for 27 cycles and each cycle consisted of 95°C for 45 sec, 56°C for 45 sec and 72°C for 45 sec, followed by a final 5-min elongation at 72°C. The final polymerase chain reaction (PCR) products were electrophoresed on a 1.5% agarose gel. Real-time RT-PCR was performed using a LightCyler rapid thermal cycler system (Roche Diagnostics Ltd, Lewes, United Kingdom) according to the manufacturer's instructions. Complementary DNA obtained from 2 μg RNA and 0.3 μl primers were included in the LightCycler-FastStart DNA Master Sybr Green I mix (Roche Diagnostics). The reaction condition was 40 cycles with denaturation for 10 min at 95°C, annealing for 30 s at 58°C,

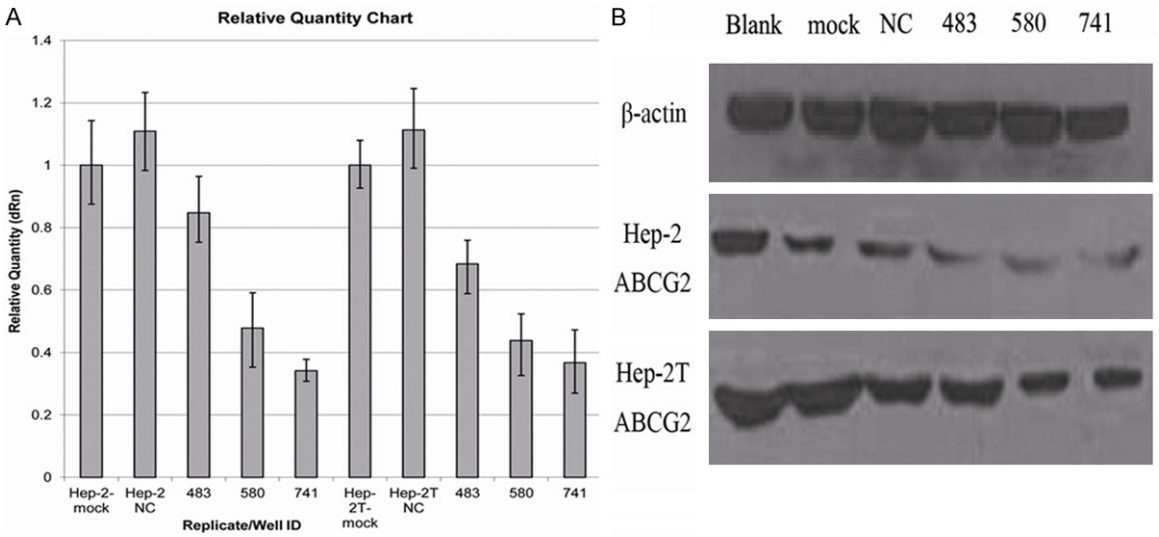

Figure 1. siRNA knockdown of ABCG2 mRNA (A) and protein (B) expression in Hep-2 and Hep-2T cell lines. Hep-2 and Hep-2T cells were simultaneously transfected with 10 nM siRNA using lipofectamine 2000 according to the manufacture's protocol. After 48 hours, cells were harvested for total RNA and protein as described in Materials and Methods. (A) Real-time quantitative was used to analyze the mRNA level in different groups. Relative ABCG2 mRNA expression level were normalized against β-actin mRNA expression levels. (B) Western blot analysis of ABCG2 expression resulting from anti-ABCG2 siRNA expression in both cell lines, using the mAb BXP-21. As control for equivalent protein loading, the filter was simultaneously incubated with a mouse mAb directed against β-actin. NC was siRNA-scramble. Representative of three independent experiments.

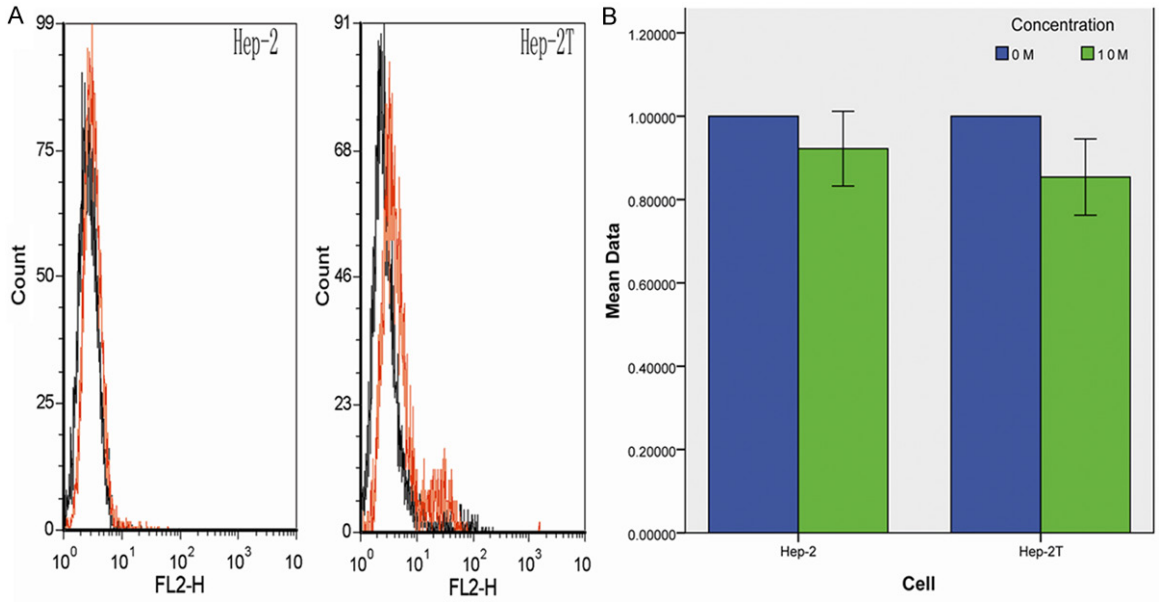

Figure 2. Effect of 10 μM AG1478 on the protein and mRNA of expression of ABCG2 in Hep-2 and Hep-2T cell lines. A. Both cell lines were treated with 10 μM AG1478 for 48 hours and were analyzed by Flow cytometry. Black lines were blank control, while red lines were group treated. B. The mRNA expression of ABCG2 in treated cell lines were analyzed by QRT-PCR and calculated as the following formula: Value of the ABCG2 gene copy/value of the β-actin gene copy.

and extention for 45 s at 72°C. To represent the ABCG2 expression level, we used the ABCG2 mRNA expression index calculated from the following formula: the values of the ABCG2 gene copies divided by the values of the β-actin gene copies.

Table 1. Effect of AG478, FTC and siRNA-ABCG2 on ABCG2-mediated resistance to paclitaxel, 5-Fu, doxorubicin, cisplatin and mitoxantrone

	IC_{50}±SD (μg/ml)	
Chemotherapeutic drugs	Hep-2	Hep-2T
Paclitaxel	4.46×10^{-3}±0.37 (1.0)[a]	0.37±0.32 (82.96)
+10 μM AG1478	4.17×10^{-3}±0.41 (0.93)	0.31±0.28 (69.51)
+5 μM FTC	4.08×10^{-3}±0.32 (0.91)	0.39±0.39 (87.44)
+siRNA-ABCG2	3.86×10^{-3}±0.40 (0.87)	0.36±0.42 (80.72)
5-FU	32.98±2.96 (1.0)[a]	83.46±1.92 (2.53)
+10 μM AG1478	25.90±2.42 (0.79)*	57.51±1.33 (1.74)**
+5 μM FTC	25.96±1.76 (0.79)*	59.87±1.60 (1.82)**
+siRNA-ABCG2	22.56±2.76 (0.68)**	59.17±1.64 (1.79)**
Doxorubicin	1.27±0.03 (1.0)[a]	55.47±2.26 (43.68)
+10 μM AG1478	0.47±0.04 (0.37)**	17.26±1.08 (13.59)**
+5 M FTC	0.56±0.04 (0.44)**	18.47±1.04 (14.54)**
+siRNA-ABCG2	0.48±0.03 (0.38)**	16.55±0.84 (13.03)**
Cisplatin	2.64±0.10 (1.0)[a]	3.74±0.11 (1.42)
+10 μM AG1478	2.63±0.14 (1.0)	3.61±0.07 (1.34)
+5 μM FTC	2.55±0.15 (0.97)	3.64±0.07 (1.42)
+siRNA-ABCG2	2.60±0.11 (0.98)	3.63±0.09 (1.34)
Mitoxantrone	1.74±0.13 (1.0)[a]	1.87±0.15 (1.07)
+10 μM AG1478	0.36±0.06 (0.21)**	0.28±0.09 (0.16)**
+5 μM FTC	0.42±0.04 (0.24)**	0.27±0.04 (0.21)**
+siRNA-ABCG2	0.23±0.04 (0.13)**	0.24±0.07 (0.19)**

To examine the effects of AG1478, FTC and siRNA-ABCG2 on ABCG2-medited resistance to paclitaxel, 5-Fu, doxorubicin, cisplatin and mitoxantrone, cells were pre-incubated with or without AG1478, FTC and siRNA-ABCG2 for 24 h and then incubated with various concentrations of paclitaxel, 5-Fu, doxorubicin, cisplatin and mitoxantrone. Cell survival was determined by the MTT assay as described in Section 2. Data are means ± SD of at least three independent experiments performed in triplicate.

Cytotoxicity assay for cell survival

3-4,5-dimethylthiazol-20yl-2,5-diphenyltetrazolium bromide (MTT) assay was employed to assess cell viability. MTT assay was based on the ability of the viable cells to reduce soluble yellow MTT to blue formazan crystals. In this assay, optical density (OD) values represented the absorption of formazan dissolved by dimethyl sulfoxide at 570 nm. Cells (5×10^3 per well) were seeded in quintuplicate in a 96-well plate containing 150 μl culture complete medium. After 24 hours of incubation, mitoxantrone, doxorubicin, cisplatin, paclitaxel or 5-Fu was added into designated wells in dilution series in triplicate in the presence or absence of siRNA, FTC (10 μM) and AG1478 (10 μM). After 48 hours incubation, 20 μl of MTT (5 mg/ml) was added to each well, and cells were further incubated for 4 hours at 37°C. The medium was removed and the product was dissolved in dimethylsulfoxide (DMSO). Absorbance was measured at 570 nm with a spectrophotometer. 10% DMSO was selected as a positive control for the cytotoxicity studies.

The IC_{50} values were calculated using SPSS 16.0 software. Each study was performed in triplicate and repeated three times.

Drug accumulation assays

Cellular mitoxantrone accumulation was determined according to the procedure originally reported by Allen et al [8], with some modifications as described previously. Briefly, 4×10^5 cells were seeded in six-well plates 24 hours prior to the experiment. After incubation with or without FTC and AG1478 (10 μM) for 1 hour at 37°C, mitoxantrone (0.5 μg/ml) was added into and incubated for 48 hours at 37°C and 70-80% cellular confluence. Cells were washed with ice-cold PBS, trypsinized, and resuspended in medium at a concentration of 4×10^5 cells/ml. Intracellular fluorescence of mitoxantrone was determined by flow cytometry (Becton Dickinson). The cells were excited at 480 nm and emission was measured at 630 nm. A minimum of 10^4 cells was analyzed for each sample. All assays were performed at least in three independent experiments, each time with triplicate wells.

Statistical analysis

Categorical variables were evaluated by Pearson Chi-Square, Continuity Correction or Fisher's exact test. Spearman's rank correlation test was conducted to assess the correlation between ordinal variables. The statistical software SPSS16.0 was used in data processing and analyzing the significance using the student's t-test, and a $P<0.05$ denoted the presence of a significant difference.

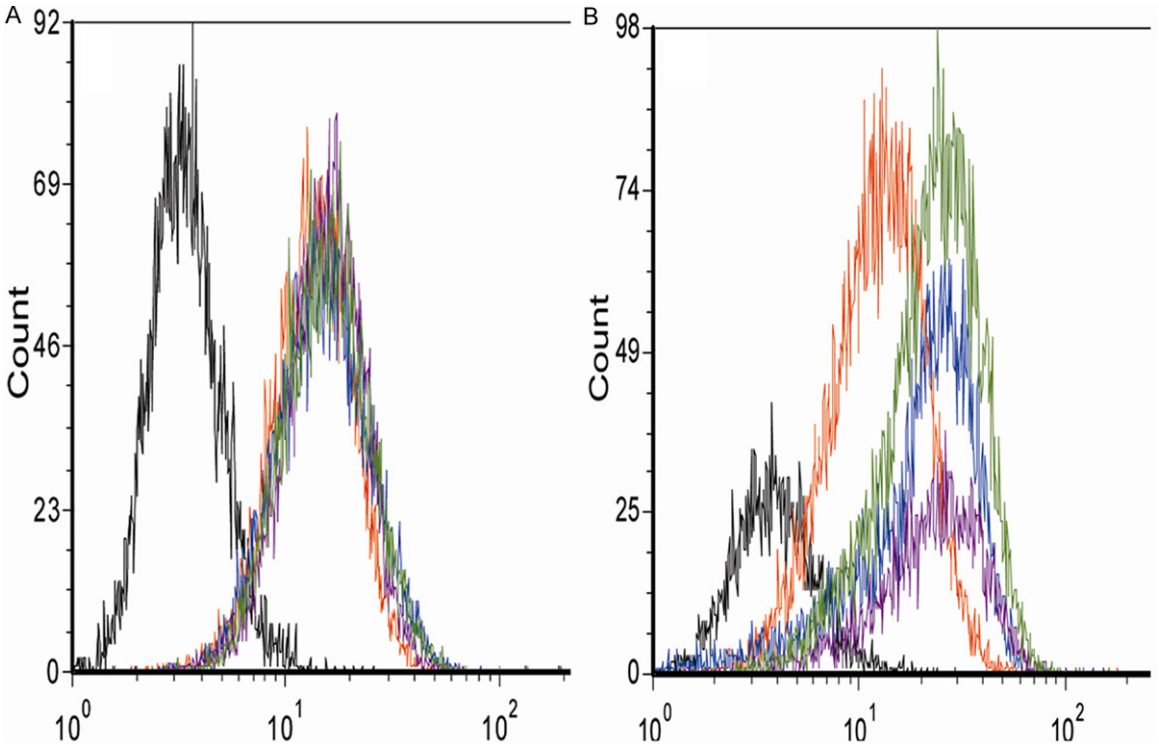

Figure 3. Effect of FTC, AG1478 and AG1478+FTC on the accumulation of mitoxantrone. A. Intracellular accumulation of mitoxantrone in Hep-2 cell lines, blank control (grey), negative control (red), 10 μM FTC (green), 10 μM AG1478 (blue) and 10 μM FTC+AG1478 (purple); B. Intracellular accumulation of mitoxantrone in Hep-2T cell lines, blank control (grey), negative control (red), 10 μM FTC (green), 10 μM AG1478 (blue) and 10 μM FTC+AG1478 (purple).

Results

Decrease in ABCG2-specific mRNA and protein expression by siRNA

Three different siRNA constructs, 483, 580 and 741, were used to decrease the expression of the ABCG2 in Hep-2 and Hep-2T cell lines. Compared to the other two groups, 741 considerably decreased the ABCG2 mRNA and protein expression level after 48 hours of siRNA treatment (**Figure 1A**, **1B**). Negative control is siRNA-scramble.

AG1478 did not affect the expression of ABCG2 protein and mRNA in Hep-2 and Hep-2T cells

Aside from directly inhibit the pump activity of ABCG2, decreasing the expression of ABCG2 protein and mRNA also could alter function of ABCG2-mediated MDR. In order to clarify the effect of AG1478 on the ABCG2 protein and mRNA expression, Hep-2 and Hep-2T cell lines were treated with AG1478 at 10 μM for 48 hours, then lysed with RIPA and Trizol buffer, and analyzed by flow cytometry and QRT-PCR. As shown in **Figure 2A**, **2B**, AG1478 did not affect the levels of protein and mRNA expression of ABCG2 in Hep-2 and Hep-2T cell lines.

AG1478 and siRNA sensitize Hep-2 and Hep-2T cell lines to some chemotherapeutic drugs

We examined the effect of FTC, AG1478 and siRNA on the sensitivity of Hep-2 and Hep-2T to drugs that are usually used into laryngeal squamous carcinoma cancer. Five anti-cancer drugs mentioned above at various concentrations were added into Hep-2 and Hep-2T cell lines for 48 hours, in the presence or absence of FTC, AG1478 and siRNA. The IC_{50} were calculated by MTT assay. Then RI of cells to anti-cancer drugs was determined. As shown in **Table 1**, compared to parental cells Hep-2, Hep-2T has 82.96-fold and 43.68-fold resistance to paclitaxel and doxorubicin, but not to 5-Fu, cisplatin and mitoxantrone. When cytotoxicity assays were repeated in the presence of the AG1478, FTC and siRNA-ABCG2 was able to decrease the IC_{50} values for 5-Fu, doxorubicin and mito-

Table 2. Effect of FTC, AG1478 and FTC+AG1478 on intracellular accumulation of mitoxantrone in Hep-2 cell lines

	Mean ± SD	95% confidence interval		P
		Lower	Upper	
0.5 μg/ml mitoxantrone	61.90±4.15	51.61	72.20	
+10 μM FTC	67.10±3.73	57.84	76.36	0.203
+10 μM AG1478	72.50±3.85	62.94	82.07	0.022*
+10 μM FTC+AG1478	73.35±6.19	79.98	90.23	0.016*

* and ** present P<0.05 and P<0.01 respectively, for values versus those in the control group. At least 3 independent experiments were performed and a representative experiment is shonw.

Table 3. Effect of FTC, AG1478 and FTC+AG1478 on intracellular accumulation of mitoxantrone in Hep-2T cell lines

	Mean ± SD	95% confidence interval		P
		Lower	Upper	
0.5 μg/ml mitoxantrone	59.87±2.85	52.78	66.94	
+10 μM FTC	81.88±2.10	76.67	87.09	<0.01
+10 μM AG1478	85.10±1.16	82.23	87.97	<0.01
+10 μM FTC+AG1478	85.10±2.06	79.98	90.23	<0.01

* and ** present P<0.05 and P<0.01 respectively, for values versus those in the control group. At least 3 independent experiments were performed and a representative experiment is shown.

xantrone in cells. AG1478 and siRNA-ABCG2 was better than FTC in decreasing the IC_{50} values for 5-Fu, doxorubicin and mitoxantrone. However, the IC_{50} values of paclitaxel and cisplatin showed no significant difference in the parental cells or the Hep-2T cells in the presence or absence of AG1478, FTC and siRNA-ABCG2.

AG1478 and FTC increase the intracellular accumulation of mitoxantrone in Hep-2 and Hep-2T cell lines

To investigate the mechanism by which AG1478 and FTC potentiate the sensitivity of Hep-2 and Hep-2T cells to chemotherapeutic drugs that are ABCG2 substrates, we examined the effect of AG1478 and FTC on the accumulation of mitoxantrone in the cells. Intracellular accumulation of mitoxantrone was measured in cells in the presence or absence of AG1478 and FTC, and the results are shown in **Figure 3**. In the presence of AG1478 or AG1478+FTC, even at 10 μM, Hep2 and Hep-2T cell lines showed an increase in intracellular mitoxantrone levels. As shown in **Tables 2** and **3**, FTC, AG1478 or AG1478+FTC all increased the intracellular mitoxantrone levels at 10 μM in Hep-2T cell lines, and significant more than that observed in its parental cell lines Hep-2.

Discussion

Chemotherapy can fail because of the development of tumor cell MDR to a large extent. ABCG2 is a major member of the ABC-transporter superfamily that mediated a variety of transport processes. In our early research, we have found that ABCG2 is not only over-expression in laryngeal squamous cell cancer cell lines, and play a key role in pathological activity [3, 4].

Molecular targeting therapy for cancer treatment is a fast-growing research field in oncology. Now, there is two most popular methods of targeting therapy, one is small interference RNA, and the other is specific inhibitor. One specific molecular target of high promise in oncology is the EGFR pathway. EGFR is a key physiological regulator of growth and differentiation of normal epithelial cells, but it is also involved in the development and progression of cancers derived from these tissues [9]. Then, EGFR has been proposed as a rational molecular target for anticancer strategies. Currently, two predominant classes of anti-EGFR targeting agents have been developed for use in the clinic including monoclonal antibodies such as cetuximab that blocks the ligand binding to the extracellular domain of EGFR and prevents receptor activation, and small molecule TKIs such as gefitinib and erlotinib that compete with ATP to bind to the intracellular receptor catalytic domain of EGFR [10-12]. AG1478 have the same structural quinazoline backbone as gefitinib, with ABCG2.

In the present study, we use the siRNA-ABCG2, FTC and AG1478 to evaluate the reverse effect of MDR in laryngeal squamous cell carcinoma. Our data show that siRNA-ABCG2 potently decreased the specific ABCG2 expression of mRNA and protein in Hep-2 and Hep-2T, but the similar effect were not seen in FTC and AG1478. Meanwhile, all of them sensitize cells to the chemotherapeutic drugs of 5-Fu, doxorubicin and mitoxantrone in cell lines. It is inconsistent with Shi [12], we found that AG1478 and siRNA-

ABCG2 was better than FTC in sensitizing cells to 5-Fu, doxorubicin and mitoxantrone. However, all of them did not alter the IC_{50} of cisplatin and paclitaxel, both of them are not substrate of ABCG2. Though, 5-Fu is not the substrate of ABCG2, cell sensitivity was still changed by inhibitor. We infer that ABC transporters superfamily may have interaction, but the mechanism is not clear. Furthermore, the results of drug accumulation study with mitoxantrone showed that AG1478 and FTC significantly enhanced the intracellular accumulation of mitoxantreone in cell expressing either Hep-2 or Hep-2T. Additionally, we examined the effect of AG1478 on the reversing MDR was stronger than FTC.

Interestingly, Hep-2T is an ABCB1 over-expressed cell lines that showed more significant effect of ABCG2-mediated MDR. We can deduce that there are interaction between ABCB1 and ABCG2. Thus, further experiments are needed to confirm the interaction of these compounds.

Conclusion

In conclusion, the present study shows that siRNA and AG1478 potently reverse ABCG2-mediated MDR by directly decreasing the expression and inhibiting their drug efflux function, resulting in the increase of the intracellular concentration of anticancer drugs in cancer cells. Our results may be useful to develop new and more effective methods as well as new combinational chemotherapeutic strategies.

Acknowledgements

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Disclosure of conflict of interest

None.

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