RESEARCH ARTICLE

Establishment and Partial Characterization of an Epirubicin-Resistant Gastric Cancer Cell Line with Upregulated ABCB1

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Abstract

Multidrug resistance (MDR) is a major impediment to successful chemotherapy of gastric cancer. Our aim was to establish an epirubicin-resistant cell subline (AGS/EPI) and to elucidate the mechanisms involved in acquired EPI resistance. The AGS/EPI cell subline developed by exposing parental AGS cells to stepwise increasing concentrations of EPI demonstrated 2.52-fold resistance relative to the AGS cell line, and mRNA expression of the ATP-dependent drug-efflux pump P-glycoprotein (Pgp), more recently known as ABCB1 protein, was similarly upregulated. An AGS/EPI cell subline could thus be effectively established, and MDR mechanism of these cells was shown to be related to the overexpression of mRNA of the *ABCB1* gene.

Keywords: Multidrug resistance - gastric cancer - ABCB1 - epirubicin

Asian Pac J Cancer Prev, 15 (16), 6849-6853

Introduction

Despite decreased incidence, gastric cancer (GC) is still the fourth most cancer and the second most common cause of cancer-related death in the world (Crew et al., 2006; Ferlay et al., 2010; Brayet et al., 2012). Even with the advancement of human cancer therapy, mechanisms that have advanced in mammals to protect cells against cytotoxic drugs in the environment will continue to function as an important obstacle to successful cancer treatments (Gottesman, 2002; Kim and Tononock, 2005; Nobili et al., 2006). Generally, the multidrug resistance (MDR) is characterized by overexpression of ATPdependent drug-efflux pumps P-glycoprotein (Pgp), more recently known as ABCB1 protein (Gottesman, 2002). The ABCB1 protein, a member of the ATP-binding cassette (ABC) transporter family, encoded by the ABCB1 gene, which considerably influences the distribution of a drug across the cell membrane as well as multidrug resistance (MDR) of anti-neoplastic drugs (Ueda et al., 1986; Richter et al., 2006).

Compounds of the anthracycline family (doxorubicin, daunorubicin, epirubicin), known as substrate for P-gp efflux pumps (Perez, 2009) and epirubicin (EPI) belongs to ECF regimen treating (epirubicin, cisplatin, fluorouracil) (Findlay et al., 1994). Importantly, this treatment regimen improved outcomes in patients with the advanced gastric cancer (Wagner et al., 2005) and patients undergoing surgery following neoadjuvant chemotherapy had a statistically improved overall survival (Cunningham et

al., 2006). Nevertheless, increased expression of mRNA *ABCB1* gene as a predictor of poor clinical outcome to epirubicin treatment exemplifies the success of studies focused on the cellular pharmacology of epirubicin resistance (Yuan et al., 2009).

Strategies for inducing *in vitro* resistance has been little explored and, furthermore, to demonstrate how such models of GC cell lines is an important step in the study of the role of *in vitro* chemoresistance assays in the GC field. A significant advance was shown recently by Chen et al., (2013) that established the TSGH-S3 oxaliplatin-resistant cell line from TSGH human gastric adenocarcinoma. Similarly, but in other tumors, a study performed by Dupuis et al. (2013) reported that vinblastine-resistant leukemia cell lines were well-established and isolated by stepwise selection of the parental drug sensitive in the existence of increasing vinblastine concentrations. Finally, Chen et al., (2013) conducted a study that established a paclitaxel-resistant breast cancer cell line to explore the mechanisms of MDR in breast cancer.

To dissect the mechanisms underlying epirubicin resistance, we established an epirubicin-resistant cell subline (AGS/EPI), from human gastric adenocarcinoma AGS cells. In the work reported here, we evaluated the chemoresistance differences between the AGS/EPI cell subline and AGS cells. Furthermore, we assessed the expression profile of mRNA of the *ABCB1* gene between cell lines, suggesting that their overexpression may also contribute to MDR development. The purpose of this study was to establish an epirubicin-resistant cell subline (AGS/

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EPI) from human gastric adenocarcinoma AGS cells, and to further elucidate the mechanisms involved in acquired epirubicin resistance of gastric cancers.

Materials and Methods

Chemicals and media

Epirubicin Hydrochloride solution was purchased from Accord Healthcare Ltd, (Middlesex, Wielka Brytania, UK). All reagents and cell culture media were from Invitrogen/Life Technologies or Gibco/Life Technologies (Carlsbad, CA, USA). All other chemical reagents were from either Ambion, Molecular Probes/Life Technologies (Carlsbad, CA, USA) or Qiagen (Valencia, CA, USA).

Cell culture and development of the in vitro chemoresistant cell subline

Human gastric adenocarcinoma cell line AGS (Rio de Janeiro Cell Bank, BCRJ, ID 0311, Brazil) was cultured in RPMI, supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO₂. The AGS/EPI cell subline was developed by exposing the parental AGS cells to stepwise increasing concentrations of anticancer drug EPI [up to the final concentration of 0.125uM and 1uM, respectively]. Both cell lines were cultured under the same conditions but with drug-free medium for 10 days before commencing the experiment.

Cytotoxicity assay

The in vitro cytotoxicity assays were performed by 2-(4,5-dimethyltriazol-2yl)-2,5diphenyltetrazoliumbromide (MTT) colormetric assay (Mosmann, 1983; Hussain et al., 1993). Approximately 1x10⁴ cells/well AGS/EPI cells subline or AGS cells were washed with PBS and seeded in 96-well U-bottom culture plates for 24hr at 37°C in a 5 % CO, humid atmosphere incubator. After incubation, cells were exposed to various concentrations of EPI. Following overnight incubation, the medium was removed and cells then cells were incubated in 100 µl of RPMI containing 5 mg/mL MTT for a further 4 hr. To develop the color 100μL of acid-isopropanol (0.04 M HCL in isopropanol) were added to dissolve the formazan crystals. The absorbance was measured on a microplate reader Elx800TM (BIO-TEK Instruments, Inc., Winooski, VT, USA) at a wavelength of 570 nm, with a reference wavelength of 630 nm, against medium only blank. Cytotoxicity was determined as the ratio between the absorbance measured for EPI treated cell samples and the absorption measured for untreated cell samples. The half maximal inhibitory concentration (IC) was based on the growth inhibition rate attained. EPI-resistance folds=IC (EPI-resistant cells)/IC (EPI-sensitive cells).

Quantitative real-time reverse transcription PCR (qRT-PCR)

Reverse-transcriptase reactions were performed using the TaqMan Gene Expression Cell-to-CT kit™ (Ambion) following the manufacturer's protocol. cDNA specific to ABCB1 mRNA was amplified with TaqMan

Gene Expression Assays were purchased for human ABCB1 (TagMan Gene Expression Assays™) Inventoried Analysis Identification Number (ID): Hs00184491_m1, and Endogenous control human β-actin (ACTB) Assay, Inventoried assay ID: Hs01060665_g1 from Applied Biosystems (ABI, Foster City, CA). In each experiment, triplicate reactions were performed in each sample. Reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. using the Step One Plus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The relative quantity of mRNA for each sample were calculated by $2^{\text{-}\Delta\Delta CT}\!,$ where $\Delta CT\!\!=\!\!(\text{mean of triplicate }CT_{ABCB1}\!\!$ mean of triplicate CT_{ACTB}) and $\Delta\Delta CT = (\Delta CT\text{-mean }\Delta CT\text{ of all }$ the samples). In pilot studies, the PCR amplicons were evaluated on agarose gels to verify product size.

DNA fragmentation

Apoptosis was analyzed by DNA fragmentation was examined by agarose gel electrophoresis as described previous (Lee and Bahaman, 2012; Simsek and Uysal, 2013). Briefly, 2×10⁶ cell/mL AGS/EPI cells subline and AGS cells were grown in 25cm² flasks and treated with EPI at IC₅₀ dose and incubated for 48 h. Control plates were also made with 0.05% DMSO at a similar period of time. Then cells were harvested and collected by centrifugation. DNA was extracted and isolated from control and treated cells using the QIAamp TM DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Three micrograms of DNA were stained with ethidium bromide and subjected to electrophoresis at 70 V for 1 h in 1.2% agarose gel. Separated DNA fragments were visualized under UVP ultraviolet light transilluminator /Cannon camera system (UVP Laboratory Products, Upland, CA, USA).

Cell viability assay

The viable cells counts were obtained by trypan blue exclusion method. Treated [with EPI at IC $_{50}$ by 48 h] and untreated AGS/EPI cells subline and AGS cells were grown to confluence and trypsinized with 0.05% Trypsin-EDTA for 5 minutes, centrifuged at 800 rpm for 2 minutes. Fifteen microlitres of cell suspension was gently mixed with 15 μ L of 0.4% trypan blue solution (Invitrogen, Carlsbad, CA, USA), and incubated for 5 min at room temperature. Ten microlitres of the stained cell suspension was placed in a CountessTM cell counting chamber slides and the number of viable was measured by trypan blue exclusion assay on a CountessTM automated cell counter (Applied Biosystems, Carlsbad, CA, USA).

Statistical analysis

All experiments were performed at least in triplicate. Statistical calculations were performed using Minitab V16.0 software (State College, PA). The data of continuous variables were expressed as mean ± standard deviation (SD). Pearson's correlation analysis was used to determine the relationship between mRNA expressions and the resistance to EPI. All other statistical differences were determinate with unpaired two-tailed t-test. Differences were considered significant for p <0.05.

Results

Cytotoxicity effect of epirubicin on AGS/EPI cell subline

The human AGS and AGS/EPI gastric cancer cell subline were verified by MTT assay to evaluate the effect of EPI on cell survival. Drug cytotoxicity in AGS/EPI cell subline and AGS cell line by comparing between cell viability, as indicated in Figure 1A. Epirubicin was able to inhibit differently in these two cell lines. In both cell lines were shown not to be similarly susceptible to EPI when contrasted with the inhibitory concentrations (IC) 10, 50 and 90% of cell survival. Comparisons between the fold-change results found by inhibitory concentration (IC) for AGS/EPI cell subline and AGS cell line were investigated. The chemoresistance to EPI increased up to 6-fold for AGS/EPI cell subline versus AGS cell line (p<0.05) corresponding to IC₁₀; 2.52-fold greater to IC₅₀

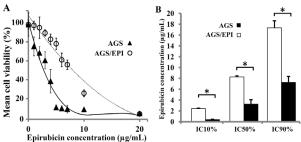


Figure 1. Epirubicin (EPI) Cytotoxicity Effects on the cell Viability of AGS and AGS/EPI gastric Cancer Cell

Lines. (A) It was verified that for EPI there was a significant differences between the viability of the AGS and AGS/EPI gastric cancer cell for all EPI concentrations. Each graph point represents the mean values based on five or six replicates of the experiment. The error bars indicates the standard deviation of the measurements. Unpaired t-test shows statistically significant differences (p<0.05). (B) Chemoresistance of EPI inhibitory concentrations (IC) of AGS-EPI and AGS cell lines. Significant statistical differences were observed between IC10 of the AGS/EPI and AGS cell line (2.41 μ g/mL±1.22 and 0.38 μ g/mL±0.56, p<0.05*); IC50 (8.27 μ g/mL±1.22 and 3.24 μ g/mL±0.56, p<0.05*); and IC90 (17.33±1.22 and 7.26±0.56, p<0.05*), for IC10. All data shown are averages±SEM of three biological replicates

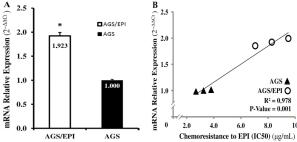
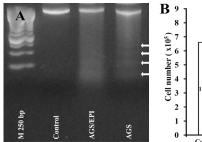


Figure 2. ABCB1 mRNA Expression Levels in AGS/EPI and AGS Gastric Cancer Cell Lines. (A) The ^{2-ΔΔ}Ct equation results value to the AGS/EPI cell line was 1.923-fold higher than that of the AGS cell line as mean±SE (7.050±0.07 vs 7.994±0.017, *p<0.05). (B) Correlation between mRNA expression of ABCB1 levels in AGS/EPI and AGS gastric cancer cell lines by chemoresistance to EPI (IC50). Pearson's correlation analysis traces a regression line showing the relation between both devices. ABCB1 mRNA expression was significantly correlated with chemoresistance to EPI



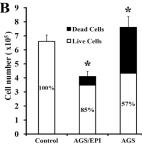


Figure 3. AGS/EPI and AGS Cells Were Treated with Epirubicin (EPI) by IC₅₀ value [3.24 μ g/mL] for 48 Hours. Control experiments without EPI treatment were performed using AGS cells. (A) DNA fragmentation assay where a concentration around 1 μ g/ μ L (13 μ L) of DNA was electrophoresed into each well and performed at 70 V for 60 min using 1.2% agarose gel along with the 250 bp ladder DNA size markers. (B) Cell viability was measured with following 0.4% trypan blue staining and CountessTM automated cell counter analysis. The cell viability was normalized as 100% in the control group before estimating the viability cell percentage. Data are reported as mean \pm S.D. of experiments performed in triplicate. *p<0.05. p values were determined using a two-tailed t-test

(p<0.05); and 2.39-fold increased to EPI for IC $_{90}$ (p<0.05), respectively, as showed in Figure 1B. The AGS/EPI cells subline were found to be resistant to a high concentration of EPI, but AGS cells were EPI sensitive.

Relationship between mRNA expressions and resistance to EPI

The quantitative real-time reverse transcription PCR (qRT-PCR) was performed to measure ABCB1 mRNA expression as illustrated in Figure 2A. Expression levels of ABCB1 mRNA in AGS/EPI cell subline and AGS cell line were quantified and we investigated the relationship between their expression and chemoresistance to EPI (IC $_{50}$). A statistically significant direct correlation between ABCB1 mRNA expression and chemoresistance to EPI (IC $_{50}$) (R 2 =0.987, p=0.001) as is demonstrated in Figure 2B.

Relationship between DNA fragmentation and cell viability assay

The DNA fragmentation assays were carried out to measure apoptosis coincided with the cells deaths results analyzed by trypan blue exclusion assay. From the figure 3A indicates that the EPI therapy showed lower decreasing of DNA fragmentation in AGS/EPI cell subline when contrasted with AGS cell line, as proof by the development of 180-200bp ladder DNA in 1.2% agarose gel. AGS/EPI and AGS cells deaths were analyzed by Countess™. Automated Cell Counter (Invitrogen). Treated cells survival was statistically higher (approximately 30%) in AGS/EPI cell subline than in AGS cell line (85% versus 57%, p<0.05) after Trypan Blue staining, as showed in Figure 3B.

Discussion

An increasing number of studies on the ABCB1/P-glycoprotein effects have been explored in the last years (Chen et al., 2013; Yang et al., 2013; Zhu et al., 2013;

Louisa et al., 2014). The MDR occasioned by increased efflux transporters, including ATP-binding cassette transporters is associated with upregulated ABCB1 expression and main cause of treatment failure (Ueda et al., 1986; Gottesman, et al., 2002; Richter et al., 2006), and can be observed in the majority of cancers (Cordon-Cardo et al., 1990). In previous studies conducted by our group (Oliveira et al., 2012), we described a case-control study of C3435T ABCB1 gene polymorphism that showed that there was no association between its polymorphism and susceptibility to gastric cancer (GC).

In the present study, we report on the possibility of establishing a GC epirubicin-resistant cell line. Most commonly, researchers have used primary cell culture studies to evaluation of intrinsic chemoresistance status in GC cells (Ying et al., 2013). We can elucidate this phenomenon in the opposite way as in the previous works, through the development of the *in vitro* chemoresistant cell model and then determine the phenotypic characteristics of cells acquired resistance to EPI.

According cytotoxicity data for treated cell lines, we have verified that the AGS/EPI cells subline were resistant to a high concentration of EPI, but AGS cells were EPI sensitive. In this study, the cytotoxicity of EPI has been shown lower for AGS/EPI cells subline than for AGS cells. Thus, we searched a correlation between our measure of EPI inhibitory concentrations (IC) on AGS cell line [3.24 μ g/mL] and the value found by others researchers on the SGC7901 gastric cancer cell line [1.9 μ g/mL] (Zhao et al., 2009). It was verified that there was a slight association between the cytotoxicities of the AGS and SGC7901 gastric cancer cell lines to IC₅₀ values of EPI.

The important role of ABCB1 transporter protein in specific tissues is an effective mechanism for detoxifying normal cells, however, as well as increased anticancer activity during chemotherapy has been widely reported. Although, its pharmacological benefit remains unclear (Gottesman, 2002). As mentioned above, we reported that the mRNA expression of ABCB1 in AGS/EPI cell subline was shown to be expressed at 1.943-fold higher levels when exposed to stepwise increasing EPI concentrations under growth conditions, than the AGS cells. Furthermore, when we compared the fold-change results found by inhibitory concentration and expression levels of mRNA of ABCB1 in AGS/EPI cell subline, these results showed a statistically significant positive correlation between ABCB1 mRNA expression and EPI resistance. This decreased cytotoxity against the AGS/ EPI cell subline was smaller than expected, seeing that the others authors (Chen et al., 2013; Chen et al., 2013; Dupuis et al., 2013) established the highest fold change to chemoresistance in parallel investigations in their own laboratories. Furthermore, our data suggest that AGS/ EPI cell subline can be used to investigate the effects of the reversal modulators to chemoresistance, as a study performed by Louisa et al., (2014) concluded that the mangiferin modulators effects were related to a reduction of the ABCB1/P-glycoprotein expressions. Our data and that of others (Redmond et al., 2008; Gillet et al., 2011) suggest that the levels of mRNA transcripts encoding ABCB1 protein may be increased in the presence of

EPI. Thus, intracellular EPI depletion resulted in posttranscriptional upregulation of ABCB1 protein on AGS surface expression.

DNA laddering assay demonstrated a nucleosomal fragmentation, which is a well documented as hallmarks of apoptosis. The evolution of *in vitro* chemotherapy resistance in a variety of cancer cells has been associated with a decreased susceptibility to drug-induced apoptosis (Plati et al., 2008). From these experiments it was determined that EPI treatment-induced showed low-decreasing of DNA chromosomal degradation in AGS/EPI cell subline when compared with AGS cell line. Likewise, the results of both analyses indicated that there was an increased level of apoptosis in AGS cell lines when compared with AGS/EPI cell subline.

In order to confirm all assays that established a GC epirubicin-resistant AGS/EPI cell subline, we performed several analyses to ensure that analytical results were acceptably reproducible. Based on these observations, we conclude that AGS/EPI cell line was successfully established in our laboratory, and which can be effectively applied to understanding of the MDR reversal mechanisms in future studies. Additionally, ABCB1 overexpression emerged as an important pharmacological risk factor for treatment failure of gastric cancer AGS cell line epirubicin-resistant.

Acknowledgements

We are thankful to Prof. Dr. Jerônimo Pereira de França and Prof. Msc. Michel Sant'Anna de Pinho who offered us the scientific support. This work was partially funded by Ministry of Education by the Coordination for the Improvement of Higher Level Personnel (CAPES).

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