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CLINICAL STUDY

miRNA-489 as a biomarker in diagnosis and treatment of cervical cancer

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ABSTRACT
miRNA-489 was shown to be a suppressor factor in many cancers, however, evidence on the effects and mechanism of miRNA-489 in progression of cervical cancer is limited. So we aimed to determine the function of miRNA-489 in cervical cancer proliferation and apoptosis in our present study. Interestingly, we found that miRNA-489 was significantly down-regulated in cervical cancer tissues. miRNA-489 overexpression inhibited the cell proliferation and improved the cell apoptosis of cervical cancer cells. Further, miRNA-489 over-expression suppressed the activation of PI3K and AKT, and stimulated P53 proteins expression. In conclusion, our results suggested that miRNA-489 may be considered as a biomarker in cervical cancer and had suppressed the cell proliferation and stimulated cell apoptosis via PI3K/AKT/P53 signaling pathway *(Fig. 5, Ref. 26)*. Text in PDF *www.elis.sk.*
KEY WORDS: miRNA-489, PI3K, AKT, P53, biomarker.

Introduction

Cervical cancer is one of the most common gynecologic malignancies, with approximately 500 thousand new cases worldwide and about 300 thousand deaths each year. In recent years, related reports had shown that the incidence of cervical cancer is moving to younger age. Cervical cancer is known to be associated with high-risk HPV infection. However, studies have found that single high-risk HPV infection is not enough to cause cervical cancer, genetic factors and immune factors also play an important role in the development of cervical cancer (1–3). The study of the mechanism of cervical cancer development and diagnosis and treatment of cervical cancer is the focus of medical research.

The discovery of miRNAs offers hope for treatment of cervical cancer patients. The relative studies found that the abnormal expression of miRNAs is closely related to the occurrence and development of most tumors (4–6). MiRNA-489 is lowly expressed in many tumors such as melanoma, breast cancer and prostate cancer, suggesting that miR-489 is a tumor related factor (7–9). Up-regulation of miRNA-489 interferes with growth factor signaling pathway and promotes cell proliferation (10, 11). However, reports on the correlation between miRNA-489 and cervical cancer are limited. In our present study, we firstly evaluated the miR-489 expression in cervical cancer and adjacent normal tissues, secondly, we studied the effects and mechanism of miRNA-489 over-expression in cell proliferation and apoptosis of cervical cancer cells in in vitro study.

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Materials and methods

Clinical data

20 pairs of tumor and adjacent normal tissues from cervical cancer patients who were admitted to Taizhou People's Hospital from 2015 to 2016 were collected. The tissues were fixed in the 4 % faure Marin solution for48h, after that the tissues were stained with H&E and were subjected to in situ hybridization (ISH).

H&E staining

After routine dehydration, soaking wax, embedding, slicing, xylene dewaxing and hydration, hematoxylin staining was performed. The alcohol was added to 0.5 % hydrochloric acid and stained with 0.5 % eosin solution; dehydrated after neutral balata. The samples were observed under an optical microscope.

ISH methods

After routine dehydration, soaking wax, embedding, slicing, glass cleaning, baking 7 h at 180 °C, and use the sterilized cotton swab on the slide under aseptic condition. After the drying, the dust is dust-free and the package is kept at 4 °C. Paraffin section at 60 °C of rewarming, routine dewaxing, rehydration; 3 % H_2O_2 at room temperature, 10 min, DEPC treated distilled water washing 3 times, each time 5 min; 3 % citric acid diluted pepsin, 37 °C 20 min digestion. 80 mL pre-hybridization droplets were added to the slides, and 20 ml 20 % glycerin was placed in the wet box to keep the moisture content in the box, and the temperature was raised at 37 °C for 4 h. Then 90 ml hybrid solution was added to

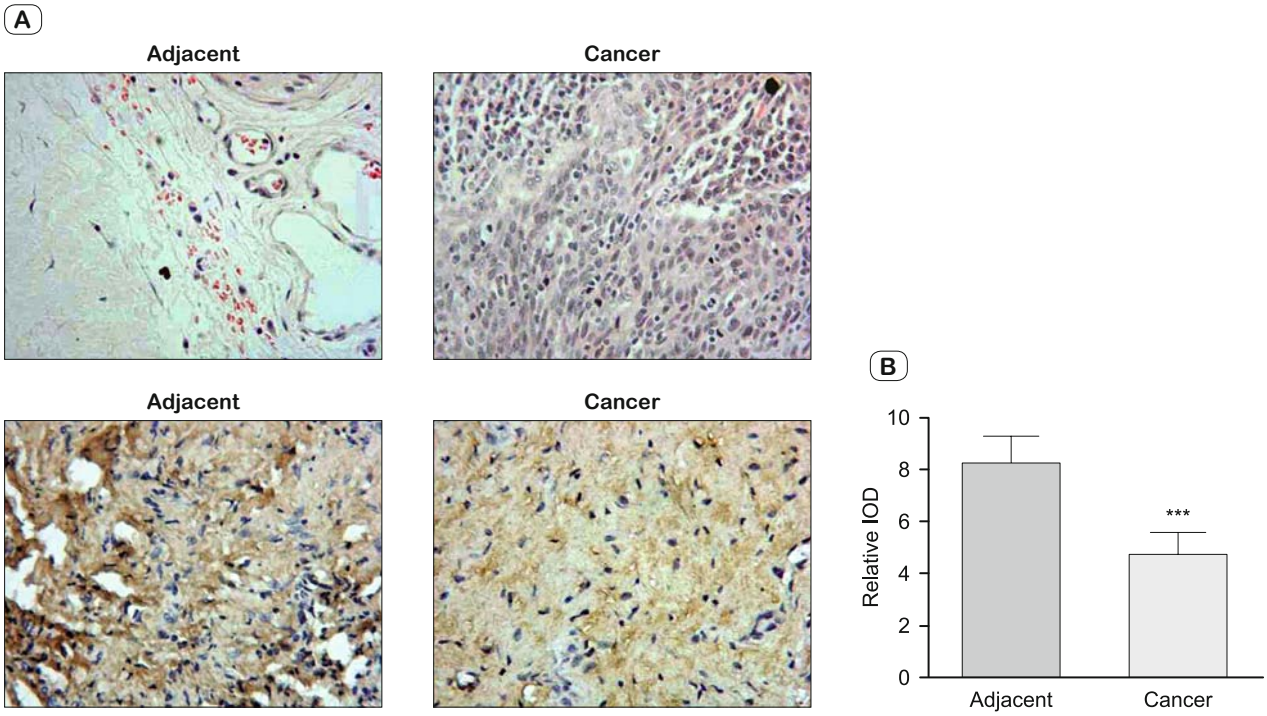

Fig. 1. The clinical data in cervical cancer. 1A: The pathology of cervical and adjacent normal tissues by H&E staining (´200). 1B. The miRNA-489 expression in cervical and adjacent normal tissues by ISH (´200). *** p < 0.05, compared with NC group.

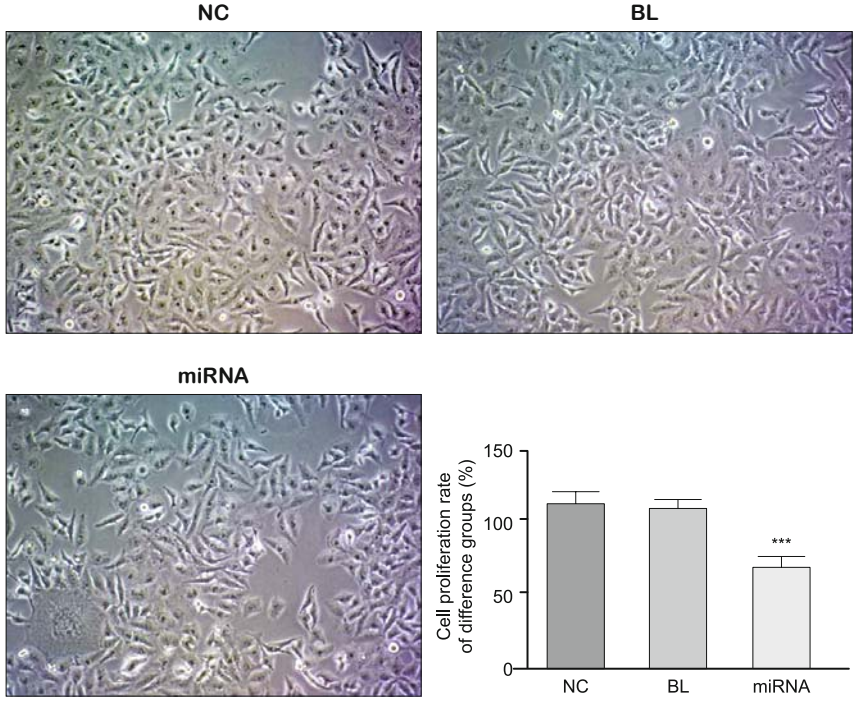

Fig. 2. The cell proliferation of different groups. *** p < 0.05, compared with NC group.

every section, cultured at 42 °C ~ 45 °C for 16~20 h. Then it was washed by preheat 2×SSC, 0.5×SSC and 0.2×SSC at 37 °C, 3 times for 5 min. Drip sealing solution, 37 °C for 30 min, Throw out the excess liquid; Biotinylated Rabbit anti digoxin, cultured at 37 °C for 60 min; After washing by PBS, adding the SABC to culture at 37 °C for 20 min; Washing by PBS and coloring by DAB, He-

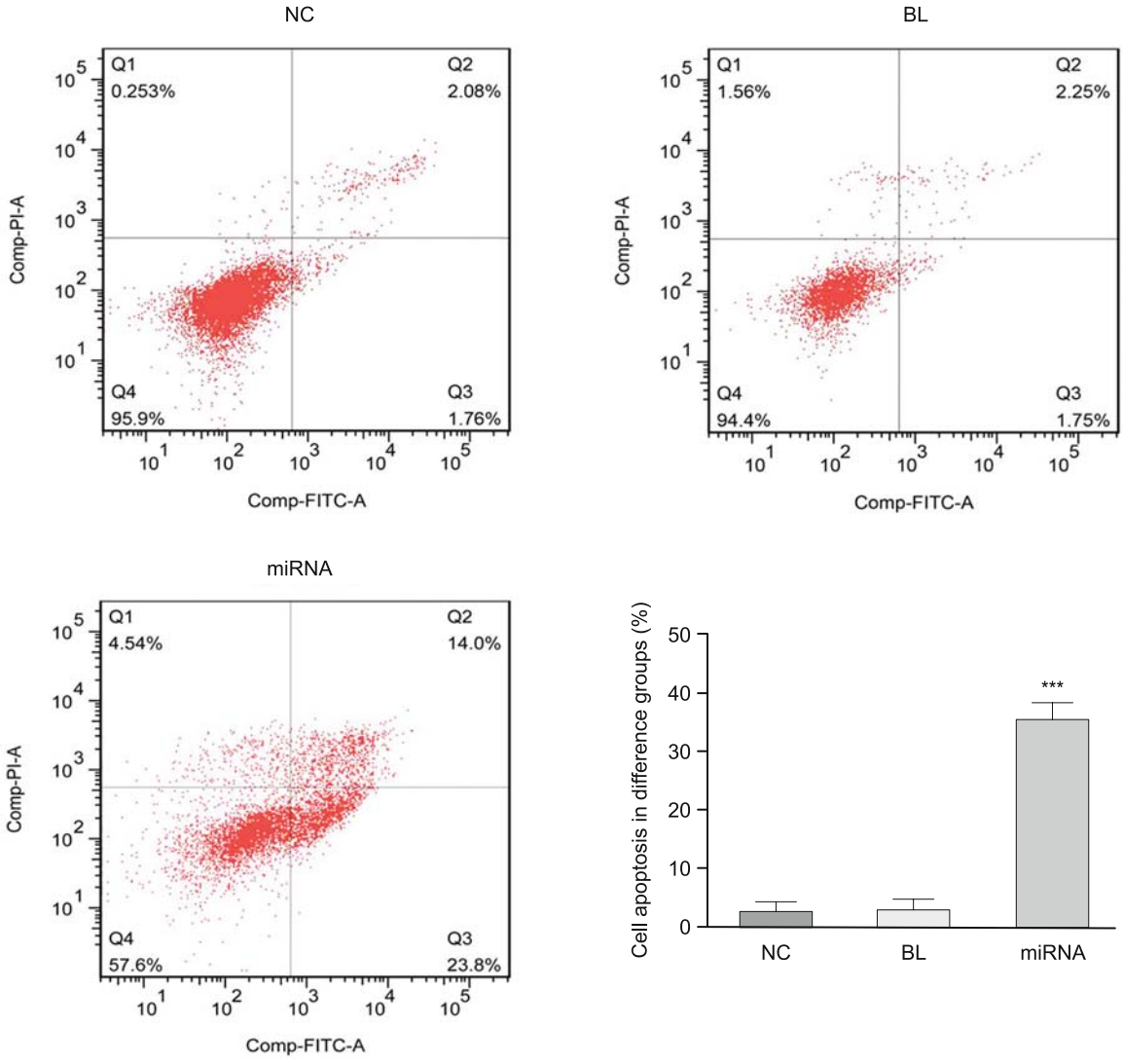

Fig. 3. The cell apoptosis of different groups. *** p < 0.05, compared with NC group.

matoxylin staining, washed back to blue, dehydrated, transparent, neutral balata, observation under the microscope.

Cell culture and grouping

Hela cells which have been purchased from ATCC (USA) were cultured in RPMI1640 culture medium containing 10 % fetal bovine serum, 100 U/ml Streptomycin and 300 mg/L glutamine and conventional subculture. The Hela were divided into 3 groups: NC group (treated with normal treatment); BL group (transfected with empty vector) and miRNA group (transfected with miRNA-489).

Cell transfection

The Hela cells in logarithmic growth phase were inoculated to the 6 pore plate at 4×10^6 cells/ pore, and each cell 2ml was divided into 3 groups after cell adherence: (1) NC group: the cells were treated with normal treatment; (2) BL group: the cells were transfected with 50 nmol/L Lipofectamine 2000; (3) miRNA group: the cells were transfected with 50 nmol/L Lipofectamine 2000 with miRNA-489.

Cell proliferation by CCK-8

The transfected Hela cells resulted in single cell suspension after 24h, and 4×10^3 cells per hole were injected into the 96 hole plate, each hole 200 mL, After cell attachment, it was cultured in the incubator for 48 h, 5 holes were set in each group, and a blank correction group (without adding PBS of the same amount of cells) was set up. At the end of culturing for 1h, 0.01 ml CCK-8 solution was added to each pore. Then the cells were cultured for 1 h, the cell proliferation was measured by enzyme labelling apparatus at 490 nm.

Cell apoptosis by flow cytometry

Collecting the cells of different groups which were cultured for 24 h it was Operated in accordance with the apoptosis of An-

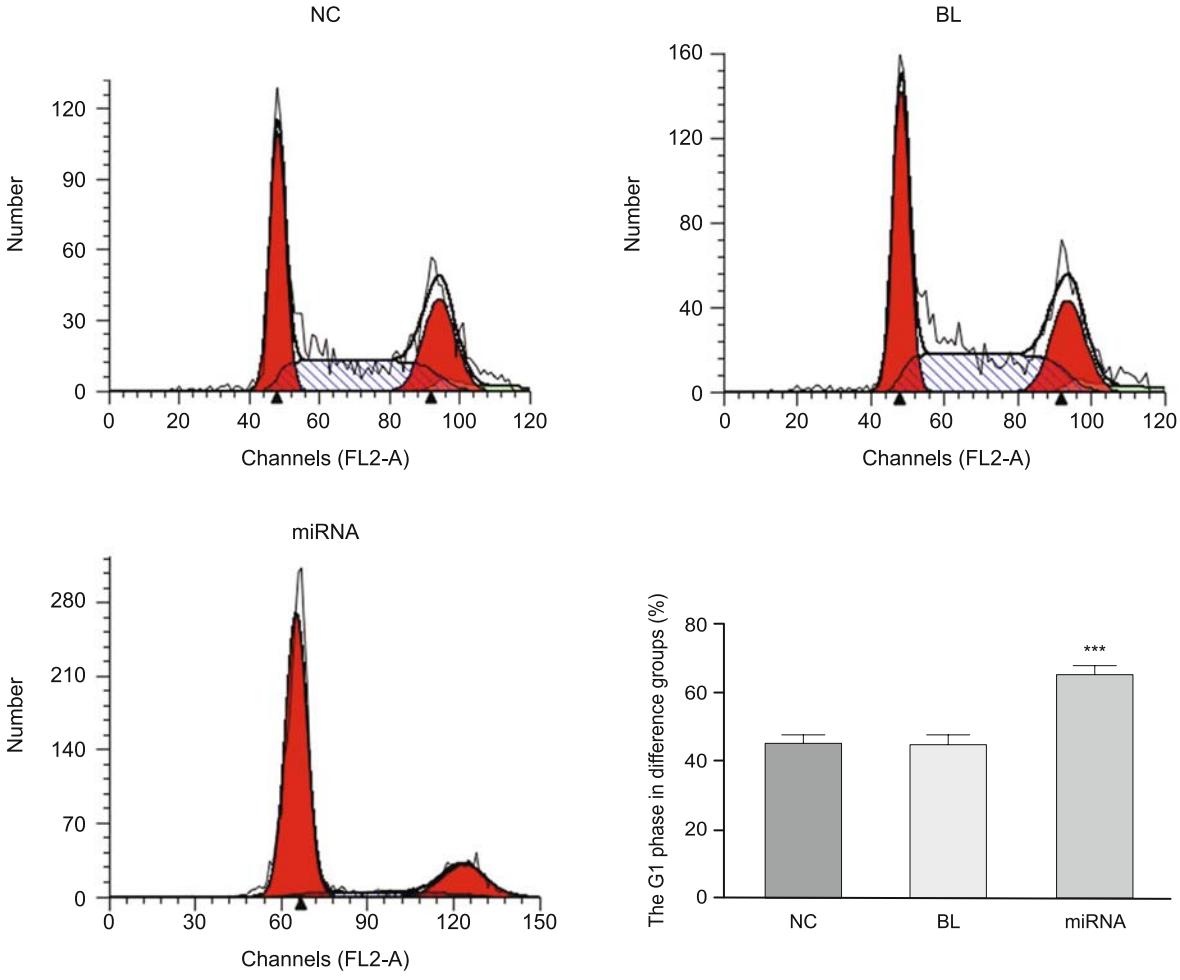

Fig. 4. The cell cycle of different group. *** p < 0.05, compared with NC group.

nexin V-FITC cells detection kit, to join the 5 μLAnnexin V-FITC after mixing, adding 5 μL propidium iodide (PI), light reaction at room temperature for 15 min and immediate detection by flow cytometry.

Cell cycle by flow cytometry

The cells of different groups were collected and were prepared into single cell suspensions and operated according to the cell cycle test kit instructions. After washing the cells with PBS to join the 10 g/ml DNaseA, 37 DEG 30 min, adding 10 g/ml PI at room temperature and light reaction of 30 min, flow cytometry was performed after using Modifit software to analyze the distribution of cell cycle in each group.

WB assay

Collecting the Hela cells of different groups, Cell lysis and separation of cell protein samples by measuring the amounts of protein Bradford concentration (20 g/ hole), conventional 8 %SDS-PAGE electrophoresis, semi dry transfer membrane transfer membrane, 5 % skim milk powder closed, with specific antibody (1:1000) to 4 °C overnight incubation, HRP labeled Goat anti rabbit IgG as second antibody (1:2500) and incubated at room temperature for 1 h, ECL color, strip exposure intensity with Quantity (Bio, RAD) One 4.6.2 analysis software, using GAPDH as internal control, and internal control through the gray ratio, the relative expression amount of the target band.

Statistical analysis and methods

The data were analyzed by SPSS 20.0 software, measurements are expressed as mean ± standard deviation, variance analysis was used between the normal distribution variables and the multiple groups, and the t test was used between the two groups, $p < 0.05$ showed significant statistical difference.

Results

Clinical and analyzing

By the H&E staining, the invasion and migration of cancer tissues were significantly enhanced compared with adjacent normal tissues (Fig. 1A). The miRNA-489 expression of cervical cancer

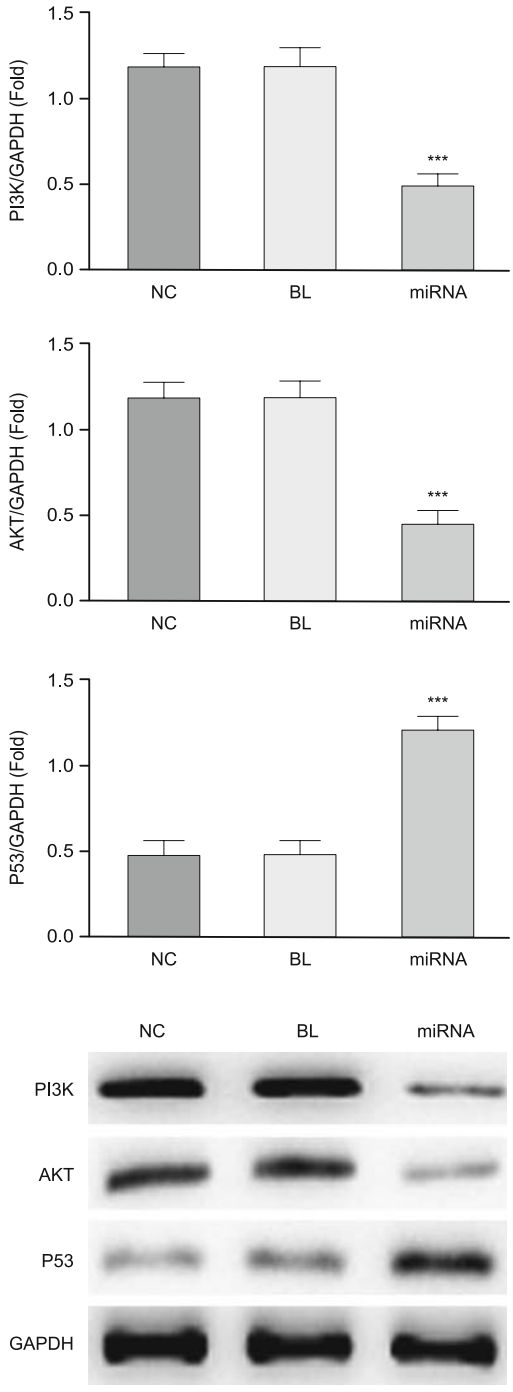

Fig. 5. The relative proteins expressions of different groups. *** p < 0.05, compared with NC group.

tissues were significantly down-regulated compared with adjacent normal tissues (p<0.05) (Fig. 1B).

Cell proliferation by CCK-8 assay

Compared with NC group, the cell proliferation rate of miRNA group was significantly suppressed (p < 0.05) (Fig. 2).

Cell apoptosis by flow cytometry

To evaluate the effects of miRNA-489 in Hela cells, the cell apoptosis rate of miRNA group was significantly up-regulated compared with NC group (p < 0.05) (Fig. 3).

Cell cycle by flow cytometry

To measure the effects of miRNA-489 in cell cycle of Hela cells, the G1 phase rate of miRNA group was significantly up-regulated compared with NC group (p < 0.05) (Fig. 4).

Relative proteins expressions by WB assay

Compared with NC group, the PI3K and AKT proteins expressions of miRNA group were significantly suppressed, however, the P53 protein expression of miRNA group was significantly stimulated (p < 0.05) (Fig. 5).

Discussion

In recent years, the relationship between miRNAs and human tumors has attracted wide attention. The research showed that the expression level of mRNA changed in many cancers, such as lung cancer, colon cancer, miRNA and Burkitt's lymphoma and chronic lymphocytic leukaemia, proto oncogenes and tumor suppressor genes may play a role (12–14). It has been found that miR-21, miR-191, miR-223, miR-let-7a and miR-106b were closely related to the development of cancer (15–18). Studies have shown that miRNA-489 is also associated with the occurrence and development of tumors (8–11). The results of our present study have shown that the expression miRNA-489 was down-regulated in the cervical cancer tissues, the Hela cells proliferation was suppressed and Hela cells apoptosis was improved with miRNA-489 over-expression. PI3K/AKT signaling pathway is an important signal transduction pathway in cells, through the activation of downstream mTOR, NF- kappa B pathway, induced inactivation of Caspase, cyclin related gene transcription or its transcriptional regulator of the formation and regulation of Bcl-2 family members Bcl-2, Bax and Bcl-XL etc. The balance of expression on cell proliferation and apoptosis play an important biological function of resistance (19–22). P53 protein is an important downstream site of PI3K/AKT signaling pathway (23, 24). P53 can affect cell proliferation and apoptosis by regulating cell division cycle (25, 26). In our present study, with miRNA-489 over-expression, the cell proliferation of Hela cells was significantly suppressed, the cell apoptosis was significantly improved through G1 phase rate increase n via regulation of PI3K/AKT/P53 pathway.

In conclusion, miRNA-489 might be a biomarker and was a suppressor factor in the cervical cancer. With miRNA-489 over-expression, the cell proliferation was suppressed and apoptosis was enhanced by G1 phase rate increase in Hela cells.

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