Original Article

Down-regulation of microRNA-20a in gemcitabine-resistant pancreatic cancer in in vitro microRNA integrative analysis

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Abstract: Objectives: The efficacy of gemcitabine-based chemotherapy remains limited due to drug resistance in advanced pancreatic cancer. This study aims to identify microRNAs (miRNA) that are associated with such resistance. This may provide important clues for further exploring the underlying mechanisms of gemcitabine resistance in pancreatic cancer. Methods: Gemcitabine-resistant strains were established by exploring BxPC-3 pancreatic cancer cell lines in intermittently increasing concentrations of gemcitabine. MiRNA that were differentially expressed in gemcitabine-resistant BxPC-3 pancreatic cancer cells and normal BxPC-3 cell control were identified by MiRNA microarray. Real-time PCR and Western Bolt were performed to validate these results. Results: Gemcitabine-resistant cell lines of BxPC-3 were successfully established. MiRNA microarray identified 34 miRNAs that were aberrantly expressed in gemcitabine-resistant BxPC-3 pancreatic cancer cells, among which 11 miRNAs were upregulated and 23 miRNAs were down-regulated. Network-control analysis showed that the highest degree hub in miRNAs was hsa-miR-20a (degree 8) and the most connected hub of its target gene was ABCG2 (degree 4). Results from Real-time PCR showed that the expression of hsa-miR-20a was reduced and ABCG2 was increased in gemcitabine-resistant BxPC-3 cells. Results from Western Blot analysis also confirmed that the ABCG2 protein was up-regulated in gemcitabine-resistant cells, comparing with the control. Conclusion: Down-regulation Has-mir-20a and up-regulation of downstream ABCG2 may play important roles in the formation of gemcitabine-resistance in pancreatic cancer.

Keywords: Gemcitabine, microRNA, hsa-miR-20a, ABCG2

Introduction

Pancreatic cancer is a relatively uncommon type of cancer worldwide. It is estimated to be the 12nd most common cancer in men and 11st in women [1]. However, it is one of the most deadly type of cancer. It has been reported that 71% of pancreatic cancer patients would die within the first year of diagnosis and 93% would die within five years of diagnosis [2]. The average life expectancy after diagnosis in patients with metastatic disease ranges from three to six months. The low survival rate is mainly due to a lack of reliable method to detect the tumor in its early stage, by which time surgical removal is still possible. However, less than 25% patients can survive more than 5 years even if the visible tumor is totally removed [3]. Compared to surgery, chemotherapy plays a more important role in pancreatic cancer therapy [4]. Combination therapy with gemcitabine is currently the leading therapeutic approach in treating pancreatic cancer. However, the treatment efficacy and patient survival rate remain limited due to the high drug resistance in the carcinoma. Identifying ways to minimize or prevent the drug resistance may increase the anticancer efficacy of gemcitabine and prolong the life span of patients, and has remained a hot topic for research in the recent years.

MicroRNAs (miRNAs) are a group of small non-coding and single-stranded RNAs with the length of 19~25 nt. MiRNAs can act as a negative regulation factor in gene expression by binding to specific sites within the 3'-UTR region, resulting in the degradation of target mRNAs or suppression of protein translation. Currently, plenty of studies indicated that miR-
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NAs can function either as oncogenes or tumor suppressors during tumorigenesis [5, 6], and they play vital roles in cancer cell metabolism, including cell proliferation, cell cycle regulation, invasion as well as metastasis [7, 9]. MiRNAs are also found to be involved in the development and progression of pancreatic cancer [10-12]. Circulating miRNAs can potentially be applied as biomarkers for early diagnosis of pancreatic cancer [13-16]. Moreover, miRNAs are found to be associated with cancer drug resistance [17-19].

Their roles in gemcitabine resistance in pancreatic cancer have been reported in previous studies [20-22]. However, the specific miRNAs associated with gemcitabine resistance remains uncertain. MiRNA microarray is a technology that enables large scale analysis of miRNA profiles. This study aims to identify the specific miRNAs that correlate with gemcitabine resistance in pancreatic cancer via a MiRNA microarray system. These findings may provide important clues for early diagnosis and targeted therapy in pancreatic cancer.

Materials and methods

Cell line and cell culture

The human pancreatic cell line (BxPC-3) was purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. BxPC-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco) in a humidified incubator at 37°C with 5% CO₂. Logarithmic (Log)-phase cells were selected and transfected in 24-well plates at 85% confluence using Lipofectamine 2000 (Invitrogen, California, USA). The cells were dissociated with 0.25% trypsin-EDTA.

The efficacy of gemcitabine on survival of pancreatic cancer cell lines via cell proliferation assay

The proliferation of BxPC-3 cells under different doses of Gemcitabine (Gene Operation Company, USA, Michigan) was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The BxPC-3 cells were seeded in 96-well plates at a density of 5×10³ to 1×10⁴ cells per well and incubated under the same condition of 37°C for 3 to 5 days. The cells were exposed to a gradient concentration of Gemcitabine 0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 µg/ml at 37°C for 48 h. Afterwards, 20 µl of MTT reagent was added into each well. Four hours later, the culture medium was discarded and 150 µl of dimethyl sulfoxide (DMSO, Sigma, USA) was added. The half maximal inhibitory concentration (IC₅₀) was defined as the concentration at which gemcitabine induced 50% inhibition on BxPC-3 cell survival. C₅₀ was estimated from the cell survival curve with the formula: LgIC₅₀ = Xm-1 (P-(3-Pm-Pn)/4), where Xm = Lg (maximum concentration), 1 = Lg (maximum concentration/adjacent concentration), P = positive inhibition rate sum, Pm = maximum inhibition rate, Pn = minimum inhibitory rate. The absorbance was measured at a wavelength of 490 nm with a spectrophotometer. All experiments were performed in quadruplicate.

Establishment of the gemcitabine-resistance BxPC-3 (BxPC-3/Gem) cell lines

BxPC-3/Gem cells were established by exposing the cells in 2 ng/ml, 10 ng/ml, 20 ng/ml and 30 ng/ml gemcitabine. The cells in Logarithmic growth phase cells were transferred to culture wells containing gemcitabine solution mentioned above, which were increased through four concentration gradients over a 25-week period. During the selective culture, dead cells were removed by changing culture medium.

MiRNAs microarrays

Total RNAs were extracted using Trizol reagent (Takara Biotechnology, Dalian, China). Microarray analysis was performed according to the manufacturer’s protocol. 5 µg of total RNA from BxPC-3 and BxPC-3/Gem cells were used for hybridization on miRNA microarray chips. The microarrays were hybridized in hybridization solution consisting of 21.5 µl RNA sample labelled with biotin, 50 µl 2× Hybridization, 15 µl 27.5% Formamide, 10 µl DMSO, 5 µl 20× Eukaryotic Hybridization controls and 1.7 µl Control Oligonucleotide B2. They were incubated at 99°C and 45°C for 5 minutes each, and were then kept at 4°C. 100 µl hybridization solution was added to the microarray chip after the solution was centrifuged for 5 seconds. The microarrays were hybridized at 48°C for 16 hours. Afterwards, the solution was removed and 100 µl of array holding buffer was added. After thorough washing, the microarrays were scanned by a Scanner with the laser at 633 nm.
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Figure 1. The growth curve of BxPC-3 cells treated with a gradient concentration of gemcitabine and the establishment of gemcitabine-resistance BxPC-3 (BxPC-3/Gem) cell lines. A. The growth curve of BxPC-3 cells treated with a gradient concentration of gemcitabine; B-I. The process of establishing BxPC-3/Gem cell strain; B. The negative control BxPC-3 cells; C. The survival of BxPC-3 cells treated with gemcitabine at a concentration 2 ng/ml; D, E. The survival of BxPC-3 cells treated with gemcitabine at a concentration 10 ng/ml (Pre-treatment (left) and post-treatment (right)); F, G. The survival of BxPC-3 cells treated with gemcitabine at a concentration 20 ng/ml (Pre-treatment (left) and post-treatment (right)); H, I. The survival of BxPC-3 cells treated with gemcitabine at a concentration 30 ng/ml (Pre-treatment (left) and post-treatment (right)).

Total RNAs were extracted using Trizol reagent (Takara Biotechnology, Dalian, China) as mentioned above. The cDNA was reverse-transcribed with PrimeScript RT Master Mix kit (Takara Biotechnology, Dalian, China). The primers for beta-actin, ABCG2, miRNA-20a and U6 were as follows: for beta-actin, 5'-CCA-TCTATGAGGTCTACGC-3' (forward) and 5'-TTTATGTCACTGCACTGATTT-3' (reverse); for ABCG2, 5'-CAGGTGGAGGCAAATCTTCGT-3' (forward) and 5'-ACCCTCTTATGACGTTTTT-3' (reverse); for miRNA-20a, 5'-GGCCTAAAGTGCTTATAGTG-3' (forward) and 5'-GTGCAGGGTGCCAGGT-3' (reverse); and for U6, 5'-CTGGTATCGCGAGCTCAGACT-3' (forward) and 5'-AACGCTTCAGAATTTG-3' (reverse). qRT-PCR was conducted in 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 60 seconds. The relative expressions of miRNA-20a, ABCG2 were normalized to beta-actin and U6 expression levels and were calculated by the comparative 2^−ΔΔCT method. qRT-PCR analysis was performed in triplicate.

Quantitative real-time PCR (qRT-PCR) assay

Western blot assay

Total protein extracts were separated by 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked and incubated with the primary antibody specific for ABCG2 (1:1000, Abcam, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, Abcam, USA) at 4°C overnight. The blots were visualized with the SuperSignal enhanced chemiluminescence kit (Pierce, Rockford, IL). The Quantity One software version 4.6.2 was used to quantify the band intensities. The experiment was repeated for three times.

Statistical analysis

All statistical analysis were performed using the SPSS 17.0 software (SPSS, Inc, Chicago, IL, USA). Data were expressed as the mean ± standard error of the mean (SEM). Student’s t test
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The IC\(_{50}\) was calculated to be 13.8 ng/ml via the formula mentioned in the methods section. Gemcitabine-resistance BxPC-3 cells were established via exposing the cells to an intermittently increasing concentrations of gemcitabine (2, 10, 20 and 30 ng/ml) (Figure 1B-I).

**Results**

The IC\(_{50}\) of gemcitabine in BxPC-3 cells and the establishment of the gemcitabine-resistance BxPC-3 cell lines

The effect of gemcitabine on the proliferation of BxPC-3 cells was shown in Figure 1A. With increasing gemcitabine concentration, the proliferation of BxPC-3 cells was gradually decreased. The IC\(_{50}\) was calculated to be 13.8 ng/ml via the formula mentioned in the methods section. Gemcitabine-resistance BxPC-3 cells were established via exposing the cells to an intermittently increasing concentrations of gemcitabine (2, 10, 20 and 30 ng/ml) (Figure 1B-I).

Gene ontology enrichment analysis (GOEA) and Kyoto encyclopedia of genes and genomes (KEGG) analysis of miRNA expression profiles in BxPC-3 and BxPC-3/Gem cells

As shown in Figure 2A and 2B, the miRNAs that were downregulated in BxPC-3/Gem cells were applied to compare results for the two groups. \(P\)-value of less than 0.05 was considered statistically significant.

![Figure 2](image-url)
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Table 1. The list of up-regulated (in yellow) and down-regulated (in green) miRNAs in BxPC-3/Gem cells compared with normal BxPC-3 cells

<table>
<thead>
<tr>
<th>Name</th>
<th>Normalized Intensity</th>
<th>CV</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BX-pc3</td>
<td>BX-pc3/Gem</td>
<td>BX-pc3</td>
</tr>
<tr>
<td>hsa-miR-3131</td>
<td>2649.39</td>
<td>770.68</td>
<td>0.03</td>
</tr>
<tr>
<td>hsa-miR-4521</td>
<td>695.82</td>
<td>246.32</td>
<td>0.05</td>
</tr>
<tr>
<td>hsa-miR-4430</td>
<td>850.21</td>
<td>301.62</td>
<td>0.03</td>
</tr>
<tr>
<td>hsa-miR-4299</td>
<td>488.49</td>
<td>188.95</td>
<td>0.05</td>
</tr>
<tr>
<td>hsa-miR-4475</td>
<td>234.97</td>
<td>90.89</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-1247-3p</td>
<td>194.95</td>
<td>77.86</td>
<td>0.08</td>
</tr>
<tr>
<td>hsa-miR-4689</td>
<td>1159.76</td>
<td>471.55</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-miR-4271</td>
<td>1581.65</td>
<td>658.77</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-miR-3907</td>
<td>713.49</td>
<td>300.98</td>
<td>0.05</td>
</tr>
<tr>
<td>hsa-miR-20a-5p</td>
<td>1561.65</td>
<td>661.88</td>
<td>0.04</td>
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<tr>
<td>hsa-miR-373-5p</td>
<td>359.04</td>
<td>154.04</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-1275</td>
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<tr>
<td>hsa-miR-3529-3p</td>
<td>187.72</td>
<td>81.55</td>
<td>0.08</td>
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<tr>
<td>hsa-miR-3974</td>
<td>160.62</td>
<td>74.19</td>
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<tr>
<td>hsa-miR-221</td>
<td>2190.04</td>
<td>1031.89</td>
<td>0.09</td>
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<tr>
<td>hsa-miR-20b</td>
<td>1157.76</td>
<td>555.16</td>
<td>0.04</td>
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<tr>
<td>hsa-miR-3654</td>
<td>150.67</td>
<td>72.47</td>
<td>0.06</td>
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<tr>
<td>hsa-miR-513b</td>
<td>276.32</td>
<td>133.93</td>
<td>0.05</td>
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<tr>
<td>hsa-miR-921</td>
<td>269.43</td>
<td>130.64</td>
<td>0.09</td>
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<tr>
<td>hsa-miR-4530</td>
<td>2088.41</td>
<td>1033.90</td>
<td>0.03</td>
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<tr>
<td>hsa-let-7b</td>
<td>2686.36</td>
<td>5742.20</td>
<td>0.12</td>
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<tr>
<td>hsa-miR-1248</td>
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<td>662.38</td>
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<tr>
<td>hsa-let-7f</td>
<td>4385.33</td>
<td>9140.53</td>
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<tr>
<td>hsa-let-7c</td>
<td>5236.22</td>
<td>11282.73</td>
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<tr>
<td>hsa-miR-4419b</td>
<td>2391.12</td>
<td>5673.41</td>
<td>0.06</td>
</tr>
<tr>
<td>hsa-miR-4498</td>
<td>599.47</td>
<td>1422.67</td>
<td>0.05</td>
</tr>
<tr>
<td>hsa-miR-668</td>
<td>234.61</td>
<td>574.65</td>
<td>0.09</td>
</tr>
<tr>
<td>hsa-miR-5096</td>
<td>162.98</td>
<td>411.00</td>
<td>0.02</td>
</tr>
<tr>
<td>hsa-miR-363</td>
<td>6319.10</td>
<td>17676.12</td>
<td>0.03</td>
</tr>
<tr>
<td>hsa-miR-3919</td>
<td>97.13</td>
<td>374.38</td>
<td>0.02</td>
</tr>
<tr>
<td>hsa-miR-4638-5p</td>
<td>1475.59</td>
<td>6718.44</td>
<td>0.15</td>
</tr>
</tbody>
</table>

The results of KEGG pathways enrichment analysis were shown in Figure 2C and 2D. The three most upregulated biological pathways in gemcitabine-resistance BxPC-3 cells were Wnt signaling pathway, Calcium signaling pathway while the down-regulated signaling pathways mainly includes Erb signaling pathway, Wnt signaling pathways and MAPK signaling pathway.

For miRNA expression profile analysis, microarray data revealed that the expression of a total of 34 miRNAs in gemcitabine-resistance BxPC-3 cells were significantly altered (≥2 folds), 23 of which was upregulated (highlighted in yellow) and 11 of which was downregulated (highlighted in green) (Table 1). The miRNA-target gene network showing the correlations between differently expressed miRNAs and their target genes were shown in Figure 2E and 2F. From the network, we found that the highest degree hub in miRNAs was hsa-miR-20a (degree 8) and the most connected hub of its target gene was ABCG2 (degree 4), indicating that miRNA-20a and ABCG2 might be the key factors involved in gemcitabine-resistance.

Differentially expressed miRNA-20a and its target gene ABCG2 in normal BxPC-3 and BxPC-3/Gem cells

The expression level of miRNA-20a and ABCG2 in BxPC-3/Gem cells relative to controls was shown in Figure 3. miRNA-20a was significantly down-regulated (Figure 3A) whereas ABCG2 was significantly up-regulated (Figure 3B) in BxPC-3/Gem cells compared with normal BxPC-

mainly involved in the process of cell differentiation, adhesion and interaction, while the upregulated miRNAs in those cells mainly play roles in protein amino acid phosphorylation, chromatin modification as well as cell adhesion. Since the drug resistance induced by gemcitabine in BxPC-3 cells mainly affected the proliferation of BxPC-3, we have focused on the miRNAs which were associated with cell differentiation and adhesion.
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3 cells. With Western Blot analysis, it was confirmed that the expression of ABCG2 in BxPC-3/Gem cells was upregulated (Figure 3C and 3D).

Discussion

Gemcitabine has been used as a standard first-line chemotherapy in advanced pancreatic cancer [23, 24]. However, the therapeutic efficacy and patient survival rate remains low due to the high drug resistance of the carcinoma. Therefore, plenty of studies have focused on the underlying mechanism of gemcitabine resistance in pancreatic cancer. It has been reported that the miRNAs play key roles in controlling drug-resistance in the tumor progression [25-28].

To identify the miRNAs associated with the formation of gemcitabine resistance in pancreatic cancer, we have chosen a primary human pancreatic tumor line (BxPC-3), which shared similar features in invasion, migration and pathological manifestations with the human cancer. The drug resistant cells were cultured and established by exposing the BxPC-3 cells in intermittently increasing concentrations of gemcitabine. With miRNA microarray, we identified a total of 34 miRNAs that were abnormally expressed in BxPC-3/Gem cells. From the network of miRNA target genes, we identified that has-mir-20a, which has a highest-degree node, as the target miRNA that may be involved in the process of gemcitabine resistance. The miRNA-20a was found to play important roles in many physiological and pathological process such as hematopoiesis, inflammation and tumor development [29-31]. Plenty of evidence showed that miRNA-20a was expressed differently in breast cancer [32], pancreatic cancer [2], lung cancer [33], as well as nasopharyngeal carcinoma [34]. In this study, we focused on its effect on gemcitabine resistance in pancreatic cancer and found that miRNA-20a was significantly down-regulated in BxPC-3/Gem cells.

Among the downstream genes of mir-20a, ABCG2 was selected for further study since it was cooperatively targeted by four (the most)

![Figure 3. Expression levels of miRNA-20a and ABCG2 in BxPC-3 and BxPC-3/Gem cells. (A) Relative expression level of miRNA-20a in normal BxPC-3 (0.986±0.231) and BxPC-3/Gem cells (0.213±0.151), P<0.001; (B) Relative expression level of ABCG2 in normal BxPC-3 (0.803±0.354) and BxPC-3/Gem cells (3.674±1.037), P<0.001; (C, D) Differentially expression of ABCG2 protein in normal BxPC-3 (0.125±0.081) and BxPC-3/Gem cells (0.268±0.098). (Data in A, B, D was expressed in mean ± SEM. Experiments were repeated in three independent experiments).](image)
miRNAs. From the results of qRT-PCR and Western Blot assay, we found that ABCG2 was upregulated in BxPC-3/Gem cells. In human, ABCG2 protein encoded by the ABCG2 gene is a membrane-associated protein in the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins function as transporters that deliver various molecules across extra- and intra-cellular membranes. ABCG2 was found to be overexpressed in hematopoietic malignancies, such as lymphoma, and solid tumors, such as lung cancer, colorectal cancer and endometrial cancer [35-46]. ABCG2 was also suggested to be differently expressed in drug-resistance tumor cell lines and stem cells [35, 47-50]. In breast cancer, ABCG2 protein, acting as a xenobiotic transporter, was associated with multi-drug resistance to chemotherapeutic agents [51]. In pancreatic cancer, Lee et al. reported that the overexpression of ABCG2 was correlated with cancer recurrence after surgery and decreased survival [52].

In conclusion, our study demonstrated that miRNA-20a was down-regulated in BxPC-3/Gem cells, resulting in the overexpression of its downstream protein ABCG2. This finding may serve as a basis for further studies on the underlying mechanism of Gemcitabine-resistance in pancreatic cancer. However, whether miRNA-20a and its target ABCG2 play roles in Gemcitabine-resistant in vivo and the underlying mechanism of how they work needed further research.

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Disclosure of conflict of interest

None.

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