EXPERIMENTAL RESEARCH OF EFFECTIVENESS OF siRNA-Her-2/neu ON DRUG SENSITIVITY OF Her-2/neu-OVER-EXPRESSING LUNG ADENOCARCINOMA CELL LINE

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ABSTRACT

Objective: Her-2/neu protein overexpression has been demonstrated in lung adenocarcinoma. Its overexpression often indicates a poor prognosis and resistance to chemotherapeutic agents. The objectives of this paper is to explore the effectiveness of double-stranded short inhibitory RNAs (siRNA) targeting Her-2/neu oncogene on the drug sensitivity of Her-2/neu-overexpressing lung adenocarcinoma cells.

Methods: Lung adenocarcinoma cell line calu-3 was transfected with siRNAs formulated LipofectAMINE 2000, and Her-2/neu protein and P-gp were determined by flow cytometry (FCM). The chemosensitivity of transfected cells to cisplatin (CDDP) was measured by MTT. Cell apoptosis detection kit (Annexin V method) was used to examine the drug induced apoptosis rate.

Results: siRNA targeting Her-2/neu greatly reduced the cell surface expression of Her-2/neu protein and had no effect on P-gp. Consequently the inhibitory rate of CDDP in combination with siRNA targeting Her-2/neu was (67.1 ± 2.3)%, while the inhibitory rates were (48.1 ± 3.5)%, (46.3 ± 5.9)% and (50.2 ± 2.9)% in untreated control, empty vector and unrelated siRNA groups, respectively. The FCM results showed that the apoptosis rate of cells treated with CDDP combined with siRNAs-Her-2/neu was elevated when compared with unrelated siRNA group and empty vector group. Conclusion: Sequence specific siRNA targeting Her-2/neu was capable of enhancing the chemosensitivity of calu-3 cell to cisplatin.

Key words: Her-2/neu; siRNA; Lung adenocarcinoma cells; Cisplatin

Lung cancer is a potentially systemic disease. Despite improvements in the detection and treatment of lung cancer in the past two decades, the overall 5-year survival remains <15%. Constitutive overexpression for Her-2/neu gene is a frequent event in a variety of human tumors, including non-small cell lung cancer (NSCLC), but not small cell lung cancer[1-5]. In recent years, many studies have explored the effects on chemosensitivity resulting from altered expression and activation of the Her-2/neu receptor, indicating that alteration of the activation status of Her-2/neu not only can lead to directly perturbation of growth regulation, but also may affect the sensitivity of cancer cells to chemotherapeutic agents.

SiRNAs 21 to 23 nucleotides (nt) in length trigger RNA interference (RNAi), resulting in post-transcriptional message degradation[6]. The use of exogenously supplied siRNAs for targeted RNA knockdowns has become widespread[6]. In this study, we used synthetic, ready to use siRNA targeting Her-2/neu to silence Her-2/neu gene expression in lung adenocarcinoma cells, exploring the enhancement of chemosensitivity to cisplatin by
RNAi.

**MATERIALS AND METHODS**

**siRNAs**

Synthetic, ready to use siRNA homologous to regions of the Her-2/neu exon were custom synthesized (Catalog # 51010, Ambion), Nonsilencing control siRNAs were kind gifts from Doctor Zhang Renli. Before use in experiments, the Silencer™ siRNAs were reconstituted in annealing buffer as per manufacturer's instructions to obtain a 20 μmol/L solution.

**Cell Lines**

The human lung adenocarcinoma cell line calu-3, was obtained from Basic Medical Sciences Cell Bank of Peiking Union Medical College. The cells were routinely maintained in MEM/NEAA (Cat. No. SH30050.01, Hyclone) with 10% fetal bovine serum (Cat. No. SH30088.01, Hyclone).

**Immunohistochemical Assay**

The slide embedded by polylysine was put into culture dish, later, calu-3 cells were seeded at 3 × 10⁵ cells. After an additional incubation of 24 h, slide was detached from the culture dish and fixed by acetone for 20 min. SABC routine immunohistochemical method was used to detect the expression of Her-2/neu protein.

**Transfection**

All transfection assays were done using LipofectAMINE 2000 following the manufacturer's protocol. The day before transfections, the cells were seeded at 3 × 10⁵ cells per well into 6 cell plates in the absence of antibiotics so that they were 40%~60% confluence on the day of transfection. Transfection volume was 500 μl, siRNA was transfected at a final concentration of 100 nmol/L. Empty vector group and nonspecific siRNA group were set up as control.

**Flow Cytometry**

The cells were harvested by trypsinization 48 h after transfection, and all the following steps were performed on ice. The cells were washed twice with 1×PBS, and then incubated with Her-2/neu monoclonal antibody (1:20) for 30 min at 4°C. After that, they were washed twice again before they were stained with FITC-conjugated rabbit anti-mouse antibody for 30 min in the dark. The homologue mouse IgG was used as negative control. Her-2/neu protein expression was analysed on a FACSscan flow cytometry.

Similarly, the harvested cells 48 h after transfection were washed twice with 1×PBS and resuspended in 50 μl of 1×PBS, then, 20 μl of P-gp-PE was added and incubated for 15 min in the dark at room temperature. After washed once with 1×PBS, P-gp protein expression was analysed on a FACSscan flow cytometry.

**Chemosensitivity Assay**

The effects of Synthetic siRNA-Her-2/neu on the chemosensitivity of calu-3 to cisplatin were evaluated using MTT assay. Briefly, the cells were harvested by trypsinization 48 h after transfection, counted and seeded in 100 μl at a density of 5×10⁴ cells/ml into 96-well plates. Cisplatin at a final concentration of 1 μg/ml was added. Each group included tricouple parallel wells. The cells were incubated at 37°C in a CO₂ incubator for 96 h until they were ready to assay. MTT (5 mg/ml) was added to the wells (100 μl/well) at the end of experimental period. After a 4 h incubation at 37°C, medium were removed from the wells, and dimethylsulfoxide were added to each wells (100 μl/well). The plates were agitated at room temperature for 5 min. Absorbance (volume A) of each well at 570 nm wavelength was read on an enzymelinked immunosorbent assay reader. The results were the means of triplicate data.

**Cell Apoptosis Assay**

Detection of apoptosis was done according to the manufacturer’s protocol. Briefly, cells were stained with annexin V-FITC and PI. After cells were stained in the dark for 15 min at room temperature, their fluorescence was analyzed via FACSscan flow cytometry.

**Statistical Analysis**

SPSS 10.0 software was used to perform statistical analysis, ANOVA test was used for comparison of variance among several groups. P<0.01 or P<0.05 was considered significant.

**RESULTS**

**Immunohistochemical**

Cell membrane of calu-3 cells showed positive brownish yellow staining (Figure 1).
Flow-cytometric Detection of the Expression of Her-2/neu Protein

Calu-3 cells 48 h after transfection with chemically synthesized siRNAs showed markedly less Her-2/neu protein according to flow-cytometric analysis. The Her-2/neu protein positive expression rates in Her-2/neu siRNA group, untreated control group, empty vector group and unrelated siRNA group were (25.04±1.56)%, (98.24±2.23)%, (95.67±1.98)% and (94.79±0.87)%, respectively. There was significant difference between Her-2/neu siRNA group and the other three groups (P<0.01).

Flow-cytometric Detection of the Expression of P-gp Protein

There was very low expression of P-gp protein in the four groups. The P-gp protein positive expression rates in Her-2/neu siRNA group, untreated control group, empty vector group and unrelated siRNA group were (4.24±1.01)%, (5.11±2.98)%, (6.98±2.47)%, and (5.59±3.66)%, respectively. There was no significant difference among the four groups (P>0.05).

Cisplatin Sensitivity

The inhibitory rate of CDDP in combination with siRNA targeting Her-2/neu was (67.1±2.3)%, while the inhibitory rates were (48.1±3.5)%, (46.3±5.9)%, and (50.2±2.9)% in untreated control, empty vector and unrelated siRNA groups, respectively. There was significant difference between Her-2/neu siRNA group and the other three groups (P<0.01) (Figure 2).

Cisplatin Induced Apoptosis in Her-2/neu Silenced Cells

The FCM results showed that the apoptosis rate of CDDP combined with siRNAs-Her-2/neu was elevated when compared with unrelated siRNA group and empty vector group. When cisplatin at a final concentration of 5 μg/ml was used, the apoptosis rate of CDDP in combination with siRNA targeting Her-2/neu was (35.6±3.4)%, while the apoptosis rates were (8.8±0.5)%, (9.6±1.7)% and (11.3±1.8)% in untreated control, empty vector and unrelated siRNA groups, respectively. There was significant difference between Her-2/neu siRNA group and the other three groups (P<0.01) (Table 1).

Table 1. Effect of CDDP in combination with siRNA targeting Her-2/neu on apoptosis rate of calu-3 cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Apoptosis rate (%) (x±s)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control group</td>
<td>8.8±0.5</td>
<td></td>
</tr>
<tr>
<td>Empty vector group</td>
<td>9.6±1.7</td>
<td></td>
</tr>
<tr>
<td>Unrelated siRNA group</td>
<td>11.3±1.8</td>
<td></td>
</tr>
<tr>
<td>Her-2/neu siRNA group</td>
<td>35.6±3.4</td>
<td>P&lt;0.01*</td>
</tr>
</tbody>
</table>

DISCUSSION

RNAi technology is a potent new technology. RNA interference (RNAi) is a process in which double stranded RNA acts as a signal to promote degradation of mRNA with sequence identity[6]. It has been demonstrated that small, synthetic, double stranded RNA can effectively mediate gene silencing through RNAi mechanism[7]. The decrease in expression of targeted gene products can be extensive, with 90% silencing induced by a few molecules of siRNA. Kern and Tateishi reported significantly inferior survival in patients overexpressing Her-2/neu. Cancer cells that overexpress Her-2/neu may also render resistant to cisplatin[2, 3]. At present, the mechanism by which p185neu confers intrinsic chemoresistance remains nuclear. Several lines of evidence suggest that high-p185neu-expressing cancer cells may possess an enhanced DNA repair capacity. Pietras suggested that NER activity may play an
important role in the cisplatin resistance of NSCLC cells[8,9].

Our study represented the first report on the use of siRNA to silence the Her-2/neu gene and enhance tumor cells chemosensitivity to cisplatin. We chose calu-3 cells transfected with siRNAs formulated LipofectAMINE 2000. The results showed that Her-2/neu protein was evidently inhibited in Her-2/neu siRNA group and was still very high in other three groups; there was very significant difference in the four groups (P<0.01). Simultaneously, we detected the expression of P-gp protein by flow cytometry (FCM), there was very low expression in P-gp protein and no significant difference among the four groups (P>0.05).

Chemotherapeutic agents based on cisplatin, which was a commonly used DNA-damaging anticancer agent, play an important role in NSCLC treatment. The anticancer effect of cisplatin is related to dosage. The larger the side effects are; the larger the dosage is used. Therefore, cisplatin dosage limits its usage. In addition, intrinsic chemoresistance profiles in human cancers has been a very difficult problem disturbing clinics. In our study, we showed that chemically synthesized siRNA against Her-2/neu mediated efficient and selective inhibition of Her-2/neu expression in carcinoma cells overexpressing Her-2/neu and substantially increased inhibition rate and apoptosis of cisplatin to calu-3 cells, therefore, enhanced the chemosensitivity of calu-3 cells to cisplatin. Our results were consistent to Funato’s results in gastric cancer cell lines[10].

P-gp is a drug efflux pump, which lies in cancer cell cytoplasm membrane and connects with multidrug resistance. Our results suggested that the level of P-gp was not changed while Her-2/neu protein was significantly decreased after calu-3 cells were transfected with siRNAs formulated LipofectAMINE 2000. Calu-3 cells expressed very low level of P-gp, the effect of inhibiting the expression of Her-2/neu protein on P-gp would require further study.

Although knockdown of the target gene by siRNA is not permanent, a single siRNA transfection leads to a prolonged inhibition of the target protein in the parents as well as progeny cells. Moreover, the development of vectors that allows the stable, long-term expression of constructs that give rise to siRNA in vitro. In turn, this may permit development of therapeutic approaches based on the RNAi technology.

In summary, we have shown that Her-2/neu-specific siRNA mediates efficient and selective inhibition of Her-2/neu expression in carcinoma cells overexpressing Her-2/neu. The degree of suppression was sufficient to enhance the chemosensitivity of calu-3 cells to cisplatin, therefore providing a potential novel strategy for human lung cancer.

REFERENCES