The ABCC4 gene is a promising target for pancreatic cancer therapy

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1. Introduction

Pancreatic cancer is a malignant neoplasm of the pancreas that usually has a poor prognosis. The investigation of targets that effectively inhibit pancreatic cancer cell proliferation should provide a fundamental basis for the clinical application of gene therapy. Here, high expression levels of ABCC4 protein in thirty-six pancreatic cancer specimens were quantified using an immunohistochemical assay, and the potential of ABCC4 as a therapeutic target for pancreatic cancer was investigated. Inhibition of ABCC4 expression at the mRNA and protein levels was achieved in Panc-1 and BxPC-3 pancreatic cancer cells infected with a lentivirus expressing an ABCC4 short hairpin RNA (shRNA). The downregulation of ABCC4 expression in Panc-1 and BxPC-3 cells significantly inhibited their proliferation and colony formation in vitro, compared to cells infected with mock control (p<0.05). Moreover, the specific downregulation of ABCC4 led to the accumulation of cells at the G1 phase of the cell cycle. Our findings reveal that the ABCC4 gene promotes pancreatic cancer cell growth and represents a promising target for gene therapy in pancreatic cancer.

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2. Materials and methods

2.1. Cell lines and tissue samples

The Panc-1 and BxPC-3 human pancreatic cancer cell lines were purchased from ATCC (Manassas, VA, USA). Panc-1 and BxPC-3 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) or RPMI 1640, respectively, both of which contained 10% fetal bovine serum (FBS) and 100 units/ml of penicillin and streptomycin. Cells were cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

A total of 36 patients with pancreatic cancer were selected from our outpatient and inpatient services at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. The development and pathogenic progression of pancreatic cancer were diagnosed and classified by histopathological examination according to the WHO criteria. Informed consent was obtained from individual subjects. The experimental protocols were approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine.

2.2. Immunohistochemistry

Paraffin-embedded tissue sections from pancreatic cancer specimens were stained with a goat polyclonal anti-ABCC4 antibody (Abcam, Cambridge, MA, USA; ab77184) at 4 °C overnight. After being washed, the sections were incubated with a polyclonal secondary antibody to goat IgG (Abcam, Cambridge, MA, USA; ab6880) at room temperature for 1 h and visualized using peroxidase-conjugated streptavidin (Histostain-Bulk-SP Kit, Zymed, San Francisco, CA, USA) and diaminobenzidine, followed by counterstaining with Mayer’s hematoxylin.

The percentage of positive cells in a total of 10 fields from each slide was examined and graded as 0 (0–5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), or 4 (over 75%). The intensity of the immunohistochemical staining was graded as follows: 0 (no staining), 1 (bright yellow), 2 (orange), or 3 (brown). The staining was quantified according to the sum of the positive cells and the intensity of the immunohistochemical staining as 0, 1–2, 3–4, or 5–7, which represent negative (−), weakly positive (+), positive (++) or hadero-positive (+++), respectively.

2.3. Lentiviral construction and infection

To generate a lentivirus containing shRNA against the ABCC4 gene, the RNAi design strategy was based on the conserved cDNA fragments within the coding region of the human ABCC4 gene. The shRNA sequences were 5′-GACATCTTTAATAACAAAGAGA-3′ for human ABCC4 and 5′- TTCTCCGAAGTTGTCAGT-3′ for the control. The sequences were cloned into the Age I and EcoR I sites of the pGCSIL-GFP lentiviral plasmids to generate an ABCC4 shRNA-expressing lentivirus (ABCC4-shRNA-lentivirus) or a control shRNA-expressing lentivirus. Panc-1 and BxPC-3 cells were plated in 6-well plates (5×10⁴ cells/well), grown to 40% confluence, and treated with titered viral supernatant at an m.o.i (multiplicity of infection) of 10.

2.4. Quantitative real-time RT-PCR

After five days, the total RNA was extracted from Panc-1 and BxPC-3 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The expression of ABCC4 mRNA was detected using the standard SYBR Green RT-PCR Kit (Takara, Otsu, Japan) according to the manufacturer’s instructions. The cDNA was used as the template in triplicate reactions containing the IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) that were subjected to an initial denaturation at 95 °C for 15 s, followed by 45 sequential cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 30 s. The specific primer sequences for ABCC4 were 5′- GTTCTTCTGTGGCGCTAATCC-3′ and 5′- GGCCTCTGTGGCATATCTC-3′. β-Actin was used as an internal control, and the primer sequences were 5′-GGCGCAGACCCACATGTACCT-3′ and 5′- AGGGGGCCCGACTGTCATACT-3′. The relative expression of ABCC4 normalized to the control was calculated using the 2−ΔΔCt method.

2.5. Western blotting

After seven days of infection, the cells were lysed in 0.2 ml lysis buffer (0.1% SDS, 1% NP-40, 50 mM HEPS, pH 7.4, 2 mM EDTA, 100 mM NaCl, 5 mM sodium orthovanadate, 40 μM p-nitrophenyl phosphate, and 1% protease inhibitor mixture set I). The supernatant was collected by centrifugation and denatured. Proteins were separated on a 10% SDS-PAGE gel, electroblotted onto a polyvinylidene difluoride membrane, and blocked for 1.5 h at room temperature in 5% BSA, followed by overnight incubation at 4 °C with goat anti-ABCC4 (Abcam, Cambridge, MA, USA; ab77184) or anti-GAPDH (Santa Cruz, Biototechnology, Santa Cruz, CA, USA; sc-32233) antibodies. Membranes were rinsed and incubated for 1 h with a polyclonal secondary antibody to goat IgG (Abcam, Cambridge, MA, USA; ab6880) or goat anti-mouse IgG (Santa Cruz Biototechnology, Santa Cruz, CA, USA; sc-2005). Chemiluminescent detection was performed using an ECL kit (Pierce Chemical Co., Rockford, IL, USA).

2.6. MTT assay

Cell viability and proliferation were evaluated using a modified MTT assay after five days of lentiviral infection. Panc-1 and BxPC-3 cells in exponential growth were plated in 96-well plates at a final concentration of 4×10³ cells/well. The cell viability was assessed on days 1, 2, 3, 4, 5 and 6 after seeding. Briefly, 20 μl of MTT (5 mg/ml) was added to each well. After an additional 4 h of incubation, 100 μl of DMSO was added, and the optical density (OD) of each well was measured at 570 nm using an ELISA reader.

2.7. Colony formation assay

After five days of lentiviral infection, the effect of ABCC4 silencing on the colony formation of Panc-1 and BxPC-3 cells was analyzed by performing colony formation assays. Approximately 300 cells were plated in 6-well plates and cultured in medium containing 10% PBS for two weeks. The colonies were fixed in 4% paraformaldehyde, stained with Giemsa, and then washed twice with ddH₂O. Visible colonies with more than 50 cells were manually counted under a fluorescent microscope.

2.8. Analysis of cell cycle by flow cytometry

After five days of lentiviral infection, the impact of ABCC4 silencing on Panc-1 and BxPC-3 cells was examined by flow cytometry analysis. Cells were washed twice with ice-cold PBS and fixed with 70% ethanol overnight at 4 °C. The cells were then digested with 50 μg/ml RNase A in 100 μl of PBS and stained with 20 μg/ml propidium iodide (PI). Cell cycle was analyzed on a FACSCalibur (BD Biosciences, San Jose, CA, USA).

2.9. Statistical analysis

Data were expressed as means ± SEM. The difference between two groups was analyzed by the Student’s t-test and Chi-square test using SPSS software (Release 11.0, SPSS Inc.). A value of p < 0.05 was considered statistically significant.
3. Results

3.1. Higher levels of ABCC4 expression in pancreatic cancer

To determine the ABCC4 expression in human pancreatic cancer tissues, the levels of ABCC4 were determined by immunohistochemical analysis. The overall distribution of the ABCC4 protein in the different histological tissue types is summarized in Table 1. Strong staining with antibodies against human ABCC4 was observed in the pancreatic tumor tissues, compared to the paired tissues adjacent to the pancreatic tumor area (Fig. 1). When the different histological types were classified by tumor diameter, tumor location within the pancreas, tumor differentiation, perineural invasion, and peripancreatic invasion, there was a clear correlation between the expression of ABCC4 and the differentiation of pancreatic cancer cells (Table 2). Therefore, these data clearly indicate that high levels of ABCC4 expressed selectively in human pancreatic tumor tissues may contribute to the development of high-risk pancreatic cancer.

3.2. Downregulation of ABCC4 expression by lentivirus-mediated shRNA

To determine the impact of ABCC4 expression on the growth and proliferation of human pancreatic cancer cells, we generated lentiviruses expressing shRNA against ABCC4 and infected Panc-1 and BxPC-3 cells. More than 90% of cells showed GFP expression that serves a marker for successful infection after 5 days of infection (Fig. 2A). The mRNA and protein levels of ABCC4 were downregulated in cells infected with the ABCC4-shRNA-lentivirus, compared to cells infected with the control lentivirus (p < 0.05, Fig. 2B, C). The successful establishment of an ABCC4 gene silencing lentivirus provided a useful tool for investigating the function of ABCC4 in the growth of Panc-1 and BxPC-3 cells.

3.3. Effect of downregulated ABCC4 expression on pancreatic cancer cell growth

To investigate the possible role of ABCC4 in the growth of Panc-1 and BxPC-3 cells, MTT assays and colony formation assays were performed. After 5 days of lentiviral infection, the cell viability was significantly lower in cells infected with the ABCC4-shRNA-lentivirus compared to cells infected with the control lentivirus following a 6-day incubation (Fig. 3A). The colony number was decreased by 93.9% compared to the control (p < 0.05, Fig. 3B). For BxPC-3 cells, it was hard to identify colonies containing more than 50 cells even after two weeks (data not shown). Therefore, the low viability and the low colony number for cells treated with ABCC4 RNAi demonstrate that downregulation of ABCC4 expression inhibited the growth of human pancreatic cancer cells in vitro.

3.4. Effect of downregulated ABCC4 expression on cell cycle

To explore the potential mechanism underlying the action of ABCC4 on the growth of Panc-1 and BxPC-3 cells, the cell cycle of cells that had been infected with a lentivirus for 5 days was characterized using a flow cytometry assay. As shown in Fig. 4, a significant percentage of cells infected with ABCC4 RNAi were in the G1 phase (55.0%), with a lower frequency of progression to S phase (22.2%) compared with cells infected with the mock control. These data indicate that downregulation of ABCC4 expression led to cell cycle arrest at the G1 phase, which may contribute to the inhibition of growth of Panc-1 and BxPC-3 cells in vitro.

4. Discussion

The ABC transporters, also known as multidrug transporter proteins, are best known for their contributions to cytotoxic drug resistance and

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**Table 1** Expression pattern of ABCC4 in pancreatic cancer tissues and adjacent tissues.

<table>
<thead>
<tr>
<th>Type of tissues</th>
<th>Number of cases</th>
<th>Negative (−)</th>
<th>Weakly positive (+)</th>
<th>Positive (++)</th>
<th>Hadro-positive (+++)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic cancer tissues</td>
<td>36</td>
<td>8</td>
<td>10</td>
<td>12</td>
<td>6</td>
<td>0.014&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adjacent tissues</td>
<td>36</td>
<td>18</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Means χ² test.

**Table 2** Associations between ABCC4 expression and clinicopathological characteristics of pancreatic cancer.

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>ABCC4 expression</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor diameter (cm)</td>
<td>3.10</td>
<td>0.175&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&lt;5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>&gt;5</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Tumor location of within pancreas</td>
<td>0.858&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Tail</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td>0.039&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Middle</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Perineural invasion</td>
<td>0.361&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>No</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>Peripancreatic invasion</td>
<td>0.654&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>No</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

<sup>a</sup> Means χ² test.

Fig. 1. Characterization of ABCC4 expression in human pancreatic cancer tissues and paired adjacent tissues by immunohistochemical analysis. Representative images from thirty-six human pancreatic cancer specimens are shown. The left and middle panels are at 100× magnification; the right panel is at 200× magnification.
Fig. 2. Knockdown efficiency of lentivirus-mediated shRNA against ABCC4. (A) Micrograph of Panc-1 and BxPC-3 cells infected with ABCC4-shRNA-lentivirus for five days in bright and fluorescent fields (100× magnification). (B) Analysis of ABCC4 mRNA transcripts by real-time RT-PCR. Data are expressed as the fold changes of ABCC4 mRNA transcripts in Panc-1 and BxPC-3 cells infected with control lentivirus or ABCC4-shRNA-lentivirus. Data shown are the mean results±SEM from three independent experiments performed in triplicate. ** indicates p<0.01. (C) Western blotting results showing the expression levels of ABCC4 protein in Panc-1 and BxPC-3 cells infected with control lentivirus or ABCC4-shRNA-lentivirus.

Fig. 3. Inhibition of human pancreatic cancer cell proliferation and growth after ABCC4 downregulation. The proliferation of Panc-1 and BxPC-3 cells following infection with control lentivirus or ABCC4-shRNA-lentivirus was assessed by MTT and colony formation assays. (A) The viability of Panc-1 and BxPC-3 cells following infection with ABCC4-shRNA-lentivirus was significantly decreased. (B) Colony formation of Panc-1 cells in vitro. Cells infected with control lentivirus or ABCC4-shRNA-lentivirus were cultured in 6-well plates for 2 weeks and stained with Giemsa. The Panc-1 cells infected with ABCC4 RNAi formed fewer colonies than cells infected with control lentivirus. Data are the number of colonies formed expressed as mean±SEM from three separate experiments. ** indicates p<0.01.
for the failure of multiple chemotherapeutic agents because of their ability to increase the efflux of chemotherapeutic agents, which results in the reduction of intracellular drug levels and consequent drug insensitivity (Fletcher et al., 2010). The high expression of ABC transporters in cancer stem cells has been found to protect them from chemotherapeutic agents (Dean et al., 2005). However, a considerable body of evidence also indicates that the significance of ABC transporters in cancer extends beyond drug transport to fundamental functions in tumor biology. Several observations from clinical studies showed that ABC transporters were linked to the progression of malignant cancers that exhibit more aggressive behaviors (Luo et al., 2007). Unlike other family members, ABCC4 has only been identified in a few cancers to date. Cell line data have shown low ABCC4 expression in lung, kidney, bladder and colon cancers (Kool et al., 1997). ABCC4 expression in pancreatic cancer and childhood acute lymphocytic leukemia was found to be unrelated to treatment outcomes (Konig et al., 2005; Steinbach et al., 2003). Elevated ABCC4/MRP4 expression has been found in malignant prostate cancer and has been significantly associated with poor clinical outcomes of neuroblastoma (Ho et al., 2008; Norris et al., 2005). The upregulation of ABCC4 in cancer cells suggests the possibility that ABCC4 could play a functional role in cancer development.

Pancreatic cancer is a malignant neoplasm of the pancreas, and the low success rate of routine therapeutic methods justifies efforts to develop new approaches. In previous studies, the quantification of ABCC4 mRNA levels demonstrated that ABCC4 mRNA levels did not change significantly between normal pancreatic tissue and pancreatic carcinoma specimens with different tumor grades (Konig et al., 2005). Interestingly, the immunohistochemical analysis presented herein demonstrates that ABCC4 protein expression was significantly higher in pancreatic cancer tissue compared to normal pancreatic tissue. Although the inducible expression of ABCC4 during dedifferentiation is only demonstrated in a small group of patients, ABCC4 expression may be an important factor for prognosis.

Moreover, we found that ABCC4 promoted human pancreatic cancer cell proliferation and colony formation in vitro by modulating cell cycle. Our study will contribute to delineating the precise role of ABCC4 in the tumorigenesis of pancreatic cancer. However, elucidation of the underlying mechanism responsible for the inhibition of proliferation and the cell cycle arrest of ABCC4 silenced cells warrants further investigation.

More evidence is clearly required to determine the importance of ABCC4 in pancreatic cancer.

Cancer poses a tremendous therapeutic challenge worldwide, highlighting the urgent need for developing novel therapeutics. Gene therapy, a form of molecular medicine designed to introduce genetic material with therapeutic intent into target cells, has become a promising treatment modality for malignant tumors (Luo et al., 2007). Gene silencing results in the highly specific and effective downregulation of target gene expression, and its therapeutic effects have been evaluated in a wide variety of diseases, including cancer (Martinez et al., 2002; Rubinson et al., 2003; Brummelkamp et al., 2002; Crnkovic-Mertens et al., 2003). In our study, a lentiviral vector was used for ABCC4 gene knockdown in two human pancreatic cancer cell lines, Panc-1 and BxPC-3. Lentiviruses can safely be introduced into human cells, as no adverse events have been reported (Sinn et al., 2002; Crnkovic-Mertens et al., 2003). Analysis of Panc-1 and BxPC-3 cell cycle. Cells infected with control lentivirus or ABCC4-shRNA-lentivirus were fixed with 70% ethanol, stained with PI, and analyzed by FACS. The relative frequency of different cell cycle phases was expressed as the mean±SEM of three independent experiments. ** indicates p<0.01.

Acknowledgments

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Fig. 4. Analysis of Panc-1 and BxPC-3 cell cycle. Cells infected with control lentivirus or ABCC4-shRNA-lentivirus were fixed with 70% ethanol, stained with PI, and analyzed by FACS.


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