Original Article

c-Myc silencing impedes cell proliferation and enhances cytotoxicity of cisplatin in non-small cell lung cancer

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Abstract: We investigated the expression of c-Myc in non-small cell lung cancer (NSCLC) tissues and explored the effect of c-Myc silencing on cell proliferation and cisplatin cytotoxicity. mRNA expression levels of c-Myc in 84 NSCLC tissue samples and corresponding para-cancerous tissues were detected by real-time PCR. c-Myc small interfering RNA (siRNA) was transfected into NSCLC H1299 cells by Lipofectamine 2000. Real-time PCR was carried out to validate the transfection efficiency. Cell Counting Kit-8 was used to evaluate cell proliferation. Cisplatin toxicity experiments using CCK-8 assays showed cytotoxicity of cisplatin plus c-Myc gene silencing in NSCLC cells. The c-Myc mRNA level in NSCLC tissues was significantly higher than that in corresponding para-cancerous tissues (P < 0.05). c-Myc siRNA was successfully transfected into H1299 cells. The proliferation rates of H1299 cells after c-Myc silencing at 24, 48 and 72 h were lower than those of control groups (P < 0.05). The combination of c-Myc silencing and cisplatin had the most cytotoxic effect on H1299 cells compared with individual and control groups (P < 0.05). c-Myc may act as an oncogene in NSCLC and could be a potential target for NSCLC gene therapy.

Keywords: c-Myc, non-small cell lung cancer, cell proliferation, cisplatin

Introduction

Lung cancer is the most common cancer and the leading cause of cancer mortality worldwide for both men and women. At diagnosis, most patients will be at a late stage and have a very low 5-year relative survival rate, even with current standard therapies [1]. More than 80% of lung cancer cases are non-small cell lung carcinoma (NSCLC) that includes adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and NSCLC that cannot be classified further [2]. Treatment of NSCLC depends on the tumor stage and the patient’s overall condition. Patients with early stage disease are generally treated with curative intent by surgery, chemotherapy, radiation therapy, or a combined modality approach. Unfortunately, lung cancer is usually metastatic at the time of diagnosis, and thus requires systemic therapy rather than local.

Much attention has been paid to gene therapy of NSCLC in recent years. Realization, analysis, and integration of various genes related to the development of NSCLC are the foundation for NSCLC gene therapy. The c-Myc gene is an important member of the myc gene family, which is related to the development of a variety of tumor types. Amplification or overexpression of the c-Myc gene is found in stomach cancer, breast cancer, colon cancer, cervical cancer, Hodgkin’s disease, and head tumors [3, 4]. In recent years, some studies have found abnormal expression of the c-Myc gene in NSCLC [5, 6]. Our study further confirmed abnormal expression of the c-Myc gene in NSCLC and explored the possible prospects of c-Myc gene silencing for NSCLC gene therapy.

Materials and methods

Patients and tissue samples

The study was performed using 84 randomly selected NSCLC patients (59 adenocarcinoma and 25 squamous cell carcinoma). The patients were treated at The First Affiliated Hospital with...
Nanjing Medical University between December 2011 and June 2012. All specimens were collected under a protocol approved by the Human Ethics Committee of Nanjing Medical University. Each patient participated after providing informed consent. Most patients were men (79%). A total of 168 tissue specimens were included in the study with one tumor sample and one corresponding adjacent sample (5 cm or more from the cancer tissue edge and pathologically confirmed as normal lung tissue) from each patient. All specimens were immediately snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. The tumors were histologically classified by two professional pathologists according to the World Health Organization classification (Table 1).

### Table 1. Relationship of c-Myc expression and clinical characteristics of NSCLC patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number</th>
<th>Relative mRNA level ($2^{\Delta\Delta Ct}$)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 50</td>
<td>13</td>
<td>10.66±6.180</td>
<td>0.6933</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>71</td>
<td>12.63±1.816</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>67</td>
<td>12.53±2.199</td>
<td>0.8236</td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>11.52±1.907</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A¹</td>
<td>25</td>
<td>11.87±4.156</td>
<td>0.8692</td>
</tr>
<tr>
<td>S²</td>
<td>59</td>
<td>12.52±1.874</td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>50</td>
<td>13.17±2.601</td>
<td>0.8532</td>
</tr>
<tr>
<td>II</td>
<td>26</td>
<td>11.08±2.917</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>8</td>
<td>11.13±1.848</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>30</td>
<td>10.88±3.490</td>
<td>0.551</td>
</tr>
<tr>
<td>No</td>
<td>54</td>
<td>13.13±2.021</td>
<td></td>
</tr>
</tbody>
</table>

Note: 1, adenocarcinoma; 2, squamous cell carcinoma.

The ABI Prism7900 Sequence Detector System (ABI, USA) and $2^{\Delta\Delta Ct}$ method were employed to determine the relative levels of mRNAs in tumor and adjacent tissues. Quantitative real-time PCR (qRT-PCR) analysis of c-Myc and β-actin was performed with SYBR Premix ExTaq™ (TaKaRa) according to the manufacturer’s instructions. PCR was performed in a final volume of 20 µl using 10 µl of 2× Premix buffer, 0.4 µl of each primer, 2 µl of sample, and 7.2 µl distilled water. The PCR conditions were 50°C for 5 min, 95°C for 10 min and then 45 cycles at 95°C for 15 sec and 60°C for 2 min. The following primers were used: c-Myc forward primer, 5'-GGCTCCTGGCATAAGGTCAG-3' and c-Myc reverse primer, 5'-AGTTGTGTGCTGTGTA-3'; β-actin forward primer, 5'-GTGCCCATCTACGAGGGGTATGC-3' and β-actin reverse primer, 5'-GTTACATGGTGTGCGCAGACA-3'. Fluorescence detection was performed at the end of the extension phase. To discriminate specific and nonspecific cDNA products, a melting curve was obtained at the end of each run.

#### Cell culture and small interfering RNA (siRNA) transfection

The H1299 cell line (American Type Culture Collection) was employed in the present study.

DEPC-treated water was incubated at 37°C for 15 min and then 85°C for 5 s. cDNA was stored at 4°C until use.

The following primers were used: c-Myc forward primer, 5'-GGCTCCTGGCATAAGGTCAG-3' and c-Myc reverse primer, 5'-AGTTGTGTGCTGTGTA-3'; β-actin forward primer, 5'-GTGCCCATCTACGAGGGGTATGC-3' and β-actin reverse primer, 5'-GTTACATGGTGTGCGCAGACA-3'. Fluorescence detection was performed at the end of the extension phase. To discriminate specific and nonspecific cDNA products, a melting curve was obtained at the end of each run.
c-Myc silencing in NSCLC

Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified incubator at 37°C with 5% CO₂. For c-Myc silencing, H1299 cells were seeded into 6-well plates (1.5×10⁵ cells/well) and incubated for 24 h. The H1299 cells were then transfected with 25, 50 and 75 nmol/L Signal Silenc™ c-Myc siRNA (#6341, Cell Signaling Technology, USA) or Signal Silenc™ control siRNA (#6568, Cell Signaling Technology) using Lipofectamine (lip) 2000 reagent (Invitrogen, USA) following the manufacturers’ protocols. After 6 h of incubation, the siRNA and Lipofectamine 2000 solutions were gently mixed and added to the cells. The cells were incubated in fresh complete medium until analysis. Non-specific control siRNA was used as a negative control. To evaluate the effects of siRNA on gene silencing, transfections (5×10⁵ cells/well) were performed in 6-well plates for 24-72 h, and then mRNA expression was determined by qRT-PCR.

Cell proliferation assay

H1299 cells were seeded in 96-well plates (2×10³ cells/well) and transfected with 50 nmol/L c-Myc siRNA. The cells were divided into five groups including blank, control, lip, control siRNA, and c-Myc siRNA. Cell viability was assessed by Cell Counting Kit-8 (CCK-8) assays (Beyotime Institute of Biotechnology, China). Cells were subjected to CCK-8 assays before transfection and at 0, 24, 36, 48, 60 and 72 h after transfection. After 4 h of incubation in CCK-8 solution, the optical density (OD) of each well was read on a spectrophotometer (Thermo, Pittsburgh, PA, USA) at 450 nm (A450). The following formula was used to analyze the results: cell proliferation rate = (the average value in the experimental group - that in the control group)/that in the control group × 100%. Three independent experiments were performed in quintuplicate.

Drug toxicity test

H1299 cells were seeded in 96-well plates (4×10³ cells/well). After 24 h, the cells were transfected with 50 nmol/L c-Myc siRNA and/or treated with 5 µg/ml cisplatin (cis). Cells were divided into six groups including blank+cis, control, control+cis, lip+cis, control siRNA+cis, and c-Myc siRNA+cis. Cell viability was assessed by CCK-8 assays as described above.

Statistical analysis

A paired t-test was employed to analyze the expression levels of c-Myc mRNA. One-way analysis of variance was applied to the results of cell proliferation and cisplatin cytotoxicity experiments. All analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). P < 0.05 was considered as statistically significant.

Results

c-Myc mRNA expression in NSCLC tissues is higher than that in corresponding adjacent lung tissues

c-Myc mRNA levels were analyzed in NSCLC specimens and corresponding adjacent samples from 84 patients by qRT-PCR. Significantly higher c-Myc mRNA levels were detected in tumor specimens than in adjacent areas (P < 0.05) (Figure 1; Table 1). Moreover, the of c-Myc mRNA expression in NSCLC tissues (6.28×10³) was 1.77-fold higher than that in adjacent tissues (3.54×10³) and had no correlation to sex, age, histological type, clinical stage, or lymph node metastasis.

Transfection of c-Myc siRNA into NSCLC H1299 cells

H1299 cells were successfully transfected with c-Myc siRNA (Figure 2). After 48 h, expression
of c-Myc mRNA in H1299 cells was significantly inhibited and proportional to the concentration of c-Myc siRNA. The inhibition disappeared after about 72 h.

**C-Myc silencing suppresses the proliferation of H1299 cells**

The proliferation rates of H1299 cells after transfection with c-Myc siRNA for 48 and 72 h were significantly lower than those of cells without transfection (P < 0.05). There were no significant differences in control, lip, or control siRNA groups at each time point.

**Combined c-Myc silencing and cisplatin treatment have the most cytotoxic effect on H1299 cells**

Results of drug toxicity testing showed that the cytotoxicity of cisplatin and c-Myc siRNA+cisplatin groups was significantly higher than that of the control group at 60 h after c-Myc siRNA transfection (P < 0.05). Cytotoxicity of the c-Myc siRNA+cisplatin group was significantly higher than that of the cisplatin group (P < 0.05).

**Discussion**

Although the c-Myc gene has been investigated in recent studies, its specific function in NSCLC remains unclear. In this study, we found that the mRNA level of c-Myc in NSCLC specimens was markedly higher than that in adjacent tissues. c-Myc gene silencing significantly suppressed the proliferation of H1299 cells in vitro. The effect of c-Myc gene silencing was comparable to that of cisplatin. In particular, c-Myc silencing combined with cisplatin had the strongest cytotoxicity. Therefore, c-Myc might play an important oncogenic role in NSCLC and may be a new target for NSCLC gene therapy.

With the development of molecular genetics and gene transfer technology, gene therapy to change the genetic structure of cells for therapeutic purposes has made significant progress and gradually proceeded to clinical application. The occurrence and development of NSCLC are a multistep process that involves multiple genes, including activating mutations and inactivation of tumor suppressor genes. Oncogenes are involved in initiation, development, and prognosis of each tumor stage [7, 8]. The genetic characteristics of NSCLC has been reviewed previously, and more than 50 genomic regions are frequently added or deleted in human lung cancer [9].

However, so far, the vast majority of gene therapy strategies for cancer, including NSCLC,
remain in the conception and laboratory stages. Few gene therapies have been tested in clinical trials. Strategies for gene therapy have mainly included replacement of tumor suppressor genes, antisense therapy, immunotherapy approaches, and suicide gene therapy [10].

Replacement of tumor suppressor genes involves the use of a gene therapy vector to encode a tumor suppressor gene that is absent or mutated in the cancer. The mechanisms include mutation, deletion, methylation, or their combination. Theoretically, gene therapy for NSCLC by replacement of a tumor suppressor would lead to inhibition of tumor growth or increase tumor cell mortality in vivo [10]. Currently, the tumor suppressor gene p53 is the most widely studied, and its mutation is at the highest frequency among genetic alterations in lung cancer. p53 gene mutations are detected in 45-75% of NSCLC cases [11, 12].

A wide variety of molecules can promote tumor growth, and reducing these growth factors can significantly inhibit the growth of tumor cells. It is rational to use anti-sense therapy for NSCLC. For example, experience with drugs targeting vascular endothelial growth factor (VEGF) and epidermal growth factor receptor is summarized elsewhere [13, 14]. In recent years, research of the molecular biology of tumors has made great progress. Researchers can design and synthesize certain materials for the development of tumor pathways that regulate tumor cell growth, proliferation, foreign invasion, and angiogenesis [15].

Tumor cells can synthesize certain proteins that can enhance the innate immune system to recognize and kill the tumor cells, which is the theoretical basis of immunotherapy [16]. The mechanism of suicide gene therapy is that genes encoding certain enzymes are transfected into tumor cells to increase sensitivity to drug treatment [17].

The c-Myc gene is an important member of the MYC gene family. MYC proto-oncogenes include N-Myc, c-Myc, and L-Myc [18]. The c-Myc gene is a transpositional gene that is not only regulated by a variety of substances, but also promotes cell division and decreases cell mortality, leading to cell longevity [19, 20]. The protein function of c-Myc located at chromosome 8q24 is related to the regulation of various processes, including tumor cell growth, proliferation, differentiation, and apoptosis. Overexpression of c-Myc protein promotes tumorigenesis [21].

In recent years, an increasing number of researchers are becoming concerned about “driver mutations” in cancer therapy, because the success of targeted cancer therapy depends on the presence of driver mutations. Epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) are two important driver mutations in lung cancer therapy [22]. EGFR is the positive choice and causes tumorigenesis. It can provide advantages in terms of the growth, proliferation, and transformation of cells, and drives normal cells toward cancer cells [23]. Recently, some findings have suggested that c-Myc might be involved in the progression of early stage lung adenocarcinoma, especially in conjunction with EGFR and ALK pathways [24].

High expression of the c-Myc gene is closely related to increases in the density of vascular and lymphatic vessels. The ability of c-Myc to induce angiogenesis has been observed previously in lung and other tumor models. MYC has been shown to induce angiogenesis in conjunction with hypoxia inducible factor-1α via VEGF [25]. Tumor tissue hypoxia, necrosis, and expression of the c-Myc gene can promote the expression of VEGF [26]. High expression of VEGF can promote tumor tissue angiogenesis and the expression of other vascular growth factors, which plays an important role in the occurrence, development, and prognosis of a variety of tumors including lung cancer [27].

Despite recent advances in NSCLC treatment, the clinical outcomes of these patients are still far from satisfactory. For example, cisplatin is active against many types of tumors and is toxic to cochlear hair cells, renal convoluted tubule cells, dorsal root ganglion neurons, and gastrointestinal enterochromaffin cells, but is not toxic to most rapidly dividing normal tissues. In conclusion, it is very important to develop more effective NSCLC treatments with fewer side effects, and the c-Myc gene is a suitable target of study for such purposes.

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**Disclosure of conflict of interest**

None.

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