

Dynamic quantitative detection of ABC transporter family promoter methylation by MS-HRM for predicting MDR in pancreatic cancer

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Abstract. The main focus of the present study was to evaluate whether ABC transporter family promoter methylation predicted multidrug resistance in gemcitabine-resistant cancer cell lines (BxPC-3/Gem and PANC-1/Gem). Using low concentrations of gemcitabine, the cell lines acquired drug resistance with different initial gemcitabine concentrations. A novel technology, methylation-sensitive high-resolution melting, was used to monitor the dynamic changes of ABC transporter family promoter methylation, including ATP binding cassette subfamily B member 1 (ABCB1), ATP binding cassette subfamily C (ABCC) and ATP binding cassette subfamily G member 2 (ABCG2) mRNA expression. It was revealed that, with elevation of initial gemcitabine concentration, expression of ABCB1, ABCC and ABCG2 mRNA and corresponding downstream proteins was increased while promoter methylation was decreased. These discoveries indicate that promoter methylation of ABCB1, ABCC and ABCG2 may be a valuable indicator of drug-resistance characteristics in BxPC-3/Gem and PANC-1/Gem cells via quantitative and simultaneous detection. These results also implied that MDR in pancreatic cancer not only arises from gene mutation, but also originates from promoter methylation.

Introduction

Pancreatic cancer is one of the most malignant tumors and is characterized by a poor prognosis. An increasing number of patients are diagnosed with *de novo* pancreatic cancer each year (1). The majority of patients with pancreatic cancer when diagnosed with distant metastasis, require comprehensive treatment, including chemotherapy. However, patients who have undergone radical resection have experience a poor prognosis and a high rate of recurrence (2,3). Additionally, patients still require conventional adjuvant chemotherapy in order to minimize the risk of postoperative recurrence and metastasis. Therefore, chemotherapy currently serves an important role in the comprehensive treatment of pancreatic cancer and gemcitabine-based chemotherapy, a first-line chemotherapy option, has been indicated to markedly prolong survival time in patients with pancreatic cancer (4).

However, multidrug-resistance (MDR) in pancreatic cancer often occurs during chemotherapy treatment due to the biological characteristics of the tumors, leading to a decline in the clinical efficacy of chemotherapy over time (5). Of the mechanisms of pancreatic cancer drug resistance, the most important is that the transporters on the tumor cell membrane mediate drug efflux and inactivation (6). Abnormal expression of ATP-binding cassette (ABC) transporters [ATP binding cassette subfamily B member 1 (ABCB1), ATP binding cassette subfamily C (ABCC) and ATP binding cassette subfamily G member 2 (ABCG2)] in patients with resistant pancreatic cancer has confirmed that these transporters are associated with MDR in pancreatic cancer (7).

Previous epigenetic studies have revealed that the level of ABC transporter promoter methylation in pancreatic cancer is negatively correlated with chemoresistance in patients with pancreatic cancer (8,9). ABC transporter promoter methylation has also served as an indicator of drug resistance in certain types of solid tumor (10). However, promoter methylation studies concerning MDR in pancreatic cancer are insufficient. There have been a number of relevant studies suggesting that the level of ABC transporter promoter methylation in pancreatic cancer is negatively correlated with drug resistance in pancreatic cancer (11,12). However, there is a lack of further

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quantitative simultaneous analysis and evaluation with regards to predicting MDR induced by methylation.

During the process of inducing the gemcitabine-resistant cell lines, PANC-1/Gem and BxPC-3/Gem, the present study aimed to use methylation-sensitive high-resolution melting (MS-HRM) to quantitatively and simultaneously detect promoter methylation of the ABCB1, ABCC and ABCG2 genes, while monitoring the expression of downstream mRNA and protein expression. Then, the changes in ABC transporter DNA promoter methylation of pancreatic cancer were investigated and the potential for using the promoter methylation level to predict acquired drug resistance was evaluated in order to provide a theoretical experimental basis for clinical research concerning the mechanisms underlying MDR and the treatment of pancreatic cancer.

Materials and methods

Cell culture and drug preparation. The human pancreatic cancer cell lines, BxPC-3 and PANC-1, were acquired from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cell lines were characterized as authentic by short tandem repeat profiling and were passaged in the laboratory for <6 months following receipt. All cell lines were grown in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 mg/ml ampicillin and 100 mg/ml streptomycin. The cultures were incubated at 37°C in a humidified atmosphere with 95% O₂ and 5% CO₂. Gemcitabine was purchased from Eli Lilly and Company (Indianapolis, IN, USA) and dissolved in sterile saline to form a 50 g/l stock solution.

Establishment of the resistant cell lines BxPC-3/Gem and PANC-1/Gem. The gemcitabine-resistant cell lines, BxPC-3 and PANC-1, were incubated in different initial gemcitabine concentrations of 0, 10 and 20 μM for the PANC-1 cell line (PANC-1, PANC-1/10, PANC-1/20, respectively) and 0, 6, 20, 40 and 70 μM for the BxPC-3 cell line (BxPC-3, BxPC-3/6, BxPC-3/20, BxPC-3/40, BxPC-3/70, respectively), to select cells with natural resistance to gemcitabine. The cells were incubated in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) without drugs following cultivation of the BxPC-3 cells and PANC-1 cells in this medium for 72 h. When the cells entered the logarithmic growth phase, they were passaged twice and cultivated with increasing concentrations of gemcitabine (1, 2, 5, 10, 20, 50, 100, 200, 500 and 1,000 μM) over a 10-month period, then resistant cell lines BxPC-3/Gem (BxPC-3, BxPC-3/6, BxPC-3/20, BxPC-3/40 and BxPC-3/70) and PANC-1/Gem (PANC-1, PANC-1/10 and PANC-1/20) were acquired. The cells were then cultivated in RPMI-1640 medium without gemcitabine for 2 months.

Sensitivity analysis of gemcitabine-resistant cell lines BxPC-3/Gem and PANC-1/Gem. The logarithmic phase cells were grown in 96-well plates (4x10³/well) for 24 h. Following adherence, cells were cultured in varying concentrations (1, 2, 5, 10, 20, 50, 100, 200, 500 and 1,000 μM) of gemcitabine for 72 h, with 6 wells per concentration. After 72 h, the media

was removed and 180 μl media and 20 μl MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were added to each well. The media was removed and 200 μl dimethyl sulfoxide was added to each well 4 h later to dissolve the formazan crystals. The cells were then agitated on a microplate shaker for 10 min. Absorbance (A) was read at 530 nm on a microplate reader. This experiment was repeated 4 times and equal amounts of DMSO were used as a blank control. The cell inhibition of each drug was calculated using the following formula: Inhibition=1-(dosing group A/control group A) x100%. Data were plotted on a semi logarithmic curve with drug concentrations on the X-axis and cell inhibition on the Y-axis. SPSS version 21 software (IBM Corp., Armonk, NY, USA) was used to calculate the half maximal inhibitory concentration (IC₅₀) and the resistance index (RI). The formula used to calculate RI was as follows: RI=IC₅₀ of resistant cell line/IC₅₀ of sensitive cell line.

Western blot analysis of ABCB1, ABCC and ABCG2 protein expression. BxPC-3 and PANC-1 cell lines were untreated. BxPC-3/Gem and PANC-1/Gem cells incubated for 24 h were collected and lysed with radioimmunoprecipitation lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, ethylenediaminetetraacetic acid (EDTA), leupeptin, and 1 nM phenylmethylsulfonyl fluoride] for 20 min on ice. Cells were then centrifuged at 20,817 x g and 4°C for 10 min and the supernatant was collected. The total protein concentration was then detected with a bicinchoninic acid assay (Beyotime Institute of Biotechnology, Shanghai, China) and was adjusted to 2.5 μg/μl with the lysis buffer. Proteins were electrophoresed on 20% SDS-PAGE, and then a constant current of 30 mA overnight at 4°C was used to electro-transfer the proteins onto polyvinylidene fluoride membranes (PVDF; EMD Millipore, Billerica, MA, USA). The PVDF membranes were blocked with 5% milk at room temperature for 1 h prior to being incubated with rabbit anti-ABCB1 mAb (cat. no. 13342S), rabbit anti-ABCC mAb (cat. no. 72202S) and rabbit anti-ABCG2 mAb (cat. no. 42078S) primary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C. Following washing with 1X Tris-buffered saline (TBST; 0.1% Tween-20) 3 times, horseradish peroxidase-conjugated secondary antibodies (goat anti-Rabbit IgG (H+L), dilution: 1:1,000; cat. no. A0208, Beyotime Institute of Biotechnology) were used to incubate the PVDF membranes for 3 h at room temperature. Following washing with TBST 3 times, enhanced chemiluminescence reagents (GE healthcare, Chicago, IL, USA; cat. no. RPN2106) were used to detect the bound antibody complexes and Tanon 5200 multi automatic chemiluminescence image analysis system (Tanon Science & Technology Co., Shanghai, China) was used to image. The same experiment was implemented on the untreated and gemcitabine-treated PANC-1 cell lines. An anti-β-Actin mouse monoclonal antibody (dilution, 1:2,500; cat. no. M1000170; Beyotime Institute of Biotechnology) was used as a loading control.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis of gene expression of ABCB1, ABCC and ABCG2. TRIzol reagent (Invitrogen; Thermo

Table I. Sequences of primers and the size of the sequences used for RT-qPCR.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')	PCR product (bp)
ABCB1	TATGCTGGAGCAGTTCCTCA	CCAGCTCCTCCTCCTTCTTT	149
ABCC	GAAGGAAGCAAAGCAAATGG	CCTGCTGATGTCCCCACTAT	109
ABCG2	CGGAAGGTGTCCTGCTACAT	CTTGACCATTTCCCTTCTGC	129
β -actin	TGCGCAGAAAACAAGATGAG	GTCACCTTCAACCGTTCAGT	116

PCR, polymerase chain reaction; bp, base pairs; ABCB1, ATP binding cassette subfamily B member 1; ABCC, ATP binding cassette subfamily C; ABCG2, ATP binding cassette subfamily G member 2; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

Fisher Scientific, Inc.) was used to extract the total RNA from cultured cells according to the manufacturer's protocols. The RNA content was measured by UV spectrophotometry at 260 nm. The sequences of primers and the size of the sequences are presented in Table I (TaqMan probe; Generay Biotech Co., Ltd, Shanghai, China). cDNA was synthesized according to the protocols of the first cDNA strand synthesis kit (Bioline Reagent Ltd., London, UK). PCR amplification was performed according to manufacturer's protocols (MyTaq™; Bioline Reagents Ltd.). The amplification cycling conditions were as follows: Stage 1: 1 cycle of 95°C for 5 min; stage 2: 40 cycles of 95°C for 15 sec and 60°C for 45 sec; and stage 3: 1 cycle of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. The PCR products were analyzed using ABI Prism 7500 SDS Software version 1.4 (Applied Biosystems, Thermo Fisher Scientific, Inc.) and the expression level of mRNA was calculated by the $2^{-\Delta\Delta Cq}$ method (13).

Detection of promoter methylation of ABCB1, ABCC and ABCG2 via MS-HRM. PANC-1 and BxPC-3 cells were grown in 96-well plate at a density of 5×10^3 cells per well for 24 h and washed with phosphate buffered saline 3 times. Total DNA was extracted from cultured cells and inverted using a DNA extraction kit (Generay Biotech Co., Ltd.) according to the manufacturer's protocols. MS-HRM was performed with a Methylated Cytosine Mapping kit (Shanghai Genmed Co., Ltd., Shanghai, China) in a total volume of 50 μ l containing: 2 μ l modified template DNA, 32.5 μ l PCR Master mix (PCR kit; Applied Biosystems, Thermo Fisher Scientific, Inc.), 0.5 μ l primer-F, 0.5 μ l primer-R, 0.5 μ l Taq-1, 0.5 μ l Taq-2 and 13.5 μ l PCR grade water. The amplification cycling conditions were: Stage 1: 1 cycle of 50°C for 2 min; stage 2: 1 cycle of 95°C for 5 min; stage 3: 40 cycles of 95°C for 15 sec and 60°C for 50 sec. The PCR products were analyzed by ABI Prism 7500 SDS Software version 1.4 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Methylation % = $100 / (1 + 2^{(Cq(CQ) - Cq(TG))})$.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS version 21 (IBM Corp.). A one-way analysis of variance and Student Newman-Keuls post-hoc test was used to identify statistically significant differences between groups of data and $P < 0.05$ was considered to indicate statistically significant differences. Pearson correlation analysis was used

to test whether the methylation level of the DNA promoter was correlated with the level of mRNA.

Results

Expression of ABCB1, ABCC and ABCG2 in BxPC-3/Gem and PANC-1/Gem cell lines. PANC-1 and BxPC-3 cell lines treated with a concentration gradient of gemcitabine were induced to acquire drug-resistance and inhibition of cell viability was detected using an MTT assay (Fig. 1). The IC_{50} of primary PANC-1 and BxPC-3 cell lines was 65.81 and 6.61 μ M, respectively. Additionally, IC_{50} increased with an increasing initial gemcitabine concentration of PANC-1 (PANC-1, PANC-1/10 and PANC-1/20) and BxPC-3 (BxPC-3, BxPC-3/6, BxPC-3/20, BxPC-3/40 and BxPC-3/70), demonstrating that PANC-1 and BxPC-3 successfully acquired resistance to gemcitabine during the process of induction. The dynamic changes of ABCB1, ABCC and ABCG2 of PANC-1 and BxPC-3 cell lines with different initial gemcitabine concentration were detected by western blotting during the process of inducing resistance to gemcitabine (Fig. 2). Compared with the primary culture cells, expression of ABCC and ABCB1 was significantly increased with an increase of initial gemcitabine concentration in the BxPC-3/Gem cell line, while no significant change in expression of ABCG2 was observed. In PANC-1/Gem, expression of ABCB1, ABCC and ABCG2 was elevated with an increasing initial gemcitabine concentration.

ABCB1, ABCC and ABCG2 mRNA expression in BxPC-3/Gem and PANC-1/Gem cell lines. The mRNA expression of the 3 genes, ABCB1, ABCC and ABCG2, in the BxPC-3/Gem and PANC-1/Gem cell lines was analyzed by RT-qPCR (Fig. 3). In contrast to primary culture cells, ABCB1, ABCC and ABCG2 mRNA expression was increased with a rising initial gemcitabine concentration in the BxPC-3/Gem and PANC-1/Gem cell lines. Of the 3 drug resistant genes, expression of ABCB1 and ABCC mRNA was enhanced more markedly than that of ABCG2.

Methylation of ABCB1, ABCC and ABCG2 in BxPC-3/Gem and PANC-1/Gem cell lines. In order to further understand the expression of ABCB1, ABCC and ABCG1 in BxPC-3/Gem and PANC-1/Gem cell lines, the promoter methylation of the 3 ABC transporter genes was detected quantitatively and synchronously through MS-HRM (Fig. 4). A correlation analysis between PANC-1 cell lines (PANC-1, PANC-1/10,

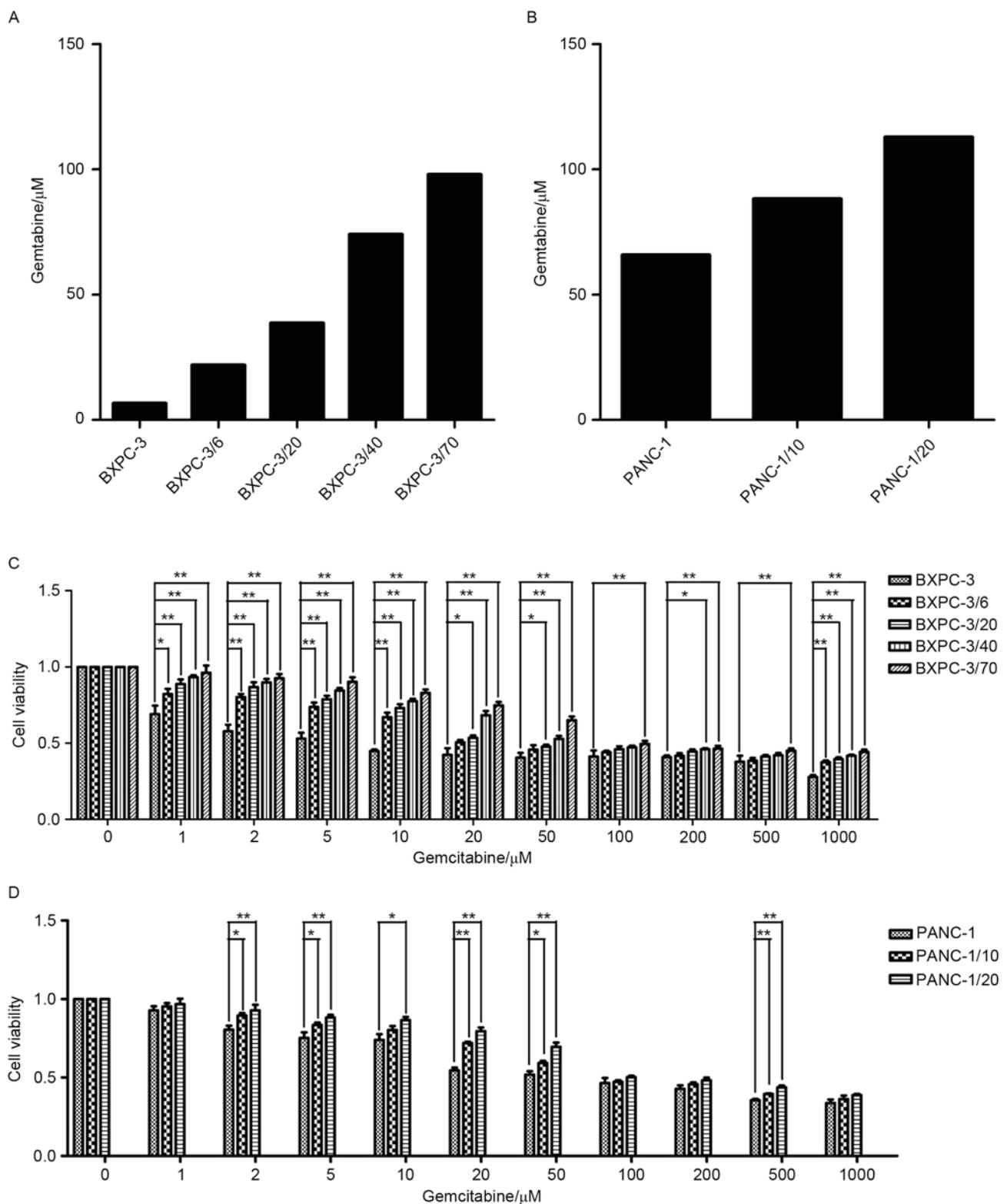


Figure 1. Establishment of BxPC-3/Gem and PANC-1/Gem cell lines and analysis of cell proliferation. (A) BxPC-3 and (B) PANC-1 cells were exposed to with increasing concentrations of gemcitabine (1, 2, 5, 10, 20, 50, 100, 200, 500 and 1,000 μM) for 72 h and the IC_{50} were calculated. Cell viability of (C) BxPC-3 and (D) PANC-1 cells with different initial gemcitabine concentration were analyzed by MTT assay. Quantification was performed by assigning a value of 100% to the untreated group of BxPC-3 and PANC-1 cells. The same volume of normal saline solution was used as a positive control, while the same volume of dimethyl sulfoxide was used as a negative control. * $P < 0.05$, ** $P < 0.01$, with comparisons indicated by lines.

PANC-1/20) and promoter methylation was conducted and between BxPC-3 cell lines (BxPC-3, BxPC-3/6, BxPC-3/20, BxPC-3/40, BxPC-3/70) and promoter methylation (Fig. 5). The results demonstrated that promoter methylation of ABCB1,

ABCC and ABCG2 was decreased with elevation of the initial gemcitabine concentration in BxPC-3/Gem and PANC-1/Gem cell lines compared with primary culture cells, suggesting that expression of ABCB1, ABCC and ABCG2 was increased in

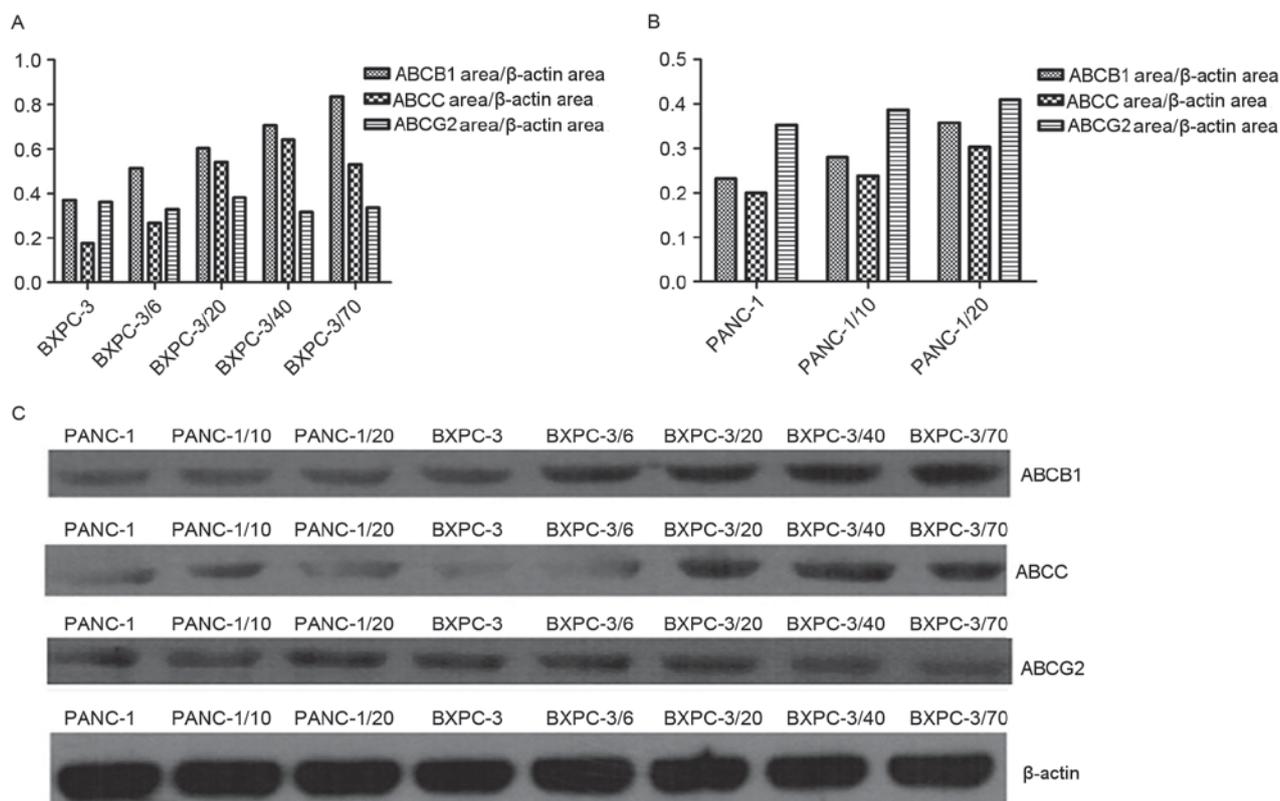


Figure 2. Protein expression differences in ABCB1, ACC and ABCG2 were detected through western blot analysis. In contrast to the parental cell lines (BxPC-3 and PANC-1 cells), protein expression of ABCB1 and ABCC was significantly increased with elevation of initial gemcitabine concentration in (A) BxPC-3/Gem cells while ABCG2 expression did not significantly change, and protein expression of ABCB1, ABCC and ABCG2 was considerably increased in (B) PANC-1/Gem cells. (C) Electrophoresis results of the 3 proteins, ABCB1, ABCC and ABCG2. ABCB1; ATP binding cassette subfamily B member 1; ABCC, ATP binding cassette subfamily C; ABCG2, ATP binding cassette subfamily G member 2.

the process of inducing gemcitabine resistance in PANC-1 and BxPC-3 cell lines. It was also revealed that ABCB1 promoter methylation was significantly reduced when the initial gemcitabine concentration was 40 in the BxPC-3/Gem cell line, suggesting that drug-resistance of BxPC-3 was markedly increased when initial gemcitabine concentration ranged from 20 to 40, and the same as for ABCG2 promoter methylation when initial gemcitabine concentration ranged from 0 to 6 in the PANC-1/Gem cell line.

Discussion

ABC transporters, which are transmembrane proteins present on the tumor cell membrane, act as a drug efflux pump to reduce the intracellular concentration of chemotherapeutic agents by binding and hydrolyzing ATP, resulting in multidrug resistance (14). Previous studies have suggested that the ABCB1 (15,16), ABCC (17) and ABCG2 (18-20) are abnormally overexpressed in tumor cells of patients with pancreatic cancer, which is clinically relevant to the reductive reaction to chemotherapy of tumors, MDR and a poor clinical prognosis. However, there is a lack of experimental data regarding the dynamic changes of the expression of these 3 proteins during the process of pancreatic cancer cells acquiring drug resistance.

In the present study, resistance to gemcitabine was successfully induced in the human primary pancreatic cancer cell

lines PANC-1 and BxPC-3 via the concentration gradient method. During the process of inducing drug resistance, it was revealed that the expression of ABCB1 and ABCC proteins in PANC-1 and BxPC-3, and the transcription of corresponding upstream mRNA, indicates a synchronous increase in line with the increased initial gemcitabine concentration. This result has confirmed the aforementioned relationship between ABCB1, ABCC and pancreatic cancer drug resistance (15-17), which also indicates that ABCB1 and ABCC are involved in the formation of the acquired drug-resistance in these pancreatic cancer cell line. Compared with ABCB1 and ABCC, the expression of ABCG2 in PANC-1 and BxPC-3 did not exhibit a marked increasing trend with an elevated initial gemcitabine concentration, and the increase in mRNA transcription of ABCG2 was not as marked as that of ABCB1 and ABCC. In studies of pancreatic cancer, ABCG2, which mainly exists in side population (SP) cells of pancreatic cancer and is highly expressed, had been recognized as a potential marker of tumor stem cells and assists SP cells in acquiring a more powerful drug efflux capacity than that of non-SP cells (21,22). The ratio of SP cells in the PANC-1 cell line is low (23) and the cells may not have proliferated to a certain percentage or demonstrated any survival advantage in drug-resistant cells due to the short time span of the experiment, which may have been the cause of the lack of statistically significant differences in the expression of ABCG2. Therefore, ABCC and ABCB1 in ABC transporters may serve a more important function

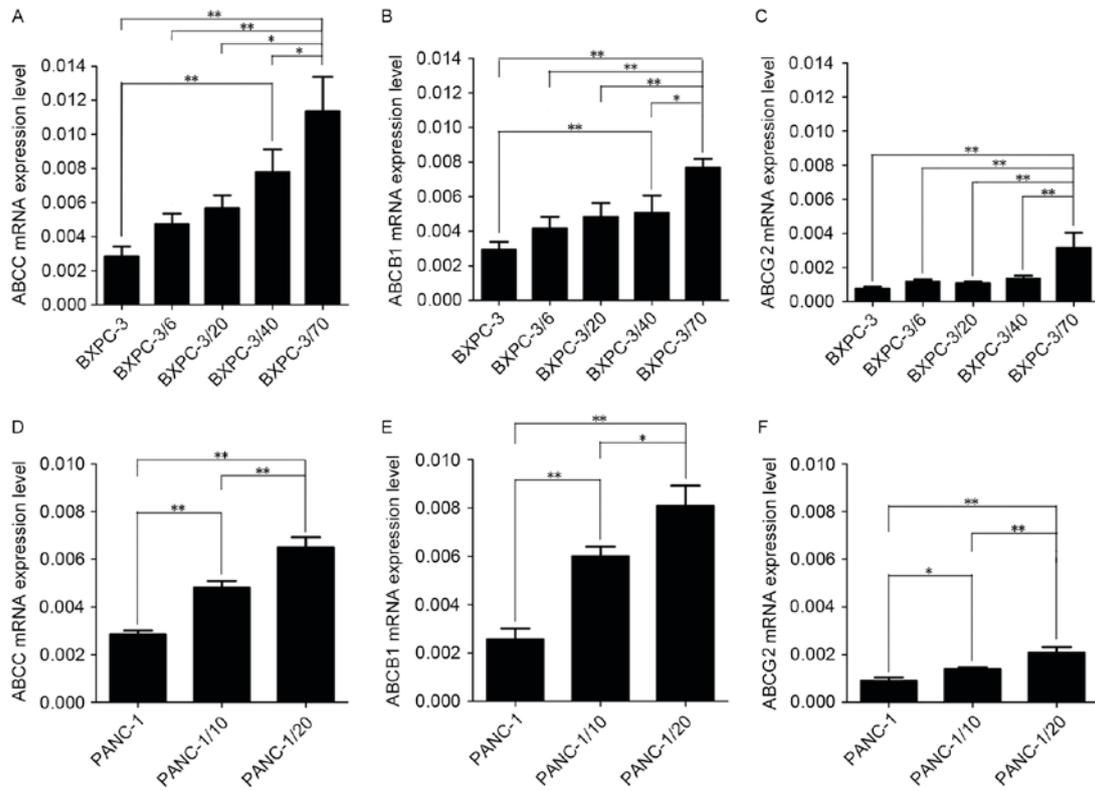


Figure 3. mRNA expression of ABCB1, ABCC and ABCG2 in PANC-1/Gem and BxPC-3/Gem cells as detected by real-time polymerase chain reaction. mRNA expression of (A) ABCC, (B) ABCB1 and (C) ABCG2 was elevated with increases of initial gemcitabine concentration in BxPC-3/Gem cells. mRNA expression of (D) ABCC, (E) ABCB1 and (F) ABCG2 was elevated with increases of initial gemcitabine concentration in PANC-1/Gem cells. * $P < 0.05$ and ** $P < 0.01$. ABCB1, ATP binding cassette subfamily B member 1; ABCC, ATP binding cassette subfamily C; ABCG2, ATP binding cassette subfamily G member 2.

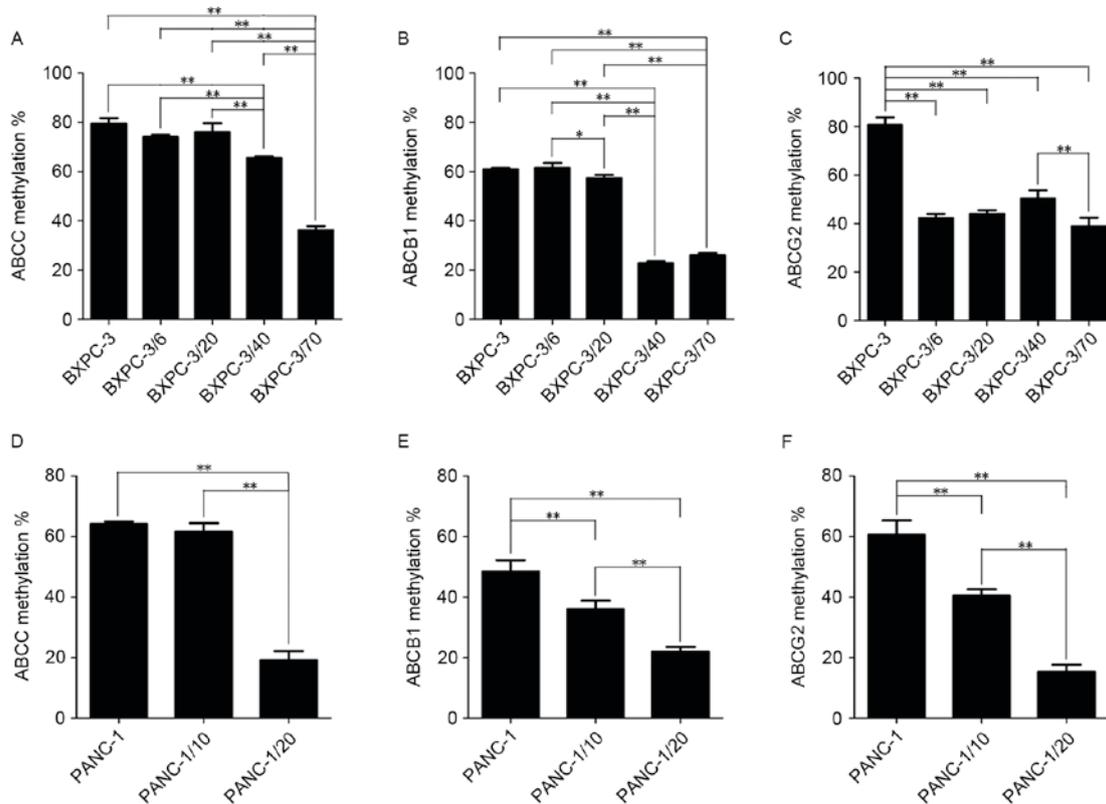


Figure 4. Promoter methylation of ABCB1, ABCC and ABCG2 in PANC-1/Gem and BxPC-3/Gem detected via MS-HRM. Through quantitative and synchronous detection, promoter methylation of (A) ABCC, (B) ABCB1 and (C) ABCG2 was markedly decreased in BxPC-3/Gem, and (D) ABCC, (E) ABCB1 and (F) ABCG2 in PANC-1/Gem, with increases initial gemcitabine concentration. * $P < 0.05$ and ** $P < 0.01$. ABCB1, ATP binding cassette subfamily B member 1; ABCC, ATP binding cassette subfamily C; ABCG2, ATP binding cassette subfamily G member 2.

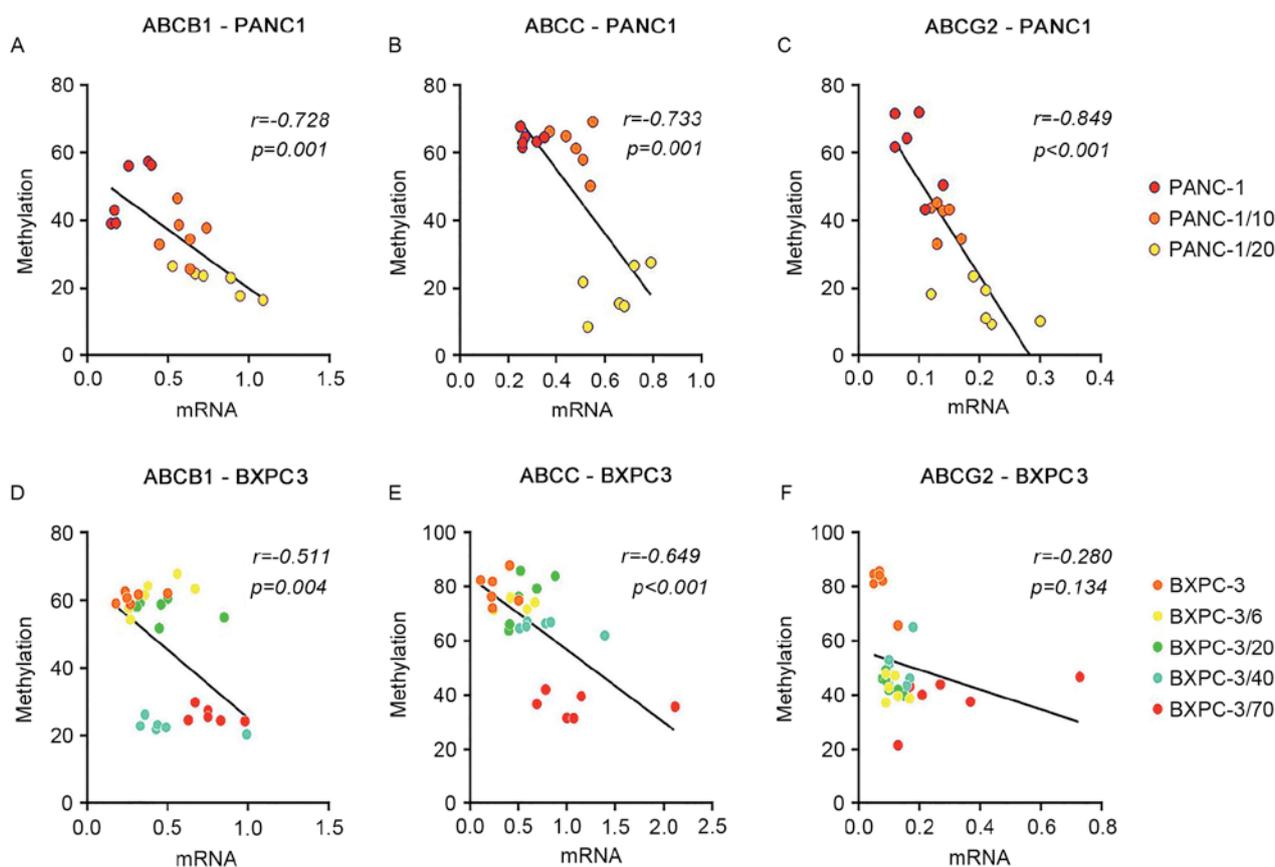


Figure 5. Correlation analysis between promoter methylation and mRNA expression. Promoter methylation of (A) ABCB1, (B) ABCC and (C) ABCG2 in PANC-1/Gem and (D) ABCB1, (E) ABCC and (F) ABCG2 in BxPC-3/Gem was negatively associated with the mRNA expression of these 3 genes. ABCB1; ATP binding cassette subfamily B member 1; ABCC, ATP binding cassette subfamily C; ABCG2, ATP binding cassette subfamily G member 2.

than ABCG2 in the early stage of establishing drug-resistant pancreatic cancer cell lines.

Methylation of DNA is one of the most essential biological epigenetic modifications. The methylation of a specific gene promoter on the DNA cut back has a direct impact on mRNA transcription and protein expression, resulting in the abnormal expression of the protein encoded by the gene (24,25). Previous studies on other malignant tumors have proposed that the methylation of ABC transporter gene promoters is negatively correlated with chemoresistance in tumor cells (26-28). In a study on pancreatic cancer, Chen *et al* (29) observed that the promoter methylation level of the ABC transporter family (ABCB1, ABCC and ABCG2) in a gemcitabine-resistant cell line (SW1990/GZ) was significantly lower than that in the primary cell line (SW1990), while resistance to gemcitabine of the cell line increased 33.3 times. The present study revealed that, during the process of establishing gemcitabine resistance in PANC-1 and BxPC-3 cell lines, the promoter methylation level of the ABC transporter family drug resistance genes, ABCB1, ABCC and ABCG2, decreased gradually with an increased initial gemcitabine concentration, and an increase in the expression of the corresponding downstream mRNA and protein. This was consistent with the effect of epigenetic methylation modification, suggesting that the promoter hypomethylation modification of the chemo-resistance genes, ABCB1, ABCC and ABCG2, was also involved in the acquisition of the

drug resistance in the pancreatic cancer cells. This provides evidence of the feasibility of predicting MDR of pancreatic cancer through independent indicators-the promoter methylation level of ABCB1, ABCC and ABCG2. However, the specific mechanisms underpinning this require further experimental verification and discussion.

The present study discovered that the change of the resistant gene methylation not only reveals drug resistance of pancreatic cancer, but may also be used as an evaluation indicator to assess the drug resistance of tumors that are treated with chemotherapy. Previously, pancreatic tumor tissues were obtained mainly from surgical resection and thus, the majority of tumor samples were from patients with resectable tumors (30). However, there is no clinical significance in detecting the methylation of resistant genes for such patients. In recent years, with the development of medical technology, endoscopic ultrasound and fine needle aspiration is widely applied in clinical practice and the tumor tissues of patients with unresectable tumors who always require long-term chemotherapy are accessible using this technique (31). Additionally, it is also possible to obtain tumor cells from the blood and to use these to detect resistance gene methylation. Therefore, the detection of resistance gene methylation may be used to monitor tumor drug resistance in patients undergoing chemotherapy, so as to aid clinicians in modulating chemotherapy regimens according to the changes of tumor drug resistance.

Certain studies have proposed the novel concept of cancer evolution, wherein the tumor follows the same selection principle of nature, as with other living creatures (32). With the mutation of tumor cell genes, tumor cells that are resistant to treatment survive and expand, and are able to evolve and adapt. At present, owing to the advanced sequencing technology and accumulation of a large volume of clinical data, scholars have drawn the map of cancer evolution and have revealed the origin of the drug resistance (33). According to the map of cancer evolution, the trunk mutations of tumor cell genes may be discovered during the process of cancer evolution as these types of mutation are present in all tumor cells. Notably, there are also branch mutations and these types of mutation are only present in certain tumor cells. We hypothesized that the epigenetic changes of the ABC transporter family is one of the trunk mutations in the present study. Through the detection of ABC transporter family methylation, changes in the chemoresistance of pancreatic cancer cells were observed, which in turn may be a closely monitored process of tumor evolution and adaptation and, as mentioned previously, may provide further basis for clinicians to adjust chemotherapy regimens in sufficient time. Future studies should also focus on further exploring branch mutations in pancreatic cancer to refine the evolutionary map of pancreatic cancer and provide more treatment options for clinicians.

It was also observed that not all resistance genes were altered to the same degree as tumor chemoresistance, and certain resistance genes served a leading function while others served a minor function or no function at all. These findings may also aid clinicians in determining more accurate therapies to target tumors. Although there is a shortcoming of the present study, namely the low detecting precision of MS-HRM, this method is economic, efficient, convenient and also has the advantage of distinguishing tumors from normal tissue samples more accurately and thus, is capable of being widely used in clinical practice (34). However, the lack of animal experiments is a drawback of the present study and animal experiments were not completed for a number of reasons. Firstly, the survival time of genetically engineered mouse models of pancreatic cancer is relatively short, and achieving a change in tumor drug resistance often requires a longer time period and therefore it was difficult to observe the variety of drug resistance in the tumors. Additionally, it is well known that pancreatic cancer is a stromal-rich malignant tumor and therefore, if a subcutaneous transplantation tumor model of pancreatic cancer in nude mice were to be selected, it would not be able to completely simulate human pancreatic tumor tissue and the results would be unreliable. Future studies will aim to find a suitable mouse model and to undertake animal experiments.

To conclude, it is presumed that the pathway to reduce the reaction of pancreatic cancer cells to drugs includes the increased expression of downstream mRNA and protein through the promoter hypomethylation modification of the ABCB1, ABCC and ABCG2 genes in the ABC transporter family and the enhanced drug efflux capacity of the transporters. Additionally, with the evolution of drug resistance in pancreatic cancer cells, the promoter methylation of ABCB1, ABCC and ABCG2 gradually decreases. Therefore, the

promoter methylation level is capable of quantitatively and dynamically reflecting the progression of chemo-resistance in pancreatic cancer cells.

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Availability of data and materials

All data generated and analyzed during the present study are included in the published article and available for use.

Authors' contributions

LY and JG performed all the data analysis and drafted the manuscript. YM and XZ performed basic experiments and collected the data. XW helped to edit the manuscript and provided support for experiments. DF and CJ performed independent reviews of all studies and data, and JL designed this study and executed the analysis.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. *CA Cancer J Clin* 66: 115-132, 2016.
2. Gillen S, Schuster T, Meyer Zum Büschenfelde C, Friess H and Kleeff J: Preoperative/neoadjuvant therapy in pancreatic cancer: A systematic review and meta-analysis of response and resection percentages. *PLoS Med* 7: e1000267, 2010.
3. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2015. *CA Cancer J Clin* 65: 5-29, 2015.
4. Hidalgo M: Pancreatic cancer. *N Engl J Med* 362: 1605-1617, 2010.
5. Neoptolemos JP, Stocken DD, Friess H, Bassi C, Dunn JA, Hickey H, Beger H, Fernandez-Cruz L, Dervenis C, Lacaine F, *et al*: A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med* 350: 1200-1210, 2004.
6. Tanaka M, Okazaki T, Suzuki H, Abbruzzese JL and Li D: Association of multi-drug resistance gene polymorphisms with pancreatic cancer outcome. *Cancer* 117: 744-751, 2011.
7. Polgar O and Bates SE: ABC transporters in the balance: Is there a role in multidrug resistance? *Biochem Soc Trans* 33: 241-245, 2005.
8. Douet V, Heller MB and Le Saux O: DNA methylation and Sp1 binding determine the tissue-specific transcriptional activity of the mouse *Abcc6* promoter. *Biochem Biophys Res Commun* 354: 66-71, 2007.

9. Bram EE, Stark M, Raz S and Assaraf YG: Chemotherapeutic drug-induced ABCG2 promoter demethylation as a novel mechanism of acquired multidrug resistance. *Neoplasia* 11: 1359-1370, 2009.
10. Gao P, Yang X, Xue YW, Zhang XF, Wang Y, Liu WJ and Wu XJ: Promoter methylation of glutathione S-transferase p11 and multidrug resistance gene 1 in bronchioloalveolar carcinoma and its correlation with DNA methyltransferase 1 expression. *Cancer* 115: 3222-3232, 2009.
11. Pang L, Word B, Xu J, Wang H, Hammons G, Huang SM and Lyn-Cook B: ATP-binding cassette genes genotype and expression: A potential association with pancreatic cancer development and chemoresistance? *Gastroenterol Res Pract* 2014: 414931, 2014.
12. Chen M, Xue X, Wang F, An Y, Tang D, Xu Y, Wang H, Yuan Z, Gao W, Wei J, *et al*: Expression and promoter methylation analysis of ATP-binding cassette genes in pancreatic cancer. *Oncol Rep* 27: 265-269, 2012.
13. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
14. Cascorbi I: Role of pharmacogenetics of ATP-binding cassette transporters in the pharmacokinetics of drugs. *Pharmacol Ther* 112: 457-473, 2006.
15. Suwa H, Ohshio G, Arao S, Imamura T, Yamaki K, Manabe T, Imamura M, Hiai H and Fukumoto M: Immunohistochemical localization of P-glycoprotein and expression of the multidrug resistance-1 gene in human pancreatic cancer: Relevance to indicator of better prognosis. *Jpn J Cancer Res* 87: 641-649, 1996.
16. Lu Z, Kleeff J, Shrikhande S, Zimmermann T, Korc M, Friess H and Büchler MW: Expression of the multidrug-resistance 1 (MDR1) gene and prognosis in human pancreatic cancer. *Pancreas* 21: 240-247, 2000.
17. König J, Hartel M, Nies AT, Martignoni ME, Guo J, Büchler MW, Friess H and Keppler D: Expression and localization of human multidrug resistance protein (ABCC) family members in pancreatic carcinoma. *Int J Cancer* 115: 359-367, 2005.
18. Lee SH, Kim H, Hwang JH, Lee HS, Cho JY, Yoon YS and Han HS: Breast cancer resistance protein expression is associated with early recurrence and decreased survival in resectable pancreatic cancer patients. *Pathol Int* 62: 167-175, 2012.
19. Wang F, Xue X, Wei J, An Y, Yao J, Cai H, Wu J, Dai C, Qian Z, Xu Z and Miao Y: hsa-miR-520 h downregulates ABCG2 in pancreatic cancer cells to inhibit migration, invasion, and side populations. *Br J Cancer* 103: 567-574, 2010.
20. Hamada S, Satoh K, Hirota M, Kanno A, Umino J, Ito H, Masamune A, Kikuta K, Kume K and Shimosegawa T: The homeobox gene *MSX2* determines chemosensitivity of pancreatic cancer cells via the regulation of transporter gene *ABCG2*. *J Cell Physiol* 227: 729-738, 2012.
21. Olempska M, Eisenach PA, Ammerpohl O, Ungefroren H, Fandrich F and Kalthoff H: Detection of tumor stem cell markers in pancreatic carcinoma cell lines. *Hepatobiliary Pancreat Dis Int* 6: 92-97, 2007.
22. Miranda-Lorenzo I, Dorado J, Lonardo E, Alcalá S, Serrano AG, Clausell-Tormos J, Cioffi M, Megias D, Zagorac S, Balic A, *et al*: Intracellular autofluorescence: A biomarker for epithelial cancer stem cells. *Nat Methods* 11: 1161-1169, 2014.
23. Zhou J, Wang CY, Liu T, Wu B, Zhou F, Xiong JX, Wu HS, Tao J, Zhao G, Yang M and Gou SM: Persistence of side population cells with high drug efflux capacity in pancreatic cancer. *World J Gastroenterol* 14: 925-930, 2008.
24. Bird AP and Wolffe AP: Methylation-induced repression-belts, braces, and chromatin. *Cell* 99: 451-454, 1999.
25. Toyota M, Suzuki H, Yamashita T, Hirata K, Imai K, Tokino T and Shinomura Y: Cancer epigenomics: Implications of DNA methylation in personalized cancer therapy. *Cancer Sci* 100: 787-791, 2009.
26. Ding S, Gong BD, Yu J, Gu J, Zhang HY, Shang ZB, Fei Q, Wang P and Zhu JD: Methylation profile of the promoter CpG islands of 14 'drug-resistance' genes in hepatocellular carcinoma. *World J Gastroenterol* 10: 3433-3440, 2004.
27. Chekhun VF, Kulik GI, Yurchenko OV, Tryndyak VP, Todor IN, Luniv LS, Tregubova NA, Pryzimirskaya TV, Montgomery B, Rusetskaya NV and Pogribny IP: Role of DNA hypomethylation in the development of the resistance to doxorubicin in human MCF-7 breast adenocarcinoma cells. *Cancer Lett* 231: 87-93, 2006.
28. Tada Y, Wada M, Kuroiwa K, Kinugawa N, Harada T, Nagayama J, Nakagawa M, Naito S and Kuwano M: MDR1 gene overexpression and altered degree of methylation at the promoter region in bladder cancer during chemotherapeutic treatment. *Clin Cancer Res* 6: 4618-4627, 2000.
29. Chen M, Xue X, Wang F, An Y, Tang D, Xu Y, Wang H, Yuan Z, Gao W, Wei J, *et al*: Expression and promoter methylation analysis of ATP-binding cassette genes in pancreatic cancer. *Oncol Rep* 27: 265-269, 2012.
30. Wray CJ, Ahmad SA, Matthews JB and Lowy AM: Surgery for pancreatic cancer: Recent controversies and current practice. *Gastroenterology* 128: 1626-1641, 2005.
31. Krishna NB, LaBundy JL, Saripalli S, Safdar R and Agarwal B: Diagnostic value of EUS-FNA in patients suspected of having pancreatic cancer with a focal lesion on CT scan/MRI but without obstructive jaundice. *Pancreas* 38: 625-630, 2009.
32. Goymer P: Natural selection: The evolution of cancer. *Nature* 454: 1046-1086, 2008.
33. Jiang Y, Qiu Y, Minn AJ and Zhang NR: Assessing intratumor heterogeneity and tracking longitudinal and spatial clonal evolutionary history by next-generation sequencing. *Proc Natl Acad Sci USA* 113: E5528-E5537, 2016.
34. BLUEPRINT consortium: Quantitative comparison of DNA methylation assays for biomarker development and clinical applications. *Nat Biotechnol* 34: 726-737, 2016.



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