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CLINICAL STUDY

MicroRNA-375 regulates proliferation and apoptosis of glioma cancer cells by inhibiting CTGF-EGFR signaling pathway

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ABSTRACT

AIM: To evaluate the correlation between miRNA-375 and cell proliferation and apoptosis in glioma cancer cell.
METHODS: Collecting 30 cases of glioma cancer patients and 30 cases of cerebral infarction patients. The miRNA-375 and CTGF protein expressions were evaluated by ISH and IHC methods. In the cell experiment, the U87 cells were divided into 3 groups: NC group (the cells were treated with normal method); BL group (the cells were transfected with empty vector) and miRNA group (the cells were transfected with miRNA-375). The U87 cell proliferation and apoptosis rates and cell cycle of the different groups were measured by MTT and flow cytometry. The relative proteins (CTGF, EGFR, AKT, Erk and P21) expressions were measured by WB assay.
RESULTS: The miRNA-375 and CTGF expressions of glioma cancer tissues were significantly different compared with those of no-cancer tissues ($p < 0.05$, respectively). In the cell experiments, the cell proliferation of miRNA group was significantly decreased compared with that of NC group ($p < 0.05$); the cell apoptosis and G1 phase rate of miRNA group was significantly decreased compared with NC group ($p < 0.05$, respectively). Depending on the WB assay, the CTGF, EGFR, AKT, Erk and P21 proteins expressions of miRNA group were significantly different compared with proteins expressions of NC group ($p < 0.05$, respectively).
CONCLUSION: miRNA-375 over-expression suppresses glioma cancer cells development via CTGF-EGFR pathway *(Fig. 3, Ref. 30)*. Text in PDF *www.elis.sk.*
KEY WORDS: miRNA-375, CTGF, EGFR, glioma cancer.

Introduction

Glioma is the most common primary central nervous system tumor. 50~60 % of malignant brain tumors are gliomas (1). The current treatment strategies for glioma are surgery, radiation therapy, chemotherapy, or other adjuvant therapy (2–4). However, the high metastasis rate and the tolerance to radiotherapy and chemotherapy make the prognosis of glioma very poor (5–7). Intervention or licensing of disease from a molecular perspective provides new insights into the current treatment dilemma. microRNA (miRNA) miRNA plays an important regulatory role in tumorigenesis and development, and plays a regulatory role in the proliferation, invasion and apoptosis of cancer (8–10). In some previous studies it was shown that miRNA-375 had anti-tumor effects on some kinds of cancer (11–14). However, it has been unclear that there is a correlation between miRNA-375 and glioma. In our present study, we firstly evaluated the miRNA-375 in the normal and glioma cancer tissues, and then we assessed miRNA-375 over-expression in glioma cancer cell biological activity.

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Materials and Methods

Clinical data

The glioma cancer tissues were collected from 30 cases of glioma cancer patients who were treated in our hospital, and the normal control tissues were collected from 30 cases of cerebral infarction patients who were treated at the same periods. The tissues were fixed in 4 % paraformaldehyde, Paraffin embedded section as 4 μm.

In situ hybridization assay

Paraffin wax after dewaxing, Placed in a mixture of 0.8 % pepsin and hydrochloric acid, digest for 10 min in water bath at 37 °C, Using TBS to wash 5 min, dehydrate ethanol, and then dry at room temperature. Denaturation at 98 °C for 10min, Ice bath annealing, hybridization for 1 h at the 37 °C in the water-bath. Washing by TBS 5 min×3 times, after incubation with alkaline phosphatase labeled digoxin antibody, incubated at room temperature for 0.5 h, after washing for 2 times, adding BCIP/NBT and color in the dark, 0.3 % Nuclearfast red lining dye, dehydrated, transparent after mounting.

Immunohistochemistry (IHC)0

Monoclonal antibody CTGF SP kit was purchased from Sigma (1 : 100). Using 3 % H_2O_2 to block endogenous peroxidase, microwave heating antigen repair, 10 % normal goat serum was incubated with primary antibody at –4 °C overnight in the refrig-

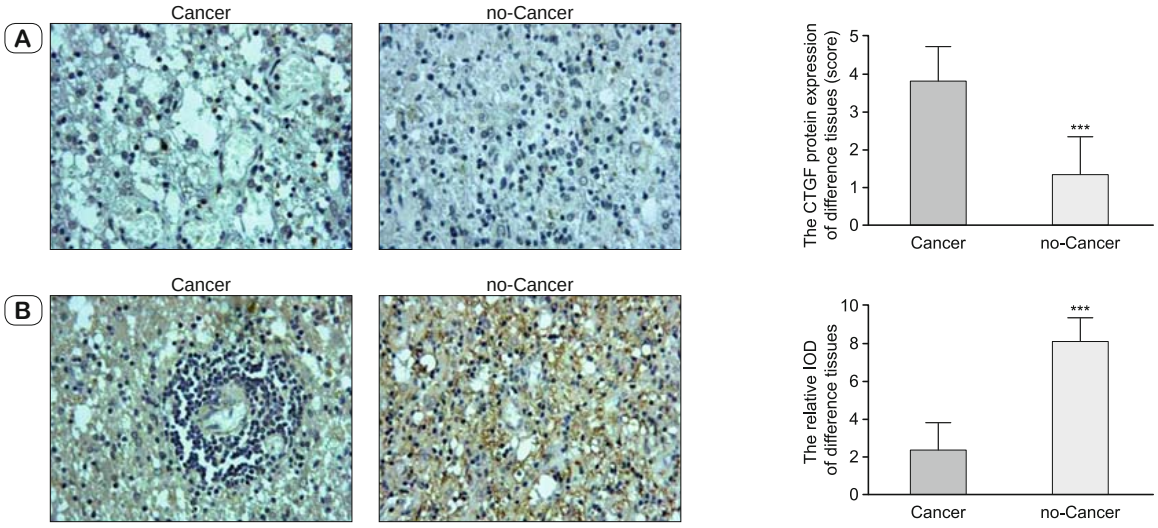

Fig. 1. Clinical data. A. The CTGF protein expression of different tissues by IHC (×200), *** p < 0.05, compared with cancer tissues, B. The miRNA-375 expression of different tissues by ISH (×200), *** p < 0.05, compared with cancer tissues.

erator. Sections were washed by PBS, after that, adding second and third anti-body, DAB color, hematoxylin staining, conventional mounting.

Cell culture and grouping

Human glioma cells U87 were cultured by RPMI-1640 with 10 % fetal bovine serum (FBS) in the incubator (37 °C, 5 % CO_2), Digested with 0.25 % trypsin, the passage was changed every 2 days. The U87 cells were divided into 3 groups: NC group (the cells were treated with normal methods); BL group (the cells were transfected with empty vector) and miRNA group (the cells were transfected with miRNA-375). After transfection for 48 h, experiments were started.

MTT assay

The cells of different groups were inoculated in 96-holes as 1×10^6/hole. 20 µl MTT (5 g/L) was added to every hole and continued to be cultured for 4 h, the culture medium was added and DMSO 150 l was added. The absorbance was detected at 490 nm with an enzyme analyzer. The cell proliferation rates of different groups were measured.

The cell apoptosis by flow cytometry

The cells were inoculated in the 6-hole plate as 1×10^5/hole. A single cell suspension was made by trypsin digestion, and PBS was used to wash 3 times. According to the instructions, the cells were stained with PI/Annexin V, and the apoptosis rate of each group was detected by light staining 20 min. The experiment was repeated 3 times, with 3 holes at each time.

The cell cycle by flow cytometry

The cells were inoculated in the 6-hole plate as 1 x 10^6/hole. A single cell suspension was made by trypsin digestion, and PBS was used to wash 3 times. After adding 1 mL PBS solution, the cells were suspended and then 2 mL 70 % ethanol were slowly dropped in (–20 °C pre-cooling). After mixing, they were fixed overnight at 4 °C. Centrifuged at 1000 r/min for 1.5 min, 70 % ethanol solution was discarded, then it was washed 2 times at 4 °C with PBS solution, then the supernatant was discarded. 800 µl of 20 µg/mL PI solution was added containing 200 µg/mL RNase, mixed, cultured at 37 °C for 30 min in dark, after that Machine inspection was done.

Western Blot assay

The cells were collected from different groups, protein concentration was measured by BCA method according to the operation of the nucleoprotein extraction kit, 12 % polyacrylamide gel and 5 % concentrated gum were prepared, and the sample was 30 µg, after the vertical electrophoresis, the gel was transferred to the PVDF film by wet process. According to the protein marker cut

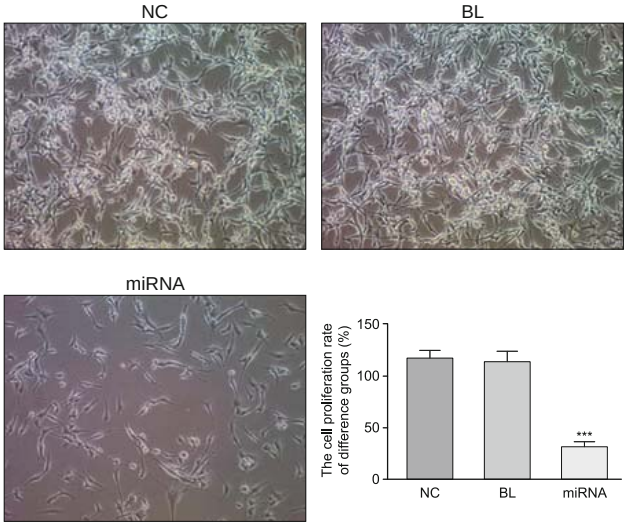

Fig. 2. The cell proliferation rate of different groups. *** p < 0.05, compared with NC group.

film, ir was washed with TBST solution, with 5 % skimmed milk powder on the shaking table atroom temperature closed for 1.5 h, and then wash away with skim milk powder solution. Adding the primary antibodies CTGF, EGFR, AKT, Erk, P21 and GAPDH (1 : 1000) to culture, The PVDF membrane and the corresponding antibody were placed in the incubation bag and incubated at 4 °C over-night. Next day, the PVDF membranes were washed by TBST (10 min x 3times). Adding goat anti mouse HRP-IgG as 1 : 5000 to culture for 1.5 h, after washing by TBST, ECL light treatment was carried out to take pictures and save the results.

Statistical analysis

All experiments were performed three times and data were analyzed with GraphPad Prism 5. Statistical evaluation of data was performed using the t-test. $p < 0.05$ was considered to be statistically significant. Spearman's nonparametric correlation test was performed to test the correlation between the expression levels of miR-181b and IGF-1R by SPSS 19.0 software.

Results

Clinical data

Compared with Cancer tissues, the CTGF protein expressions of non-Cancer tissues were significantly down-regulated and the miRNA-375 expressions of non-Cancer tissues were significantly up-regulated ($p < 0.05$, respectively). The relative data are shown in Figure 1. Depending on these results, we inferred that miRNA-375 might be in negative correlation with CTGF in cancer tissues.

miRNA-375 suppresses cell proliferation in vitro

With miRNA-375 infection, the cell proliferation rate of miRNA was significantly suppressed compared with that of NC group

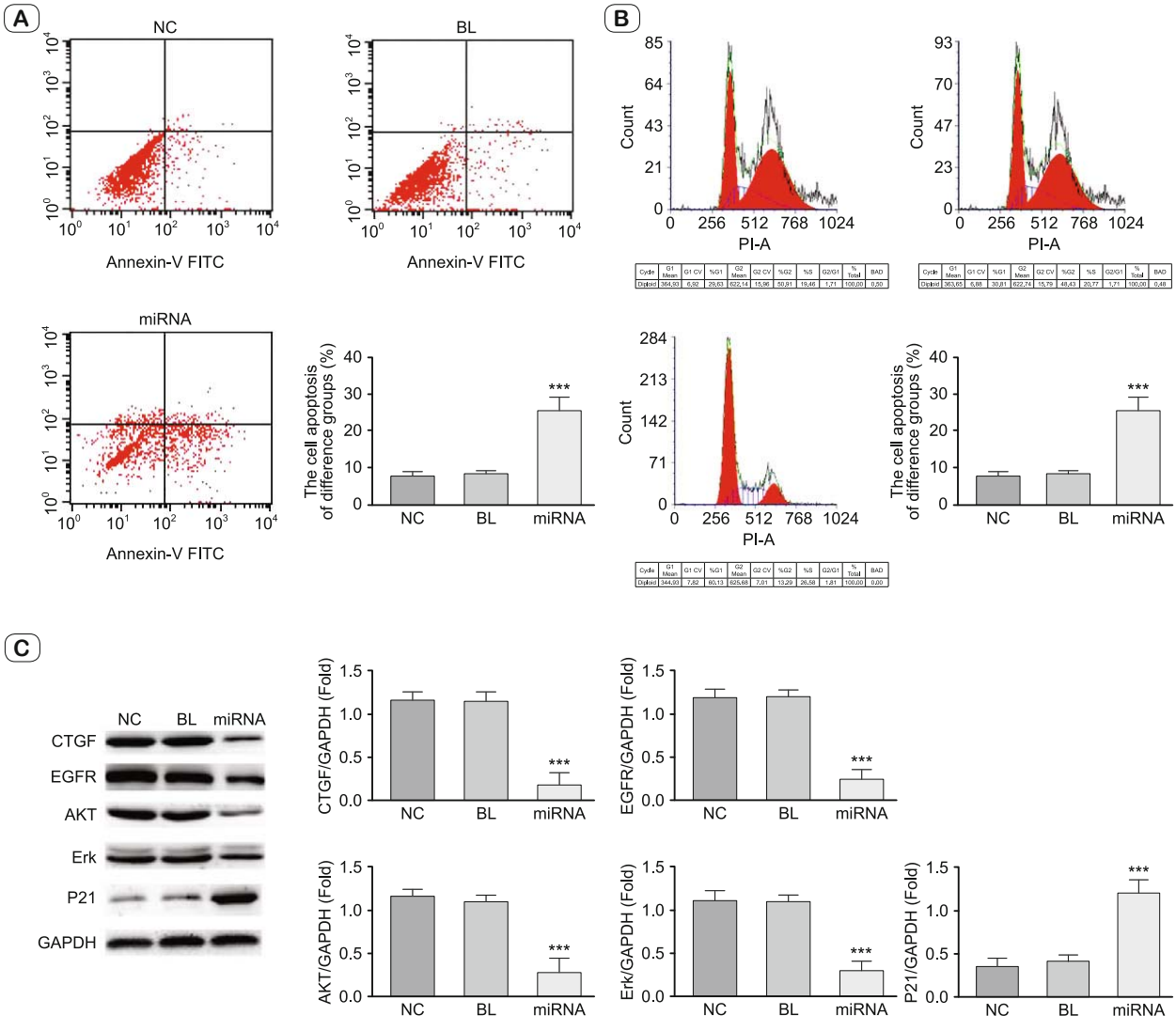

Fig. 3. The cell experiments in U87 cell. A. The cell proliferation of different groups by MTT, *** $p < 0.05$, compared with NC group, B. The G1 phase rate of different groups, *** $p < 0.05$, compared with NC group, C. The relative proteins expression of different groups by WB assay, *** $p < 0.05$, compared with NC group.

($p<0.05$), however, there were no significant differences between NC and BL groups that shows that the empty vector had no effects on cell proliferation ($p<0.05$). The relative data are shown in Figure 2.

miRNA-375 stimulated cell apoptosis rate

The data showed that the cell apoptosis of miRNA group was significantly up-regulated compared with NC group ($p < 0.05$); however, there were significant differences in cell apoptosis rates of NC and BL groups ($p < 0.05$). The relative data are shown in Figure 3A.

miRNA-375 up-regulates G1 phase in cell cycle

The G1 phase of miRNA group was significantly enhanced compared with NC group ($p < 0.05$); however, there were no significant differences between G1 phase of NC and BL groups ($p > 0.05$). The relative data are shown in Figure 3B.

miRNA-375 has effects to relative proteins

Compared with NC group, the CTGF, EGFR, AKT and Erk proteins expressions of miRNA group were significantly down-regulated ($p < 0.05$, respectively), P21 protein expression of miRNA group was up-regulated ($p < 0.05$). However, there were no significant differences between NC and BL groups in CTGF, EGFR, AKT, Erk and P214 proteins expressions ($p > 0.05$, respectively). The relative data are shown in Figure 3C.

Discussion

At present, due to the unlimited proliferation of malignant tumor cells, tumor patients are mostly incurable, have low survival rate and low quality of life, and so on, so that it has become the focus of attention world-wide. It is very important to study the mechanism of the occurrence and development of malignant tumor for its early diagnosis and targeted therapy. Connective tissue growth factor (CTGF) expression was closely correlated with the development of cancer and was involved in cell proliferation, development, adhesion, migration, angiogenesis, and predicts prognosis (15–20). miRNA regulates mRNA levels by targeting 3'-UTR of the target molecule, thereby regulating protein levels (21, 22). Previous study found that miRNA-375 had anti-tumor effects targeted by CTGF (23). However, it has the miRNA-375 expression in the glioma and the correlation between miRNA-375 and CTGF in the glioma cancer has been unclear. In our present study, depending on the clinical data, we have found that miRNA-375 had low expression and CTGF protein had high expression in the glioma tissues. Based on these results, we inferred that miRNA-375 might target CTGF in glioma. In the subsequent experiments we wanted to explain the effects and mechanism of miRNA-375 in the glioma cancer cells.

In our present study, the results have shown that miRNA-375 over-expression can suppress glioma cancer cell U87 cell proliferation and stimulate cell apoptosis by staining the cell cycle in G1 phase. In order to explain the mechanism of miRNA-375 in the development of glioma cancer cell, we evaluated the relative proteins expression at the molecular biological level.

Epidermal growth factor receptor (EGFR), a membrane receptor with tyrosine kinase activity, is expressed ubiquitously in human epidermal and stromal cells and is highly expressed in a variety of human malignancies (24). EGFR has an important role of downstream gene of CTGF (25, 26). The signal transduction effects mediated by EGFR are multi directional, including proliferation, migration, cell differentiation and the stability of the internal environment, and are closely related to cell regeneration and the occurrence and development of malignant tumors (27, 28). EGFR also mediated the AKT/Erk/P21 pathway, AKT/Erk/P21 signaling pathway has a pivotal role in cell apoptosis and in the cell development (29, 30). That pathway regulates cell apoptosis via controlling the cell cycle. In this study, we found that the AKT and Erk protein expressions were significantly suppressed and P21 protein expression was significantly stimulated in miRNA-375 over-expression group. Depending on these results, we inferred that miRNA-375 may suppress glioma cancer cell U87 cell proliferation via AKT/Erk/P21 signaling pathway.

In conclusion, miRNA-375 has anti-tumor effects via CTGF/EGFR/AKT/Erk/P21 pathway in U87 cell, a type of glioma cancer cell lines of in vitro study.

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