Introduction

Chemotherapy resistance is one of the key causal factors in cancer death and has baffled scientists and oncologists worldwide for a long time [1-2]. For cervical cancer (CC), the third most common gynecologic cancer in global women [3], chemotherapy is still an adjuvant treatment for the complement of surgery or radiation due to its insensitivity to anticancer drugs [4-5]. The molecular genetic basis of chemoresistance is complex and involves multiple processes, including drug transport and metabolism, DNA repair, and apoptosis [6]. Currently, the factors associated with chemoresistance in cervical cancer remain poorly understood.

MicroRNAs are small, single-stranded, noncoding RNAs composed of 19 to 25 nucleotides (~22 nt), which have been proven to be a key regulator in gene expression and modulated up to one-third of all genes [7-10]. Recently, an increasing number of evidence demonstrates that miRNAs may play an integral role in modulating chemosensitivity [11, 12]. For instance, upregulation of miR-138 and downregulation of miR-27a generally increased cisplatin sensitivity in advanced bladder cancer [13]; downregulation of miR-21 increased the paclitaxel sensitivity in glioblastoma cells [14]. Thus, exploration of the role of a specific miRNA and its mechanism in cancer chemoresistance may assist in discovering new approaches to reverse chemoresistance of cancers.

Given the importance role of miRNAs in modulating chemosensitivity, the present authors’ previous study identified a characteristic miRNA expression profile that was associated with paclitaxel chemosensitivity in cervical squamous cell carcinoma [15]. Among 21 differentially expressed miRNAs, miR-224 was found to be the most obviously downregulated miRNA after paclitaxel treatment in cervical cancer cells. Accumulating studies have found that miR-224 is dysregulated in various human malignancies and can potentially affect many cancer-related cellular processes, including transcription, cell differentiation, cell death, growth, and cell proliferation [16-20]. However, little is known about the role of miR-224 in drug resistance of CC. The aim of this study was to investigate the drug resistant role of miR-224 expression in CC.

Materials and Methods

Cell Culture

The HPV 16-positive human cervical carcinoma cell line SiHa was obtained from the American Type Culture Collection (ATCC) and was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO₂.

Chemotherapy inducement

Paclitaxel was used at a final concentration of five and ten nM, respectively. The cancer cell line SiHa cells were seeded 3×10⁵ per well in six-well plates and incubated overnight, and then...
Table 1. — The primers used in this study.

<table>
<thead>
<tr>
<th>miRNA reverse transcription primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>hsa-miR-224</td>
<td>5'-GTCGATCCAGAACTTCCGAGGTTCCACGGGATACGAGGACCTGTTTCG-3'</td>
<td>5'-AACGCTTCACGAAATTTGCGT-3'</td>
</tr>
<tr>
<td>U6snRNA</td>
<td>5'-AACGCTTCACGAAATTTGCGT-3'</td>
<td>5'-AACGCTTCACGAAATTTGCGT-3'</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-224</td>
<td>5'-AGCGGTGGCTCAGTTCAGCA-3'</td>
</tr>
<tr>
<td>U6snRNA</td>
<td>5'-CTCGGTTCGCGACGACA-3'</td>
</tr>
</tbody>
</table>

Abbreviation: miRNA = microRNA.

Treated with paclitaxel for 72 hours. After 72 hours of induction, cells were harvested for further experimentation.

Transfection

miRIDIAN miR-224 mimic (miR-224) and miRIDIAN microRNA mimic control 1 (negative control) were purchased. For transient transfection, SiHa cells were seeded in plates at 60% confluence overnight, then transfected with miR-224 mimic to over-express the miR-224 level using DharmaFECT 1 reagent at a final concentration of 100 nM in accordance with the manufacturer’s instructions. After overnight incubation, the culture medium was replaced with fresh Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum before further study. The expression level of miR-224 in the transfected cells at 72 hours post-transfection was determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The relative level of miR-224 in transfected cells was examined by qRT-PCR. Mock-transfected cells served as blank control and negative control mimic was transcribed as negative control.

RNA extraction and real-time RT-PCR

Total RNAs containing miRNAs were extracted from the harvested cells using one ml TRIZOL reagent following manufacturer’s instructions. The quantity and concentration of RNA were spectrophotometrically assessed by measuring absorbance at 260/280. For miRNA qRT-PCR, stem-loop RT-PCR was performed. Briefly, the RNAs were reverse-transcribed to cDNA with a miR-224-specific stem-loop-like RT primer following the manufacturer’s protocol. For miRNA quantification, each reverse transcript reaction consisted of 0.5 ug of total RNA, mixed with 2.5 ul of 5×RT buffer containing dNTPs, 0.2 ul of 10 umol/l stem-loop RT primer, 0.2 ul RNase inhibitor protein, and 0.5 ul reverse transcriptase in a final volume of 10 ul, and then incubated at 70°C for 10 minutes, 0°C for three minutes, 42°C for 60 minutes and at 70°C for 15 minutes. Real-time PCR was performed with an Applied Biosystems 7900HT Fast Real-time PCR system using the SYBR Premix Ex Taq (perfect real time). PCR volume was 20 ul, containing 1 ul reverse transcript product. Cycling conditions were 1 cycle of 95°C for 30 seconds and 40 cycles of 95°C for five seconds and 60°C for 30 seconds. PCR was performed in triplicate. Real-time PCR was performed using SYBR Premix Ex Taq. The U6 snRNA was used as endogenous control for miRNA. The sequences of the primers are given in Table 1. The ΔCt method was used to determine relative quantization of miRNA expression in samples, and the fold change was determined as 2^-ΔΔCt.

Cytotoxicity assays

Cells were triplicately seeded in flat-bottomed 96-well plates at a density of 6×103 per well in 100 ul culture medium and allowed to adhere overnight. Then the cells were transfected with miR-224 mimic or negative control mimic. After 24 hours incubation, the culture medium was replaced with fresh medium and supplemented with various paclitaxel doses (0, 5, 10, 20, 50, and 100 nM). After three days, replaced the medium with 100 ul fresh medium, 10 ul Cell Counting Kit-8 (CCK8; 1:10) was added to each well according to manufacturer’s instructions. After two hours in culture at 37°C, the cell viability was determined by measuring the absorbance at 450 nm using a 550 Bio-Rad plate-reader. Once the absorbance at 450 nm was recorded, the inhibitory concentration of 50% of cells (IC50) was calculated. The assays were conducted in triplicate and repeated at least three times.

Statistical analysis

The experiments were repeated at least three times. Results are expressed as mean ± SD. An independent Student’s t-test or an ANOVA was used to compare continuous variables. *P < 0.05 was considered as statistically significant. All analyses were performed using SPSS 16.0 software (SPSS).

Results

Paclitaxel decreases miR-224 expression in cervical cancer cells.

The SiHa cells were cultured with different amounts of paclitaxel (0, 5, and 10 nM) for 72 hours. Then the authors observed the expression of miR-224 pre- and post-paclitaxel treatment by using stem-loop real-time RT-PCR. The result showed that miR-224 was significantly downregulated with fold values at 2.130435 and 4.26087 under 5 and 10 nM paclitaxel treatments, respectively (Figure 1). This finding may suggest that paclitaxel induces a decrease in miR-224 expression in a clear dose-dependent manner.

miR-224 expression is markedly increased in SiHa cells after transfected with miRIDIAN miR-224 mimic.

MiRNA mimic or negative control was transfected into the human cervical carcinoma cell line SiHa cells and then the expression of miR-224 was detected in 72 hours post-transfection. The result showed a significant upregulation of miR-224 expression in SiHa cells transfected with miR-224 mimic compared with the negative control. miR-224 was overexpressed in SiHa cells with an increased 1605.742-fold (*p < 0.0001) compared with the negative control (Figure 2).
Upregulation of microRNA-224 sensitizes human cervical cells SiHa to paclitaxel

Exogenous miR-224 facilitates paclitaxel sensitivity in cervical cancer cells.

To confirm the involvement of miR-224 in regulating paclitaxel sensitivity in cervical cancer cells, the authors exogenously upregulated miR-224 expression using miRIDIAN miR-224 mimic and observed its impact on paclitaxel sensitivity using CCK-8. Furthermore, the drug sensitivity testing was determined with CCK-8 assay at 72 hours with different paclitaxel doses (0, 5, 10, 20, 50, and 100 nM). As shown in Figure 3, paclitaxel sensitivities were significantly increased after forced overexpression of miR-224 in SiHa cells compared with miRNA-negative controls. The IC50 value was decreased in SiHa with overexpression of miR-224 (15.72737 ± 1.887629 nM) compared with miRNA-negative control (66.08909 ± 5.966228 nM) (p < 0.0001). The present findings suggested that miR-224 positively modulates the sensitivity to paclitaxel in cervical cancer cells.

Discussion

Chemotherapy is one of the major widely used treatment methods in cancer, while many kinds of cancer are still refractory to chemotherapy [21, 22]. Deep understanding of drug resistance mechanisms is needed to improve the chemotherapy response. Nowadays, drug resistance is considered as a multifactorial phenomenon involving several major mechanisms, such as increased repair of DNA damage, reduced apoptosis, altered metabolism of drugs, and increased energy-dependent efflux of chemotherapeutic drugs that diminish the ability of cytotoxic agents to kill cancer cells [23-26]. The activation of drug resistance...
mechanisms can occur at the genetic level through gene amplification, the transcriptional level through epigenetic modifications, or the proteomic level through mutation or aberrant expression [27]. Recently, the evidence of the roles of microRNAs in determining drug sensitivity/resistance has been emerging.

MiR-224 was originally identified and ends mapped by cloning from Weri cells in human [28]. Its sequence maps to chromosome X [29]. Accumulating studies have found that miR-224 may act as a potential oncogenic miRNA. For instance, miR-224 expression is frequently upregulated in hepatocellular carcinoma [16], colorectal cancer [17], medulloblastoma [30], thyroid cancer [31], pancreatic ductal adenocarcinoma [32], prostate cancer [33], and renal cancer [34]. However, there are also some opposite findings, such as the downregulated expression of miR-224 in ovarian cancer and oral carcinoma [35-36]. This discrepancy may be related to different actions of the same miRNA in different kinds of cancer. In cervical cancer, the present authors’ previous study and Rao et al.’s study all identified miR-224 to be significantly upregulated by 2.7-3.2 fold in cervical cancer tissues compared with cervical normal tissues [15, 37]. Furthermore, miR-224 upregulation was associated with aggressive progression and poor prognosis in cervical cancer [38]. Until now, there has been no study of the association between miR-224 expression and chemotheraphy sensitivity in cervical cancer.

By comparing the miRNA microarray profiles, the present authors previously found that 21 miRNAs (including miR-224) were differentially expressed between self-paired pre- and post-chemotherapy cancer tissues. Then 5 (miR-375, miR-424, miR-181b, miR-224, and miR-27a) were selected and validated in paclitaxel-treated cervical cancer cell lines (Caski and SiHa), of which miR-224 was the most significantly downregulated with fold values range at 1.91–2.05 and 6.3–7.89 under 5 and 10 nM paclitaxel treatment, respectively. Here, the authors further observed the association between miR-224 expression and paclitaxel treatment in vitro in a clear dose-dependent manner. Moreover, they demonstrated that exogenous miR-224 facilitated paclitaxel sensitivity in cervical cancer cells in vitro and paclitaxel sensitivities were significantly increased after forced overexpression of miR-224 in SiHa cells compared with miRNA-negative controls. Thus, the present findings suggest that paclitaxel may induce an acquired drug resistance in cervical cancer cells. The authors demonstrated in this study that the upregulation of miR-224 expression by a miR-224 minic contributed to sensitizing human cervical cancer SiHa cells to the anticancer drug paclitaxel. In the clinic, the development of secondary drug resistance probably downregulated miR-224 expression in cervical cancer cells and enforced overexpression of miR-224 may be able to reverse paclitaxel resistance in cervical cancer.

In summary, the authors find for the first time, to their knowledge, that paclitaxel downregulates miR-224 expression and overexpressed miR-224 inversely reduces chemo-resistance in cervical cancer in vitro. The present study highlights a potential role of miR-224 in the development of drug resistance in cervical cancer and suggests that miR-224 might serve as a predictor for paclitaxel response or a therapeutic target in cervical cancer therapy.

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References


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