Notch1 targeted regulation of mir-224/LRIG2 signaling for the proliferation and apoptosis of cervical cancer cells

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Abstract. In the present study, we explored the participation of Notch1 targeted regulation of mir-224/LRIG2 gene signal pathway in proliferation and apoptosis of cervical cancer cells. Forty-nine cases of cervical cancer lesion samples from cervical cancer patients treated in our hospital from February 2013 to February 2015 were chosen as subjects (the observation group), and cervical samples of healthy women (42 cases) during the same period were used as the control group. We determined the mRNA and protein expression of *Notch1*, *mir-224*, and *LRIG2* genes. We also analyzed the mutual relationship between *Notch1* gene expression and cervical cancer. The *Notch1* genes in the cervical cancer cells (HeLa) were silenced and overexpressed to measure cancer apoptosis with flow cytometry. After obstruction of the Notch1 signal pathway, the mRNA and protein expression in the *mir-224* and *LRIG2* genes was also measured. It was found that in comparison to the control group, *Notch1* gene expression in the observation group was significantly higher ($p<0.05$), cell growth was suppressed in Notch1 silent cell strains but accelerated in overexpressed Notch1 cells. The silencing of *Notch1* genes can lead to the reduction of mir-224/LRIG gene and protein levels, while overexpression of the Notch1 genes increased the mir-224/LRIG gene and protein levels. In conclusion, the *Notch1* gene is positively related to cervical cancer and can promote the occurrence of the disease. The potential mechanism shows that *Notch1* gene can regulate cervical cancer cell proliferation by regulating the mir-224/LRIG2 signal pathway.

Introduction

Cervical cancer is a common type of cancer worldwide (1). According to the statistical data from China in recent years, cervical cancer has shown an increasing morbidity in females. From the end of 2012, the morbidity of cervical cancer has been ranked the first among all of the cancer types. An increasing number of young individuals are becoming affected by cervical cancer (2,3). Even after advancements in medical research and treatment methods for cervical cancer, e.g., removal by operation, chemical treatment, and radioactive therapy, there is no effective method to treat cervical cancer (4,5). The molecular mechanism for development of cervical cancer remains to be established, and there are currently not enough studies on the cervical cancer cell or its proliferation. These factors can lead to a bottleneck in the treatment of cervical cancer. The coding protein of LRIGs, as a series of genes related to tumor inside the human body is mainly located in the cell membrane (6). Previous findings showed that the expression of LRIG3 can adjust the signal pathway of EGFR, thus curbing tumor growth (7). Related studies have proved that mir-224 can regulate the primary hepatocellular carcinoma signal, and cancer cell metastasis (8). In addition, the *LRIG3/mir-224* gene may be involved in the proliferation of the cervical cancer cells (9). Notch1 is a type of transmembrane protein involved in intracellular signaling mechanism, which is closely related to the occurrence of tumor and cancer (10).

In the present study, we explored *Notch1* gene expression in the control and observation groups. We explored the relationship between the expression levels and cervical cancer. The target regulation over LRIG3/mir-224 by Notch1 in the gene signal pathway for proliferation and apoptosis of cervical cancer cell provides a certain theoretical and experimental foundation for the occurrence and transfer mechanism of the cervical cancer cells.

Patients and methods

Patients. We selected 49 cervical cancer lesion patients (average age, 48.2±23.5 years), who were hospitalized during the period from February 2013 to February 2015 as the observation group. We also selected 42 females (average age, 47.3±24.7 years) with healthy cervix uteri as the control group.

Methods

RNA extraction. The procedure was performed as published in an earlier study (11). Cryopreserved tissue (0.1 g) was thawed and 0.45 ml RNA Plus was added. The tissue was homogenized

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Table I. Fluorescent quantitation PCR primers.

Genes	Primer sequence	Fragment length (bp)
Notch1	F: 5'-CGACGCACAAGGTGTCTTCCA-3' R: 5'-CGGACTTGCCCAGGTCATCTAC-3'	179
LRIG3	F: 5'-CCAACACACTCCTTGTGTTCCCGC-3' R: 5'-TACAACCCCACCACGTACCAGATGCA-3'	138
mir-224	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'	125
GAPDH	F: 5'-GAAGGTGAAGGTCGGAGTC-3' R: 5'-GAAGATGGTGATGGGATTTC-3'	226

F, forward; R, reverse.

and 0.45 ml of RNA Plus was added again to the centrifuge tube. Subsequently, 200 μl chloroform was added and centrifuged for 15 min at 8,500 x g, at 4˚C. The supernatant was collected and mixed with the same volume of isopropanol. After mixing, the contents were centrifuged for 10 min at 8,500 x g at 4˚C. The supernatant was discarded and 750 μl of 75% ethanol was added to mix gently. The contents were centrifuged for 10 min at 8,500 x g at 4˚C. The supernatant was discarded to remove the residual ethanol, and RNA grade water was added to the pellet left at the bottom of the tube.

Fluorescent quantitative PCR. fluorescent quantitative PCR was performed as per the manufacturer's instructions (Takara, Dalian, China). The list of primers is shown in Table I.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed according to the kit instructions (12). The standard sample was diluted 1:25 with assay buffer to prepare standard curve. The samples for testing were diluted 1:100, by adding 100 μl of sample into 50 μl of solution. The solutions were incubated at 25˚C at room temperature for 2 h, and the TMB chromogenic substrate was added. We measured the light absorption value at 495 mm, and then measured the concentration of Notch1 in each sample according to the standard curve.

Western blotting. A Roche animal protein extraction kit was used to extract the overall protein in the sample (13). Subsequently, western blot analysis was carried out based on the 'Guidebook for Molecule Cloning'.

Notch1 gene silencing and overexpression. HeLa cell lines were used and the procedure was followed, as published elsewhere (13).

Flow cytometry (FCM) testing for cell proliferation and apoptosis. The procedure was conducted as per the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit instructions. HeLa 229 cervical cancer cells were used to study the gene overexpression and gene silencing. Activated HeLa cells were inoculated in the 6-well plate and incubated at 37˚C, 5% CO_2. The time-points for Notch transfection (150, 200 and 250 μg/ml) were 24, 48 and 72 h. The cells with apoptotic agent were taken as the positive control and without apoptotic agent served as the negative control group. After cell growth, the cells were washed 2-3 times in phosphate-buffered saline (PBS) after discarding the culture medium. Subsequently, 0.2% trypsin was used to digest the cells for 2-3 min before washing by PBS. Finally, the cell medium was collected and centrifuged for 5 min at 4˚C at 800 x g to discard the supernatant.

The cells were counted after PBS resuspension to obtain 1-5x10^5 resuspension cells. The supernatant was discarded after centrifugation for 5 min at 80 x g, followed by resuspension of the cell in 500 μl binding buffer. Annexin V-FITC staining was then added, and mixed to the cell suspension. PI (5 μl) was added slowly and incubated at room temperature for 15 min in the dark after mixing.

Flow cytometer (Partec AG, Arlesheim, Switzerland) was used to test the cell cycle. The excitation wavelength was 488 nm while the emission light wavelength was >630 nm. For each test sample, generally only 1x10^4 cells were tested and the test was completed within 30 min. We selected PI and Annexin V-FITC to stain the squamous carcinoma of cervical cells at the same time and referred to the result as the control group.

CellQuest software (BD Biosciences, San Jose, CA, USA) was used to analyze the results and to calculate the rate of cell apoptosis. The results were interpreted as follows: non-viable non-apoptic cell area (PI^+, Annexin V^-, the left upper area), late non-viable apoptic cell area (PI^+, Annexin V^+, the right upper area), viable cell area (PI^-, Annexin V^-, the left lower area), and early non-viable apoptic cell area (PI^-, Annexin V^+, the right lower area).

Statistical analysis. SPSS 20.0 statistical software (Chicago, IL, USA) was used to process the data. The related measurements and calculations are expressed as mean ± SD. The χ^2 test was carried out.

Results

Expression of Notch1 gene at mRNA and protein levels in the control and observation groups. We tested the *Notch1* gene expression levels in the control and observation groups, and found that in comparison to the control group, the expression

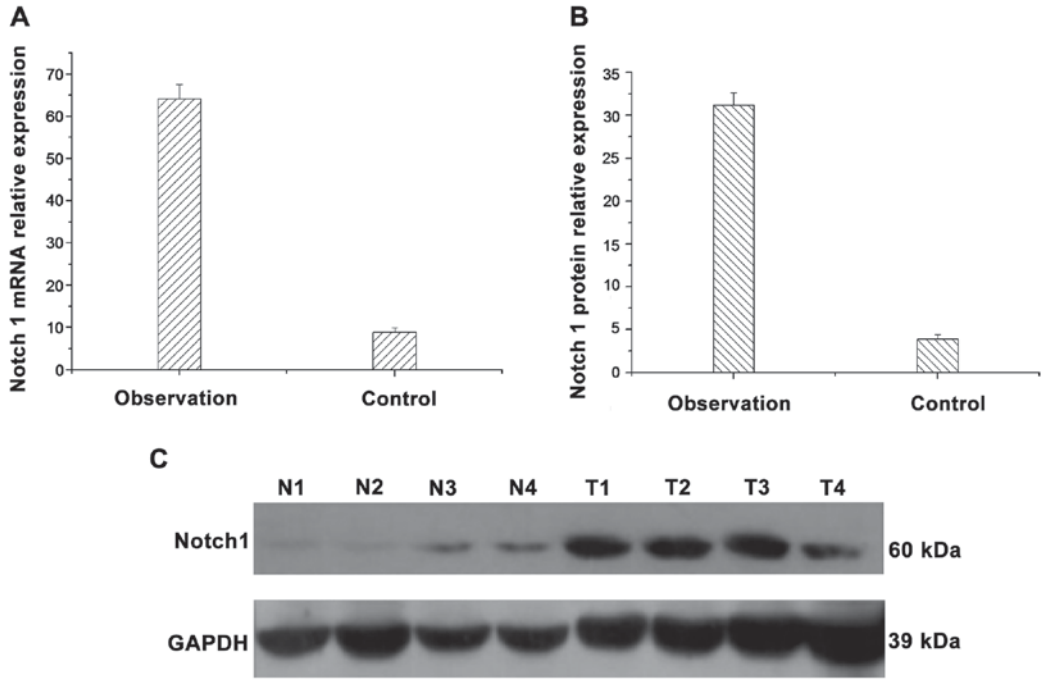

Figure 1. Gene expression levels of Notch1 in the control and observation groups (N1-N3, samples of the control group; T1-T4, samples of the observation group. (A and B) Significant difference observed. (C) Protein epxression is higher in the observation group.

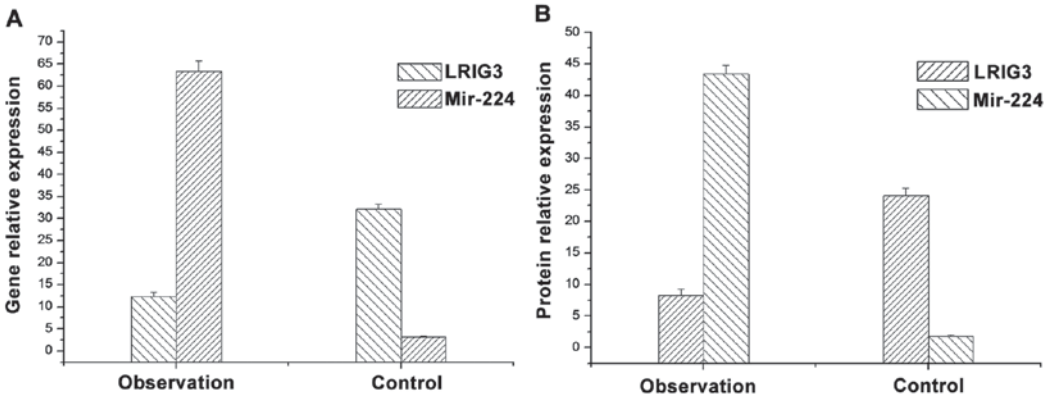

Figure 2. Expression levels of *LRIG3*/*mir-224* gene in silencing of Notch1 bacteria strains. The (A) mRNA and (B) protein levels are shown.

of mRNA of *Notch1* gene in the observation group was relatively high, with a significant difference shown between the two groups (Fig. 1A). The ELISA test also showed that there was a significant difference in *Notch1* gene between the two groups (Fig. 1B). The protein expression of *Notch1* gene in the two groups (Fig. 1C) showed that in comparison to the control group, the expression of protein product for *Notch1* gene in the observation group was significantly higher. From the mRNA and protein expression of *Notch1* gene in the control and observation groups, we developed the correlation between *Notch1* gene and cervical cancer. The high expression of *Notch1* gene was found to be associated with the occurrence of cervical cancer.

LRIG3/*mir-224 gene expression quantity in Notch1 silent bacterial strains*. After silencing the *Notch1* gene, we determined the LRIG3/mir-224 expression levels in different samples. As shown in Fig. 2, in comparison to the control group having the *Notch1* gene silenced in HeLa cells, the expression levels of mRNA (Fig. 2A) and protein (Fig. 2B) of the *LRIG3* gene was increased significantly, while the expression levels of mRNA (Fig. 3A) and protein (Fig. 3B) in *mir-224* gene was decreased significantly. It indicated that silencing of the *Notch1* gene can improve the levels of *LRIG3* gene and reduce the *mir-224* gene.

Expression quantity of LRIG3/*mir-224 gene with Notch1 overexpression*. We tested the expression quantity of the *LRIG3*/*mir-224* gene after silencing the *Notch1* gene in different samples. As shown in Fig. 3, in comparison to the control group, the expression quantity of mRNA (Fig. 3A) and protein (Fig. 3B) in *LRIG3* gene was increased significantly, while the expression quantity of mRNA (Fig. 3A and B) decreased significantly. It demonstrated that silencing of the *Notch1* gene increased *LRIG3* gene expression and decreased *mir-224* gene expression.

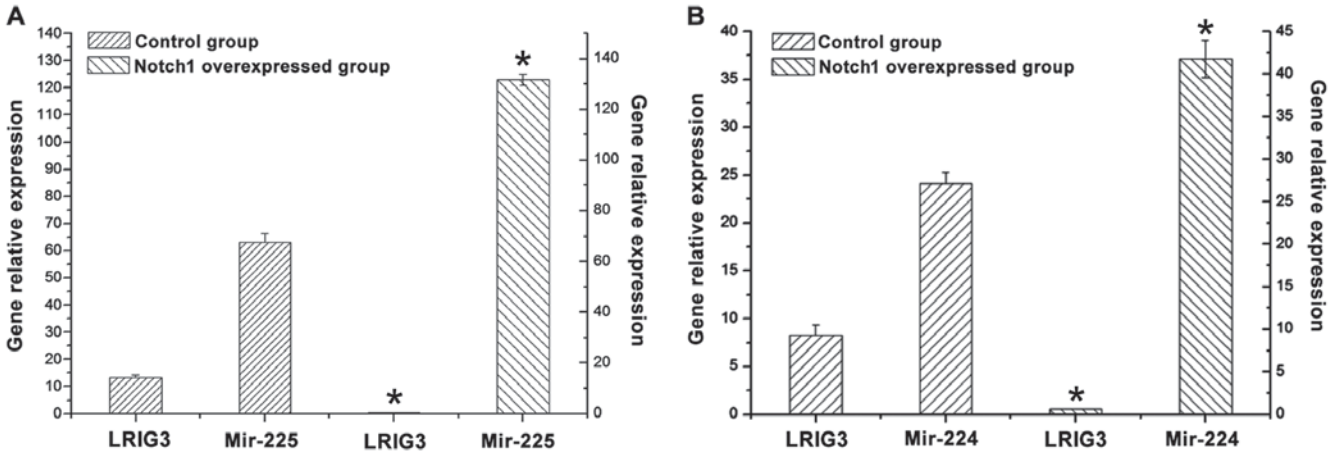

Figure 3. (A and B) Expression levels of *LRIG3*/*mir-224* gene in overexpressed Notch1 bacteria strains. *Compared with Control group, p<0.05.

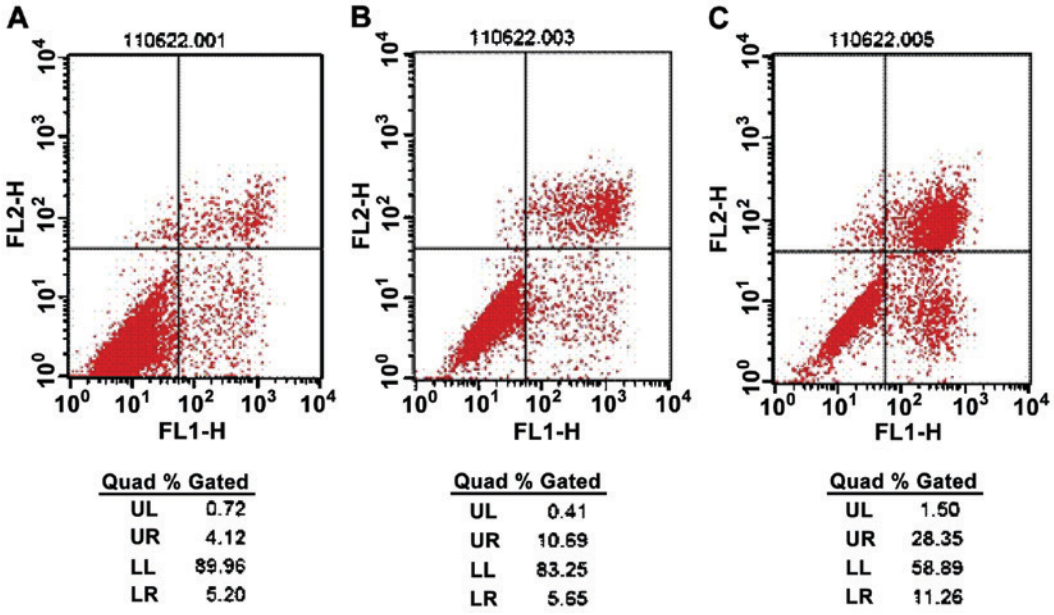

Figure 4. (A-C) Test for cell apoptosis in Notch1 bacterial strains silenced and overexpressed.

Cell apoptosis after Notch1 silencing and overexpression. The cell apoptosis by FCM after silencing the *Notch1* gene in different samples was estimated. As shown in Fig. 4B, in comparison to the control group, the apoptosis in HeLa cells after silencing *Notch1* gene was significantly decreased (Fig. 4A). The cell apoptosis by FCM after overexpression of *Notch1* gene showed that the overexpression of Notch1 facilitated apoptosis (Fig. 4C).

Discussion

The signal channel of transmembrane receptor protein Notch1 plays an important role in cell multiplication, differentiation, and apoptosis (14). It has been shown that abnormal expression of *Notch1* gene usually causes the transformation of undifferentiated cells to tumor cells to some extent (15). Notch1 can regulate the expression of many downstream genes, and can influence differentiation and metastasis of tumors and cancer cells, including HES, Hey and c-myc families (16). mir-224 is involved in metastasis and the invasive process of hepatocellular carcinoma cells (16). These results showed that there is a certain correlation between the expression of mir-224 and signal channels of PAK4 and MMP-9, and these signal channels are related to cervical carcinoma to certain extent. It has also been suggested that there is a possible association between *LRIG3* gene and the above genes, and *LRIG3* gene is involved in the two signal channels (17). However, there is still no clear study on the interaction between *Notch1* gene and signal channel of *mir-224*/*LRIG2* gene (18). We found that the expression of Notch1 in cervical carcinoma tissues was significantly higher than that in cervical tissues of normal patients. The silencing of *Notch1* gene and the expression of *mir-224*/*LRIG2* gene in related cells further influenced the cell apoptosis. This result suggested that *Notch1* gene can be targeted to regulate the involvement of signal channel of *mir-224*/*LRIG2* gene in the proliferation and apoptosis of cervical carcinoma cells. These results provide an experimental basis for the treatment of

cervical carcinoma and some indications for the treatment of advanced cervical carcinoma.

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