The role of Notch1 genes in lung cancer A594 cells and the impact on chemosensitivity

F. DENG, Z.-F. YANG, C.-Q. SUN

Department of Oncology, Dezhou People's Hospital, Shandong Province, P.R. China

F. Deng and *Z.-F. Yang* contributed equally to this work

Abstract. – **OBJECTIVE:** Expression of Notch1 gene in lung cancer A549 cells was reduced using small interfering RNA (small interfering RNA, siRNA) and the effect of Notch1 gene on proliferation and chemo sensitivity of lung cancer A549 cells was studied.

MATERIALS AND METHODS: The Notch1 siRNA was transfected into A549 cells by liposome to inhibit the expressions of Notch1 gene in A549 cells. Real-time polymerase chain reaction (RT-PCR) and Western blot were used to detect the expression of Notch1 gene and protein. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used to detect the proliferation of A549 cells. After transfection of Notch1-siRNA, cisplatin was added to each group at a concentration of 4 μg/ml, and cultured for 48 h. MTT assay and 4',6-diamidino-2-phenylindole (DAPI) staining was used to evaluate the change of apoptosis and sensitivity to chemotherapy.

RESULTS: Notch1 gene expression of A594 cells, detected by RT-PCR and Western blot was significantly reduced in transfected cells when compared with the control group ($p<0.05$). Inhibition of A594 cell proliferation was significantly decreased as detected by MTT ($p<0.05$), and the MTT assay and DAPI staining showed that Notch1 gene silencing can significantly improve the sensitivity of A549 cells to chemotherapeutic drugs.

CONCLUSIONS: The Notch1 siRNA can effectively inhibit the expression of Notch1 gene, inhibit the proliferation of lung cancer A549 cells and increase the sensitivity to chemotherapeutic drugs.

Key Words:
    Lung cancer A549 cells, Notch1-siRNA, Proliferation, Chemo sensitivity.

Introduction

Lung cancers are the most common malignant tumors in respiratory tract tumors, around the world[1]. Current treatments of lung cancer mainly include radiotherapy, chemotherapy and surgical operation. Occurrence of relapse, drug resistance and metastasis after treatment are still inevitable. Cisplatin, one of commonly used chemotherapeutic drugs in clinical practice, is widely administered to malignant lung cancer patients; however, the malignant cells tend to develop drug resistance with this agent[2-5]. Therefore, effective target gene therapy and improving sensitivity to chemotherapeutic drugs is the focus of research on lung cancer. Notch1 signal path, a highly conservative signal paths in evolution has more than one biological function, such as regulating and controlling various physiological activities of cells, like differentiation, proliferation and apoptosis[6-8]. Notch1, one of the four single-span theca cell surface receptors for Notch1, widely exists in mammals[9]. When Notch1 combines its ligand, the ligand is hydrolyzed by protease. *Notch1 intracellular domain* (NICD) first exists in intracellular region, entering the cell nucleus, initiating the transcriptional regulatory factor, and activating the transcription of target gene[10,11]. Research results have verified that Notch1 signal path plays an important role in generation and progression of multiple tumors, such as acute lymphocytic leukemia (ALL)[12], multiple myeloma[13], tumor of liver[14] and lung tumors[15]. In addition, there is evidence, which demonstrates correlation between cervical cancer cells with silent Notch1 gene and cell drug toxicity induced by cisplatin. When Notch1 gene is silenced, the cell drug toxicity induced by cisplatin will be obviously sensible, and the IC50 value reduces evidently[16]. However, there is yet little research on the sensitivity of lung cancer cells to chemotherapy with cisplatin after the interference of Notch1 gene. Hence, it is of great significance studying and illuminating the specific roles of Notch1 played in cancer cells.

Corresponding Author: Changqing Sun, MD; e-mail: hivju_443557@163.com

Materials and Methods

Materials and Reagent

siRNA was synthesized by Biomics Biotechnology Co., Ltd (Nantong, China). Lung cancer A549 cells were purchased from Union Cell Biology Institute of Chinese Academy of Science (Beijing, China); BAC protein quantification kit from Beyotime Biotechnology Co., Ltd (Shanghai, China); Notch1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); reverse transcription kit and Real-time qualification kit from TaKaRa Biotechnology (Dalian, China); lipidosome Lipofectamine 2000 and TRIzol from Invitrogen (Carlsbad, CA, USA).

Cell Culture

Lung cancer A549 cells were cultured in incubator. Out of the total, 85% of cultured cells were fused and treated with trypsinization, Dulbecco's Modified Eagle Medium (DMEM) 10% fetal bovine serum (FBS) contained (Biosharp, Hefei, China) and culture medium was used to dilute the cell suspension. Finally, the targeted concentration of cells was adjusted to $2 \times 10^8 \cdot L\text{-}1$; after counting, the cells mentioned above were inoculated on corresponding culture plate for the use in the following experiment.

Design, Synthesis and Transfection of siRNA

The sense strand of siRNA sequence of Notch1 is 5'-GCAACCUGCAGU-GUAAUAATT-3', while its antisense strand is 5'-UUAUUACA-CUGCAG-GUUGCTT-3'. siRNA (siNC) having no homology relation with Notch1 mRNA and other genes were selected as the negative control group. As lung cancer A549 cells are cultured to logarithmic phase, digestive treatment would be conducted on cells. Culture solution was used for cell suspension, and the cell concentration was adjusted to $1.5 \times 10^8 \cdot L\text{-}1$, with cells being cultured on six well plates after inoculation. Specific procedures of transfection were conducted strictly according to instructions. The culture plate was shaken gently to achieve mixing.

RT-PCR Inspection

48 h after transfection of siRNA, cells were collected to extract total RNA. Then, with total RNA inspected qualified as the template, cDNA was synthesized by reverse transcription. The reaction conditions were: incubation at 42°C for 15 min, incubation at 95°C for 3 min. Then, they were put on ice for cooling, and finally freezed

in refrigerator at -80°C for following experiment. Routine amplification was conducted based on primer sequence of Table I.

Western Blotting Inspection

48 h after transfection of siRNA, cells were collected to extract protein and detect the protein concentration. After the processing of samples, 50 μg of proteins were taken for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoretic separation. Then membrane was transferred and the separated protein was transferred electrically on polyvinylidene difluoride (PVDF) membrane (KeyGene, Nanjing, China). Confining liquid was sealed for 1 h at room temperature. After primary antibody incubation was done, the liquid was preserved overnight at 4°C. After full membrane-washing of tween/tris-buffered salt solution (TTBS), secondary antibody (1:2000) was added and TTBS was incubated for 1 h at room temperature. Colored solution was used to develop the color and get the image of TTBS after washing.

Detection of Cells Proliferation by MTT and its Sensitivity to Chemotherapeutic Drugs

A549 cells at logarithmic phase were inoculated on 96 well plates. After cells were attached to wall, Notch1-siRNA was transfected, and after 48 h of culture absorbance was measured and cells of another group were transfected with Notch1-siRNA. After another 48 h of culture, 4 μg/ml of cisplatin was added into each group. Absorbance was measured after 48 h.

DAPI Staining

The cultured cells were inoculated on 6 well plate, with 10^4 cells per well, and supernatant was absorbed after 24 h. After 48 h of transfection with siRNA, 4 μg/ml of cisplatin was added into each group. After another 48 h, cells were washed by pre-cooled PBS for three times, DAPI solution was added to each well added (1 μg/mL). DAPI solution (1 μg/mL) was added into each well. Then cells were incubated in incubator for 5 min

Table I. The RT-PCR primer sequence of Notch mRNA.

Gene	Primer sequence
Notch1	Forward: 5'-GCCTCAACATCCCCTACAAGA -3' Reverse: 5'-CCACGAAGAACAGAAGCACAAA -3'

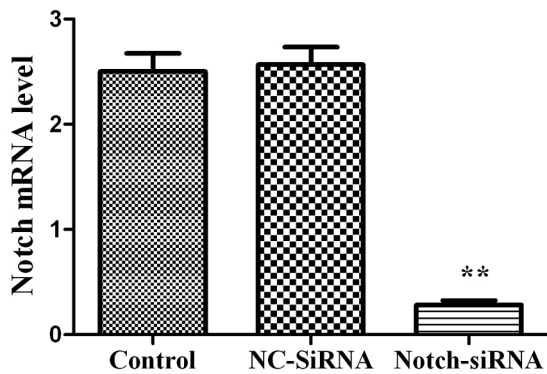Note: *, $p<0.05$ comparing between CIA group and control group.

Figure 1. Effect of Notch1-siRNA on expression level of Notch1 mRNA; $^{**}p<0.01$.

at 37°C. Fluorescence microscope was used to obtain images after washing with pre-cooled PBS.

Statistical Analysis

The data was expressed by mean value ± standard deviation, and processed with SPSS 217.0 software (SPSS Inc., Chicago, IL, USA). Comparison between groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). The difference was considered to be statistically significant when $p<0.05$.

Results

Effect of Notch1-siRNA on Expression Level of Notch1 mRNA

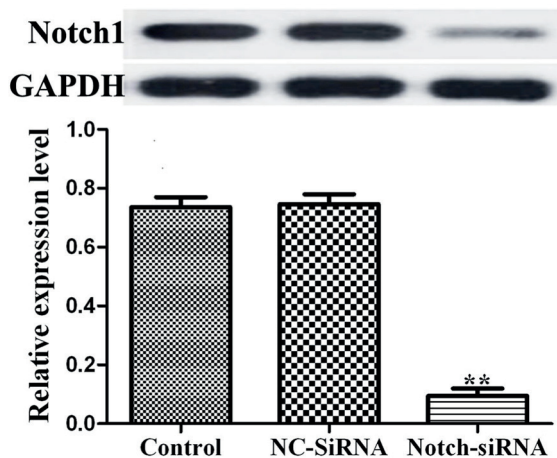There was a reduction of 91% in expression of Notch1 mRNA in lung cancer A549 cells after

transfection with Notch1-siRNA as compared to blank control group. The difference was statistically significant ($p<0.01$), suggesting that the expression of Notch1 genes was inhibited. Moreover, there was no obvious difference between blank control group and negative control group ($p>0.05$) (Figure 1).

Effect of Notch1-siRNA on Expression Level of Notch1 Protein

Total protein was extracted after 48 h from each group after A549 cells were transfected with Notch1-siRNA. The results of protein expression demonstrated that compared with control group, the protein expression level of Notch1 of transfected cells was reduced evidently. The difference was statistically significant ($p<0.01$), suggesting that the expression of Notch1 protein was inhibited obviously. Moreover, there was no difference between blank control group and negative control group ($p>0.05$) (Figure 2).

Effect of Notch1 siRNA on Proliferative Activity of A549 cells

After 48-h incubation of cells of control group, negative control group and Notch1 siRNA group, the MTT assay results showed the proliferation rate of Notch1 siRNA group was inhibited ($p<0.05$) when compared to control group and negative control group, suggesting that Notch1 gene is related to cell proliferation (Figure 3).

Inspection of Sensitivity Changes of A549 Cells to Chemotherapeutic Drugs by MTT

After 48-h incubation of cells of control group, negative control group and Notch1 siRNA group,
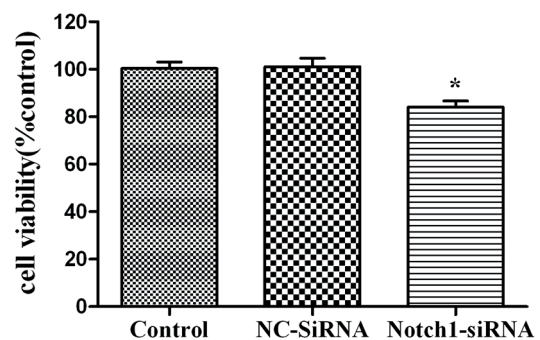

Figure 2. Effect of Notch1-siRNA on expression level of Notch1; $^{**}p<0.01$.

Figure 3. Effect of Notch1 siRNA on proliferative activity of A549 cells; $^{*}p<0.05$.

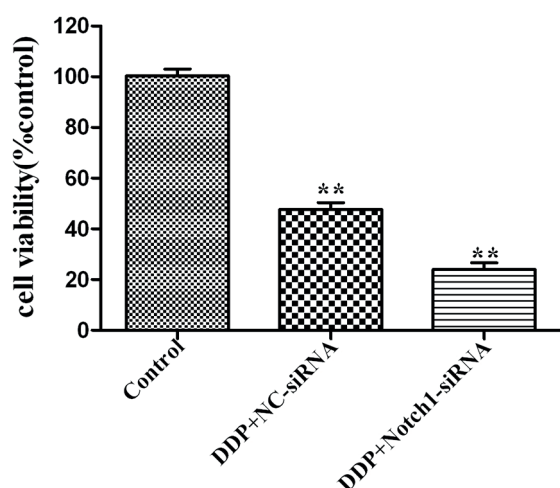**Figure 4.** Inspection of sensitivity changes of A549 cells to chemotherapeutic drugs by MTT; **p <0.01.

4 µg/ml of cisplatin was added to each group, and cells were cultured for further 48 h. MTT assay results illustrated that when compared with control group and negative control group, the cell viability of negative control group was reduced to 45%, while that of Notch1 siRNA group reduced to 25% ($p<0.01$), suggesting that inhibition of Notch1 gene improved the sensitivity of cells to chemotherapeutic drugs (Figure 4).

Inspection of Sensitivity Changes of A549 Cells to Chemotherapeutic drugs by DAPI

A549 cells were cultured for 48 h after being transfected with Notch1 siRNA and 4 µg/ml of cisplatin was added. The results indicated that nucleus of most cells from control group presented even light blue, while nucleus of most cells from Notch1 siRNA group showed pyknosis or shattering, and gave out strong blue-fluorescence, with

reduction of cell number (Figure 5). This indicates that the sensitivity of cells to chemotherapeutic drugs was enhanced by inhibiting Notch1.

Discussion

Clinically, the chemotherapy treatment for many malignant tumors are not satisfactory with poor prognosis; moreover, tumor cells that survive from chemotherapy tend to develop drug resistance to different degrees, resulting in decreased sensitivity of tumor cells to chemotherapeutic drugs, which triggers relapse and metastasis of malignant tumors. The underlying cause is the drug resistance of tumor cells[17,18]. As documented by previous studies, there is certain relationship between the signal path regulated by Notch1 receptor, the tumorigenesis and tumor metastasis. There is association of abnormal expressions of downstream signal path, Notch1 receptor and ligand in leukemia and multiple malignant tumors[19,20]. In recent years, increasing researches have indicated that Notch1 gene is related to drug sensitivity. The expression of silent Notch1 gene can evidently improve the sensitivity of tumor cells to chemotherapeutic drugs in most tumors, including pancreas cancer, breast cancer, colorectal cancer and prostate cancer, etc.[21-23]. There is yet little research on the relation between Notch1 in lung cancer cells and sensitivity to chemotherapeutic drugs. By siRNA interference technology, this experiment studied the expression and significance of Notch1 in lung cancer A549 cells. In this experiment Notch1 siRNA was created and targeted interference to the expression of Notch1 gene in A549 cells was conducted. Quantitative PCR and Western assay results indicated that Notch1 genes are inhibited. MTT study results in-
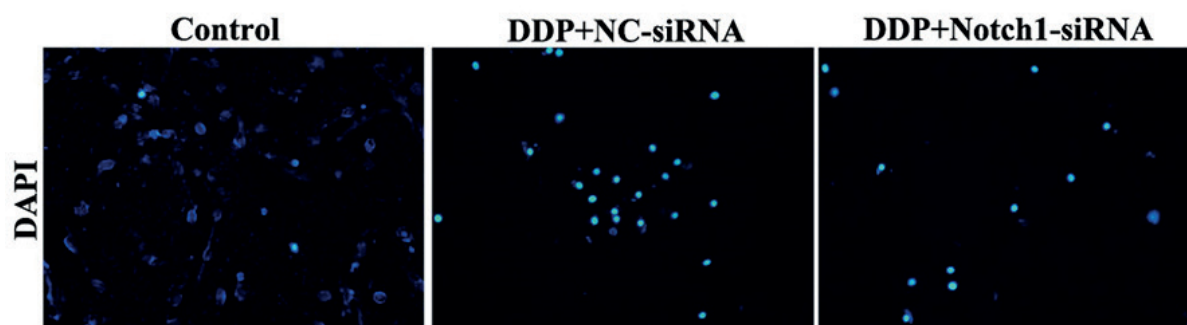

Figure 5. Inspection of sensitivity changes of A549 cells to chemotherapeutic drugs by DAPI.

dicate that silent expression of Notch1 can reduce proliferation rate of A549 cells. Also, MTT and DAPI assay results show that silent expression of Notch1 improves sensitivity of lung cancer A549 cells to cisplatin.

Conclusions

The results mentioned above indicate that after silencing of Notch1 gene, the proliferation capacity of A549 cell reduced sharply, and the sensitivity of A549 cell to chemotherapeutic drugs improved dramatically. The specific mechanism, related regulation and control signal path should be studied and illustrated in future.

Conflict of interest
The authors declare no conflicts of interest.

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