

Expression and promoter methylation analysis of ATP-binding cassette genes in pancreatic cancer

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Abstract. We investigated the relationship of *ABCB1/MDR1*, *ABCC1/MRP1* and *ABCG2/BCRP* expression and promoter methylation with pancreatic cancer tumorigenesis and drug resistance. Gemcitabine-resistant pancreatic cancer cells, SW1990/GZ (33.3-fold increased resistance), were obtained by treating SW1990 cells with gemcitabine. The expression of *ABCB1/MDR1*, *ABCC1/MRP1* and *ABCG2/BCRP* was determined by quantitative real-time RT-PCR in the cell lines, 3 normal pancreatic tissues, 15 human pancreatic cancer samples and 15 adjacent tissues. Promoter methylation was determined in cell lines by bisulfite genomic sequencing. *ABCB1/MDR1*, *ABCC1/MRP1* and *ABCG2/BCRP* were upregulated in SW1990 and SW1990/GZ compared with normal pancreatic tissue, and expression in SW1990/GZ was significantly higher than in SW1990 cells. *ABCB1/MDR1*, *ABCC1/MRP1* and *ABCG2/BCRP* were upregulated in pancreatic cancer tissues, compared to adjacent tissues. The *ABCB1/MDR1*, *ABCC1/MRP1* and *ABCG2/BCRP* promoter were hypomethylated in all the cell lines. *ABCB1/MDR1*, *ABCC1/MRP1* and *ABCG2/BCRP* expression correlated with pancreatic cancer tumorigenesis and drug resistance in a mechanism that is independent of promoter methylation.

Introduction

Pancreatic cancer is among the 10 leading causes of cancer-related deaths, with a 5-year survival <5% (1). Most patients have advanced stage disease at diagnosis. One fifth of pan-

creatic cancer patients present in the early stage and are eligible for surgical resection (2), which is a complex operation that is associated with high rates of operative morbidity. Pancreatic cancer also shows resistance to chemotherapy. Gemcitabine, the standard treatment in advanced disease, prolongs survival by 5-6 months. Chemotherapy is largely ineffective for patients with metastases as disease frequently redevelops even after surgery; therefore, there is an urgent need to understand the molecular mechanism of chemoresistance in pancreatic cancer.

One well-known mechanism of chemoresistance is the overexpression of energy-dependent pumps, such as adenosine triphosphate-binding cassette (ABC) transporters, which lead to the extrusion of drugs and their metabolites (3). P-gp, a 170,000-Da phosphoglycoprotein that consists of two ATP binding cassettes and two transmembrane regions, which is associated with chemoresistance in cancers of the gastrointestinal tract, liver and kidneys (4). The breast cancer resistance protein (ABCG2/BCRP) is a 655-amino-acid polypeptide transporter with a wide range of substrates associated with breast cancer resistance (5). Additionally, *ABCC1/MRP1* is correlated with differentiation grade and tumor size in primary hepatocellular carcinoma and breast cancer (6,7).

DNA methylation of CpG dinucleotides is the predominant epigenetic gene expression modification. More than 40% of protein coding genes have 5' CpG rich segments, termed CpG islands (8). The aberrant methylation of gene promoters is frequently observed in human cancers and correlates with carcinogenesis, tumor progression and chemosensitivity (9,10). Methylation of gene promoters is inversely proportional to transcriptional activity (11). Epigenetic silencing is mediated via methyl CpG binding proteins (MBDs) which act as docking sites for co-repressor proteins such as histone deacetylases, histone methyltransferases (12). *MDR1* mRNA transcription is inversely correlated with DNA methylation in various cancers, including prostate, gastric carcinomas (10,13). Human tumors often display altered DNA methylation, including both genome-wide hypomethylation and site-specific hypermethylation (14).

Demethylation of CpG islands in the *ABCB1/MDR1* promoter region is a mechanism of chemoresistance, which induces the expression of P-gp and the multidrug-resistant (MDR) phenotype (15). The objectives of this study were to investigate the

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expression and CpG island methylation status of ATP-binding cassette genes in pancreatic cancer, and the possible molecular mechanisms of carcinogenesis and chemosensitivity.

Materials and methods

Tissue samples and cell lines. Fifteen pairs of samples (15 pancreatic cancer tissues, 15 adjacent tissues 3-5 cm from the cancer edge) and three normal pancreatic tissues were obtained from patients suffering from pancreatic ductal adenocarcinoma and patients with pancreatic injury (experimental procedures manipulating the human tissues or human-derived cells met the principles of Declaration of Helsinki and were approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University). Each sample was cryopreserved in liquid nitrogen. The human pancreatic cancer cell line SW1990 was obtained from the Shanghai Cell Bank (Shanghai, China). Gemcitabine-resistant pancreatic cancer cells, SW1990/GZ, were obtained by treating the parental cell line SW1990 with increasing doses (maximum dose 200 μ mol/l) of gemcitabine intermittently for 24 weeks to generate stable cultures. All cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 2 mM glutamine, 100 μ g/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified chamber supplemented with 5% CO₂.

Quantitative real-time RT-PCR assays for gene expression analysis. Total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Shanghai, China) according to the manufacturer's protocol and quantified by absorption at 260 nm. *ABCB1*, *ABCC1* and *ABCG2* real-time RT-PCR primers were listed in Table I. Quantitative RT-PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (Takara, Dalian, China). Relative gene expression was normalized to the internal reference gene, glucose-6-phosphate dehydrogenase (GAPDH).

DNA methylation analysis. DNA was isolated using the Omega Tissue DNA kit (Omega, Shanghai, China) and 500 ng was subjected to bisulfite conversion using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions and prepared for PCR amplification using the Takara Taq Kit (Takara). Primers for the bisulfite-treated gene promoters (Table II) were designed using Methyl Primer Express software v1.0 (Applied Biosystems). The PCR amplification products were extracted from 2% agarose gels using the Gel Extraction Kit (Omega), cloned into the pEASY-T1 simple vector (TransGen Biotech, Beijing, China) and transfected into Transl-T1 phage-resistant chemically competent cells according to the manufacturer's instructions. The bacterial fluid containing pEASY-T1 target DNA was spread on solid LB medium, incubated overnight at 37°C and bacterial clones were picked into 1.0 ml liquid LB medium and sent to Invitrogen Corporation Shanghai Representative Office for sequencing.

Statistical analysis. Results were expressed as the mean \pm SEM of at least triplicate measurements. The Student's t-test was used to compare values with SPSS 13.0 software, and $p < 0.05$ was considered statistically significant.

Table I. *ABCB1*, *ABCC1* and *ABCG2* real-time RT-PCR primers.

| Gene | Primers | Tm (°C) |
|-------|-------------------------------------|---------|
| ABCB1 | Sense 5'-CGAAGCAGTGGTTCAGGTG-3' | 60.0 |
| | Antisense 5'-TGTGATCCACGGACACTCC-3' | |
| ABCG2 | Sense 5'-GAAGAGTGGCTTTCTACCTT-3' | 61.0 |
| | Antisense 5'-GTCCCAGGATGGCGTTGA-3' | |
| ABCC1 | Sense 5'-CGCCTTCGCTGAGTTCCT-3' | 62.5 |
| | Antisense 5'-TGCGGTGCTGTTGTGGTG-3' | |

Table II. *ABCB1*, *ABCC1* and *ABCG2* promoter bisulfite genomic sequencing primers.

| Gene | Primers | Size (bp) | Tm (°C) |
|-------|--|-----------|---------|
| ABCB1 | Sense 5'-GTAGTGGTATTGGATTATGTTG-3' | 260 | 60 |
| | Antisense 5'-TTAATACCCCAACTACTCTAAC-3' | | |
| ABCG2 | Sense 5'-AATGAGYGTGTTGGTGATTTT-3' | 256 | 60 |
| | Antisense 5'-ATTTCCCCAAATCRAAATTC-3' | | |
| ABCC1 | Sense 5'-GTTTAAGTTTTGGAGGATTTGG-3' | 176 | 60 |
| | Antisense 5'-CAACRCCRCCTAATTAACCC-3' | | |

Results

Morphological analysis and RI (resistance index) of SW1990/GZ cells. Parental SW1990 cells have a polygonal and uniform morphology (Fig. 1A). During induction of gemcitabine resistance, cells have a flat elongated morphology, with increased foot processes, with vacuoles, membrane rupture and fuzzy boundaries (Fig. 1B). In SW1990/GZ, reduced cell vacuoles and foot processes were observed in tightly packed cells (Fig. 1C). The IC₅₀ for gemcitabine, the value at which growth was inhibited by 50%, was 100 μ mol/l in SW1990/GZ cells and 3.0 μ mol/l in SW1990 cells. The RI (resistance index) of SW1990/GZ (IC₅₀ of SW1990/GZ divided by the IC₅₀ of SW1990) is 33.3.

ATP-binding cassette gene mRNA expression is increased in SW1990/GZ and SW1990 compared with the normal pancreas tissue. Expression of the ATP-binding cassette genes *ABCB1*, *ABCC1* and *ABCG2* was significantly increased in SW1990/GZ and SW1990 cells compared with the normal pancreatic tissues (Fig. 2). Additionally, expression of *ABCB1*, *ABCC1* and *ABCG2* was significantly higher in SW1990/GZ cells than SW1990 cells ($p < 0.05$). These results indicate a relationship between ATP-binding cassette gene expression and tumorigenesis and drug resistance in pancreatic cancer cells.

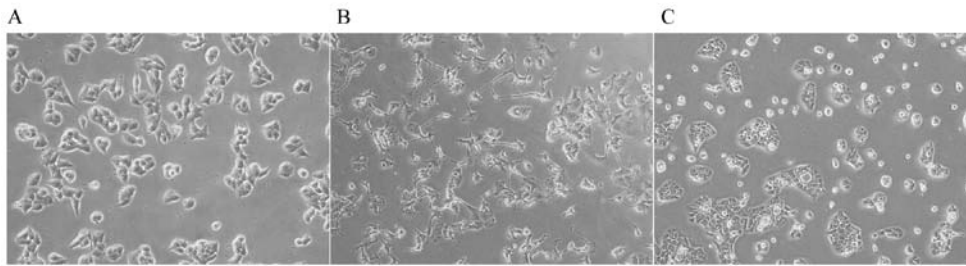


Figure 1. Pancreatic cell line morphology. (A) Parental sw1990 cells; (B) sw1990 cells in the process of gemcitabine induced resistance; (C) sw1990-GZ gemcitabine-resistant cells, x40.

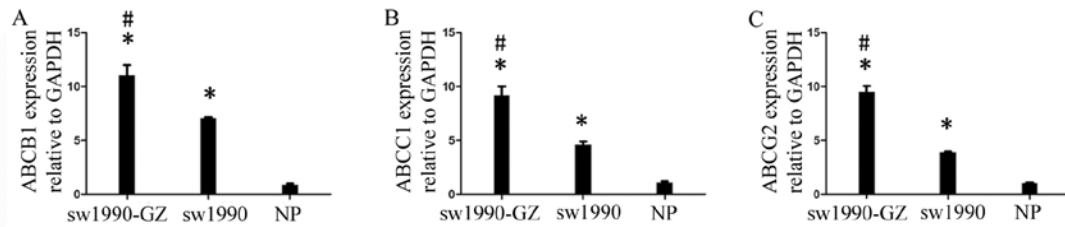


Figure 2. Quantitative real-time PCR for the normal pancreatic tissues (NP), sw1990, and sw1990-GZ gemcitabine-resistant cell lines. (A) *ABCB1*, (B) *ABCC1* and (C) *ABCG2* mRNA expressed normalized to GAPDH, * $p < 0.05$ vs. NP, # $p < 0.05$ vs. sw1990 cells.

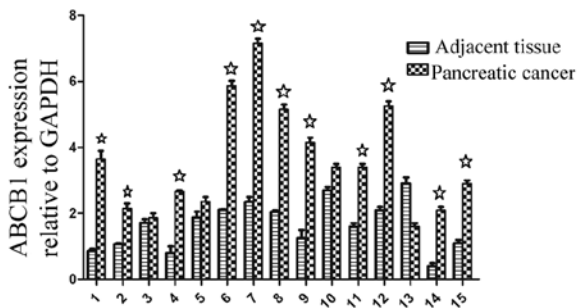


Figure 3. Quantitative real-time PCR of the ATP-binding cassette gene *ABCB1/MDR1* in pancreatic cancer and matched adjacent tissues for 15 individuals, * $p < 0.05$ vs. adjacent tissue.

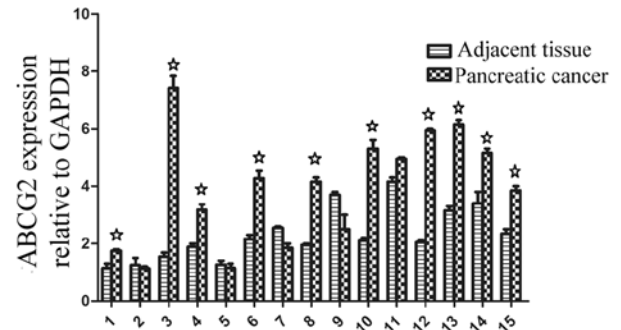


Figure 5. Quantitative real-time PCR of the ATP-binding cassette gene *ABCG2/BCRP* in pancreatic cancer and matched adjacent tissues for 15 individuals, * $p < 0.05$ vs. adjacent tissue.

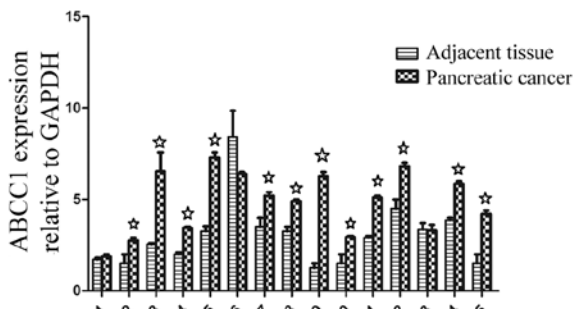


Figure 4. Quantitative real-time PCR of the ATP-binding cassette gene *ABCC1/MRP1* in pancreatic cancer and matched adjacent tissues for 15 individuals, * $p < 0.05$ vs. adjacent tissue.

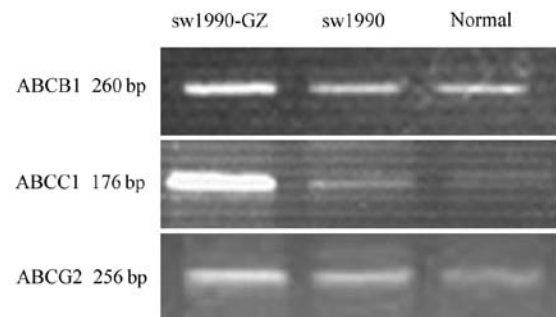


Figure 6. *ABCB1*, *ABCC1* and *ABCG2* promoter bisulfite PCR amplification in sw1990-GZ gemcitabine-resistant, sw1990 and normal pancreatic tissue.

ATP-binding cassette gene mRNA expression is increased in pancreatic cancer compared to adjacent tissue. Significantly higher *ABCB1* mRNA expression was observed in tumors compared to adjacent tissues in 73% of patients (11/15, $p < 0.05$; Fig. 3). Twelve out of 15 (80%) patients had significantly higher

levels of *ABCC1* mRNA in tumors than the adjacent tissues ($p < 0.05$; Fig. 4), and 67% of samples (10/15) showed increased levels of *ABCG2* mRNA expression in pancreatic cancer tissue samples compared to adjacent tissues ($p < 0.05$, Fig. 5). These

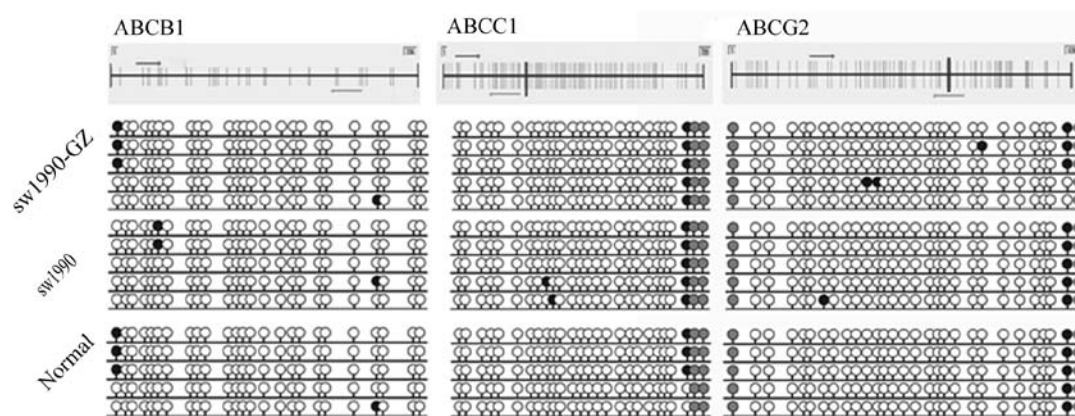


Figure 7. *ABCB1*, *ABCC1* and *ABCG2* promoter methylation status in normal pancreatic tissues, sw1990 and sw1990-GZ gemcitabine-resistant cell lines determined by bisulfite sequencing of five cloned PCR products. Arrows represent promoter bisulfite genomic sequencing primers, open circles indicate unmethylated CpG sites, filled circles indicate methylated CpG sites.

results suggest that the expression of ATP-binding cassette genes is related to tumorigenesis in pancreatic cancer.

ABCB1, *ABCC1* and *ABCG2* ATP-binding cassette gene promoters are hypomethylated in cell lines. Bisulfite analysis was performed on the *ABCB1*, *ABCC1* and *ABCG2*. The bisulfite conversion PCR products (ATP-binding cassette gene promoters) are shown in Fig. 6. The methylation status of the promoters are shown in Fig. 7. The *ABCB1*, *ABCC1* and *ABCG2* promoters were hypomethylated with no significant difference observed between SW1990/GZ, SW1990 and the normal pancreatic tissue. These results indicate that the expression of ATP-binding cassette genes in human pancreatic cancer cells was not correlated with promoter demethylation.

Discussion

In this study, we compared ABC transporter gene expression in pancreatic cancer and normal pancreas to determine the mechanisms regulating ABC transporter expression. Increased expression of *ABCB1*, *ABCC1* and *ABCG2* in pancreatic cancer compared to adjacent tissues, and also in SW1990 cells compared to normal pancreatic tissues, suggested that ATP-binding cassette genes may play an important role in pancreatic cancer oncogenesis. Additionally, the increased expression of ATP-binding cassette genes in gemcitabine-resistant cells demonstrated that these genes may contribute to pancreatic cancer drug resistance.

The ATP-binding cassette genes have been shown to be overexpressed in a subpopulation of pancreatic cancer cells with stem cell characteristics, which play an important role in oncogenesis, development and drug resistance (16-18). *ABCB1*, *ABCC1* and *ABCG2* expression were altered in a similar manner, which indicated that ATP-binding cassette genes play a joint role in oncogenesis and drug resistance.

No difference in promoter methylation status was observed in SW1990 and SW1990/GZ cells, which indicated that chemotherapy agents do not induce ATP-binding cassette gene methylation in pancreatic cancer. As the methylation status of the three ATP-binding cassette genes was not significantly different in normal pancreatic tissues, SW1990 and SW1990/

GZ cells, promoter methylation does not appear to regulate the expression of ATP-binding cassette genes. Based on the literature and our experimental results, we suggest that alternative mechanisms, such as histone modifications and microRNAs-mediated regulation of gene expression, may regulate ATP-binding cassette genes expression in pancreatic cancer tumorigenesis and drug resistance.

The major epigenetic mechanisms include DNA methylation, histone modifications and RNA-mediated gene silencing. Histone acetylation is important for the maintenance of unmethylated promoter regions and to protect against DNA methylation (19). Promoter hypomethylation is associated with histone acetylation and demethylation (20). Furthermore, histone modifications are a prerequisite for DNA methylation (21). Treatment with depsipeptide or trichostatin A, which are specific histone deacetylase inhibitors, alters DNA methylation status (20). We suggest that histone acetylation of ATP-binding cassette gene promoters may play a more important role in their activation of gene expression in pancreatic cancer than promoter methylation.

The other possible mechanism is regulation of gene expression by microRNAs, small 19-25 nucleotide single-stranded RNAs which regulate gene expression and play an important role in many cellular processes, including development, differentiation, cell proliferation and apoptosis (22) and also drug resistance (23). MiR-21 and miR-27a are overexpressed in pancreatic cancer (24). MiR-21 upregulates *MDR1/ABCB1* leading to chemoresistance in breast cancer (25), and anti-miR-21 enhances pancreatic cancer cell chemosensitivity (26,27). MiR-27a is overexpressed in MDR cancer cells, and the inhibition of miR-27a reduces *P-gp* and *MDR1* mRNA expression in ovarian cancer and esophageal carcinoma (28,29). Three microRNAs (hsa-miR-328, 519c and 520h) target *ABCG2*, and their expression negatively correlates with *BRCP/ABCG2* expression in both breast cancer and glioblastoma (30,31). Hsa-miR-520h downregulates *ABCG2* in pancreatic cancer cells, inhibiting migration, invasion and side population development (32). MiR-326 and miR-134 modulate chemoresistance via *MRP1/ABCC1* expression (33,34). On the basis of this evidence, we hypothesize that the altered expression of miR-21/miR-27a, hsa-miR-328/-519c/-520h and miR-326/miR-134, which regulate the expression of *ABCB1*/

MDR1, *BCRP/ABCG2*, *MRP/ABCC1*, respectively, may lead to pancreatic cancer tumorigenesis and drug resistance.

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