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Original Article

The effect of miRNA-210 on the biological behavior ovarian carcinoma A2780 cells

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Abstract: Objective: To explore the expression of miRNA-210 (miR-210) in human ovarian cancer and the effect of silencing of miR-210 on the biological behavior of A2780 cells. Methods: Tissue specimens of 20 patients suffering from ovarian cancer hospitalized in our hospital from June 2010 to June 2012 were collected. Real-time PCR was used to detect the expression of miR-210 in ovarian cancer and para-cancer tissues, as well as ovarian cancer cell line A2780 and normal ovarian cell line IOSE80. After miR-210 inhibitor was transfected into A2780 cells by Lipofectamine™ 2000, the proliferation of A2780 cells was detected by MTT and soft agar colony formation assay, cell cycle and apoptosis by flow cytometry and cells' migration and invasiveness by Transwell assay. A tumor model was established by inoculating A2780, A2780/NC and A2780/210 cells into BALB/C nude mice subcutaneously. Results: The expression level of miR-210 in ovarian cancer tissues and A2780 cells was evidently higher than that in para-cancer tissues and normal ovary cells ($P<0.01$). After transfection with miR-210 inhibitor, the proliferation and clonality of A2780 decreased significantly ($P<0.05$), the number of cells arrested at G0/G1 phase and the ratio of apoptotic cells increased significantly ($P<0.05$), and cell's migration and invasion were inhibited ($P<0.05$). Results of an *in vivo* tumor growth experiment showed that, when the expression of miR-210 was inhibited, the growth rate of subcutaneous tumors in mice inoculated with A2780/210 cells decreased significantly compared with that in mice inoculated with A2780 cells and A2780/NC cells ($P<0.05$). Conclusion: miR-210 was overexpressed in ovarian tissues and cells. The proliferation, migration and invasion of A2780 cells decreased significantly after transfection with miR-210 inhibitor.

Keywords: Ovarian cancer, miRNA-210, proliferation, migration, invasion

Introduction

The morbidity of ovarian cancer ranked third among various gynecology malignant tumors, while its mortality rate ranked first [1]. Despite receiving advanced surgery, conventional chemotherapy and radiotherapy, the long-term survival rate of patients with advanced ovarian cancer remained not more than 20% [2, 3]. Therefore, it is necessary to find out a new therapeutic option to improve patients' compliance and kill tumor cells.

miRNA, a short endogenous non-coding RNA, post-transcriptionally regulates the expression of tumor-related genes, and influences (influencing) tumor proliferation, invasion and migration [4, 5]. It was indicated that [6] the expression of miRNA-210 (miR-210) was up-regulated in kidney, pancreatic and colon cancer and miR-210 regulated the proliferation, migration and invasion of cancer cells in kidney cancer [7-9]. As the key factor inducing hypoxia, hypoxia-inducible factor (HIF) probably played a role in regulating miR-210. In kidney cancer cell line RCC4, miR-210 induced hypoxia by regulating the cancer suppressor gene VHL (vonHippel-Lindau) [10]. Up to now, there are few studies on the effect of miR-210 on the proliferation, migration and invasion of ovarian cancer cells. This study aims at investigating the expression of miR-210 in ovarian cancer, para-cancer tissues, ovarian cancer cells and normal ovary cells and further exploring the effect of miR-210 on the proliferation, migration and invasion of ovarian cancer cells *in vitro*.

Materials and methods

Cell culture

Human ovarian cell line A2780 and human normal ovarian cell line IOSE80 were both purchased from Shanghai Institute of Biochemistry and Cell Biology and stored in our department. Inoculated into a RPMI 1640 medium with 10% fetal calf serum (GIBCO), these cells were subcultured in a cell incubator with 5% CO_2.

Clinical data

The object of study was specimens taken from 20 patients pathologically diagnosed with ovarian cancer who underwent surgery from June 2010 to June 2012. These patients were 48-62 years old. Ovarian cancer and para-cancer tissues (normal ovary tissues at least 5 cm away from tumor edge) were taken from their specimens, preserved in liquid nitrogen within 10 min after separation and reserved for subsequent experiments. Before operation, patients were informed of the objective and methods of this study and signed informed consent forms. This study had been approved by the ethics committee of Hospital.

Main reagents

RPMI 1640 medium was purchased from Gibco (the US); fetal calf serum from Zhejiang Tianhang Biological Technology Co., Ltd.; total RNA extraction reagent, reverse transcription kit, real-time PCR kit from TaKaRa (Japan); transfection reagent Lipofectamine™ 2000 and Opti-MEM both from Invitrogen (the US); MTT from Sigma (the US); cell cycle detection kit DNA Prep™ from Beckman Coulter (the US); apoptosis antibody Annexin VPE from BD (the US); agar from Biosharp; Millicell PET film (8.0 μm) from Millipore (the US); CO_2 incubator from Thermo (the US); PCR instrument from BIO-RAD (the US); fluorescent quantitative PCR instrument from ABI (the US); centrifuger from XiangYi Centrifuge Instrument CO., LTD. (Changsha, Hunan, China); inverted microscope from Nikon (Japan); fluorescent inverted microscope from Lycra (Germany); microplate reader from Thermo (the US); and a flow cytometer from BD (the US). miR-210 primer, miR-210 inhibitor and miRNA inhibitor NC were synthesized in ChinaPeptides Co., Ltd. (Shanghai, China).

The expression of miR-210 detected by real-time PCR

Total RNA was extracted from ovarian cancer tissues and was reverse-transcribed into cDNA. The upstream primer sequence of miR-210 was CTGTGCGTGTGACAGCGGCTGA and the downstream primer was a general primer Uni-miR real-time PCR primer (produced by TaKaRa). Amplification was performed by taking cDNA as a template and U6B as an internal reference, using SYBR Primix Ex Taq II (TaKaRa kit). Three duplicates were set. The reaction condition was as follows: after predegenerating for 30 s at 95°C, degenerating for 5 s at 95°C and then annealing and extension for 31 s at 60°C (40 cycles in total). The expression level of miR-210 was expressed as $2^{-\Delta\Delta Ct}$.

A2780 and IOSE80 cells were prepared for cell recovering, culturing and subculturing. RNA was extracted from cell lines in good growth condition and reverse-transcribed to cDNA. The expression level of miR-210 in different cell lines was detected by qPCR. With U6B used as a reference gene, results obtained were analyzed by $2^{-\Delta\Delta Ct}$ method as well.

Transfecting A2780 cells with miR-210 inhibitor

After miR-210 inhibitor (sequence: UCAGCCGCUGUCACACGCACAG) and miRNA inhibitor NC (sequence: CAGUACUUUUGUGUAGUACAA; since this sequence was not non-homologous to human genome, it had no interference effect on any miRNA; miR-NC in short) powders were centrifuged, they were prepared into 20 μmol/L solution. One day before transfection, cells were laid on a 24-well plate so that the cell confluence reached up to 30%-50%. Then, 500 μl medium without antibiotics was added into the plate. The diluted miRNA and Lipofectamine™ 2000 was mixed and incubated for 20 min at room temperature. The mixture was later added into A2780 cells, which was shaken and homogenized. After that, cells were incubated at 37°C for 24 h and cryopreserved and reserved for subsequent usage.

MTT assay

According to the instructions for use of Lipofectamine™ 2000, transfection was performed in a 96-well plate and three groups, including an experimental group transfected with miR-

210 inhibitor (miR-210 inhibitor transfection group), a miR-NC control group (miR-NC transfection group) and a non-transfected group, each group was set with 6 duplicates. Results were detected 1, 2, 3, 4 and 5 days after transfection. In each well, 20 μl MTT reagents were added. After incubation in an incubater with 5% CO_2 at 37°C for 4 h, 150 μl DMSO was added in each well, which was oscillated overnight. Then, the optical density (D) value at 570 nm was measured by using a microplate reader. This assay was repeated three times.

Colony formation assay

The assay was conducted 48 h after transfection into A2780 cells. 0.6% agar and RPMI 1640 medium with 10% fetal calf serum were mixed at the rate of 1:1 (1.5 ml) and the mixture was laid on a 6-well plate. After clotting, 0.3% agar and cell suspension with 10% fetal calf serum and 1×10^3 cells/well were mixed (1.5 ml) and the mixture was laid on the upper layer. After clotting, the mixture was placed in an incubator. Then, it was stained by 0.4% crystal violet, four visual fields (×100) were selected at random and the number of colonies with more than ten cells was counted. This assay was repeated three times.

Flow cytometry assay

A2780 cells were used in this assay after transfection for 48 h. Apoptosis was detected as follows: 1×10^4 cells were collected and suspended with 100 μl PBS suspension; afterwards, 100 μl DNA Prep LPR was added; after incubation for 20 min, 1 ml DNA Prep Stain was added and the mixture was incubated for another 20 min. Cell cycle was detected by flow cytometry as follows: 1×10^4 cells were collected and washed with PBS; then, 50 μl Annexin buffer solution, 5 μl 7AAD and 5 μl AnnexinV-PE were added. After reaction for 15 min, 200 μl buffer solution was added and the result was analyzed with a flow cytometer. This assay was repeated three times.

Transwell assay

This assay was conducted 48h after transfection into A2780 cells. On the previous day, 1300 μl complete medium was added into a 24-well plate, which was laid on a chamber and then put into an incubator overnight. The cell density was adjusted to 1×10^6 cells/ml. In each well, 200 μl serum-free cell suspension with 0.2% BSA was added and 1300 μl complete medium was added into the lower plate. Then, the plate was cultured in an incubator with 5% CO_2 for 24 h at 37°C. After that, it was fixed with 70% methanol and stained by 0.4% crystal violet. At last, after mounting, four visual fields (×100) were selected at random for counting. The mean value was calculated. This assay was repeated twice.

This assay was conducted 48 h after transfection into A2780 cells. On the previous day, matrigel was diluted with a serum-free medium at the rate of 1:8. The resultant dilution was laid on a chamber (60 μl), air dried and set aside. The cell density was adjusted to 1×10^6 cells/ml. In each well, 200 μl serum-free cell suspension with 0.2% BSA was added and 1300 μl complete medium was added into the lower plate. Then, the plate was cultured in an incubator with 5% CO_2 for 24 h at 37°C. After that, it was fixed with 70% methanol and stained by 0.4% crystal violet. At last, after mounting, four visual fields (×100) were selected at random for counting. This assay was repeated twice.

In vivo anti-tumor assay

Female BAL B/C nude mice at the age of 6-8 weeks were inoculated subcutaneously 5×10^6 A2780, A2780/NC and A2780/210 cells. Then, the length and width of their tumor were measured every week and the formation and growth of subcutaneous tumor were observed. Tumor volume was calculated according to the following formula: Volume of tumor = 1/2 long diameter × short diameter^2.

Six weeks later, mice were killed by cervical dislocation. Tumor tissues were weighed and statistical analysis was performed concerning weight.

Results

The expression of miR-210 in ovarian cancer tissues and cells

In this study, the expression of miR-210 in ovarian cancer and para-cancer tissues relative to the housekeeping gene U6 was pres-

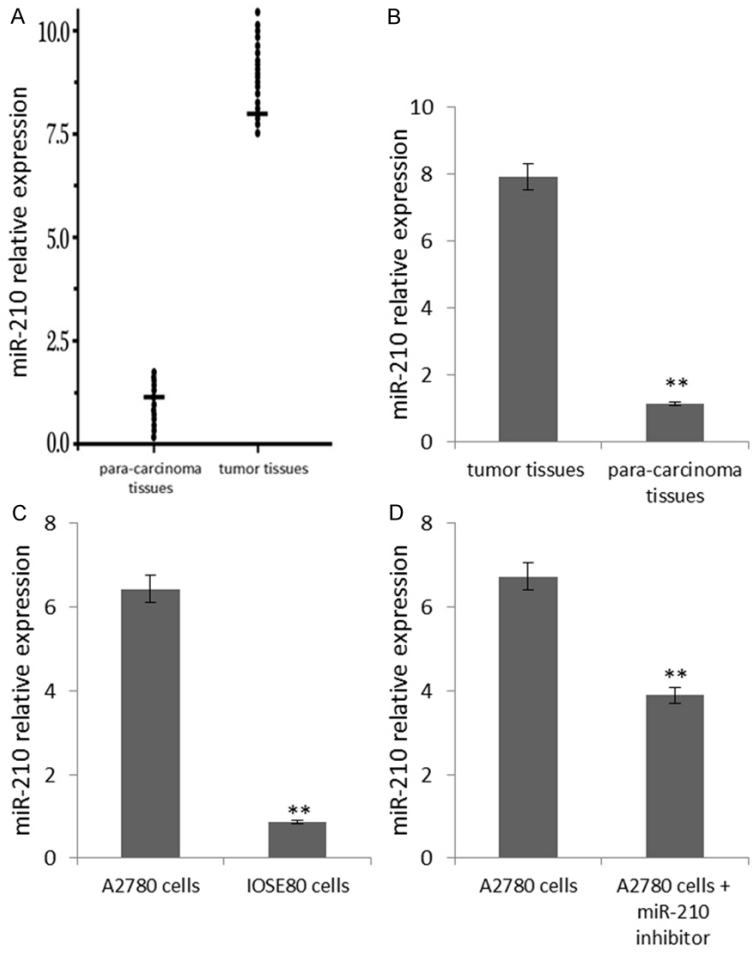

Figure 1. Expression of miR-210 in tissues and cells detected by RT-qPCR. A, B. Expression of miR-210 in cancer and para-cancer tissues in patients with ovarian cancer; C. Expression of miR-210 in ovarian cancer cell line A2780 and normal ovary cell line IOSE80; D. Expression of miR-210 after transfecting miR-210 inhibitor into A2780 cells. **P<0.05.

ented in **Figure 1A**. Results of statistical analysis showed that the expression of miR-210 in ovarian cancer tissues (**Figure 1B**) was significantly higher than that in para-cancer tissues ((7.92 ± 2.21) vs (1.15 ± 0.15), P<0.01). Besides, the relative expression of miR-210 in A2780 cells (**Figure 1C**) was also significantly higher than that in normal IOSE80 cells ((6.43 ± 1.03) vs (0.86 ± 0.11), P<0.01). These results suggested that the expression level of miR-210 in ovarian cancer tissues and cells was significantly higher than that in normal ovary tissues and cells.

The expression of miR-210 after transfecting miR-210 inhibitor into A2780 cells

Results of RT-qPCR indicated (**Figure 1D**) that after cells being transfected with miR-210 inhibitor, the relative expression of miR-210 in A2780 cells decreased from (6.73 ± 0.84) to (3.89 ± 0.27) with statistically significant difference (P<0.01). It confirmed that miR-210 inhibitor had been transfected into A2780 cells successfully.

MTT assay and cell colony formation assay

Three, four and five days after transfection, the proliferation of cells in the miR-210 inhibitor transfection group decreased significantly compared with the miR-NC transfection group and the non-transfected group. The difference was statistically significant (P<0.05). However, no significant difference was found between the non-transfected group and the miR-NC transfection group (P>0.05) (**Figure 2A**). Further, the colonies counting in the miR-210 inhibitor transfection group was (22.58 ± 2.21), which was significantly lower than that in the miR-NC transfection group (49.34 ± 3.31). The difference was statistically significant (P<0.01). Again, no significant difference was found between the non-transfected group and the miR-NC transfection group (P>0.05) (**Figure 2B**). These results suggested that miR-210 inhibitor could inhibit the proliferation of A2780 cells.

Cell cycle arrested at G0/G1 phase and apoptosis after transfecting miR-210 inhibitor into A2780 cells

The number of A2780 cells arrested at G0/G1 phase was significantly higher in the miR-210 inhibitor transfection group compared with the miR-NC transfection group ((68.32 ± 3.87)% vs (51.18 ± 1.29)%, P<0.01), while the number of cells at S phase was not evidently different from that in the miR-NC transfection group

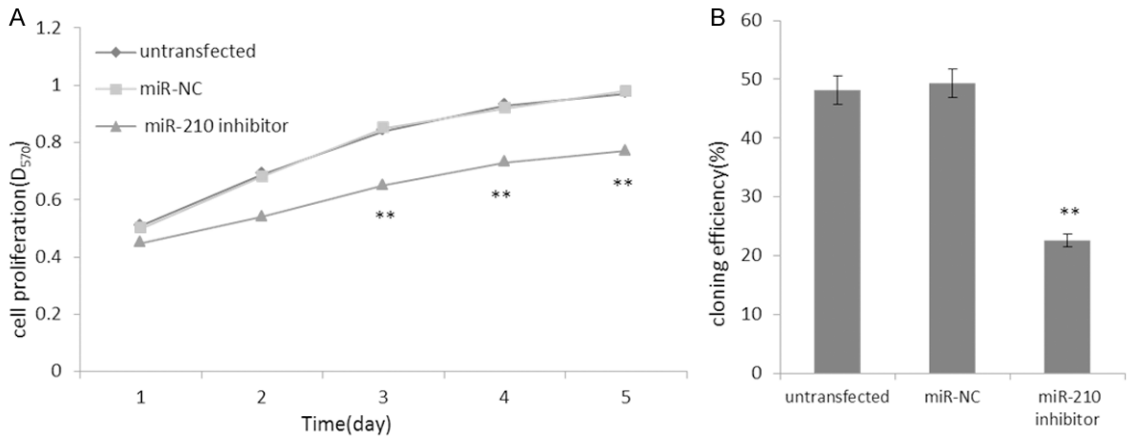

Figure 2. Effects of cell proliferation (A) and clonogenic ability (B) after transfecting miR-210 inhibitor into A2780 cells. **P<0.05 vs miR-NC group and untransfected group.

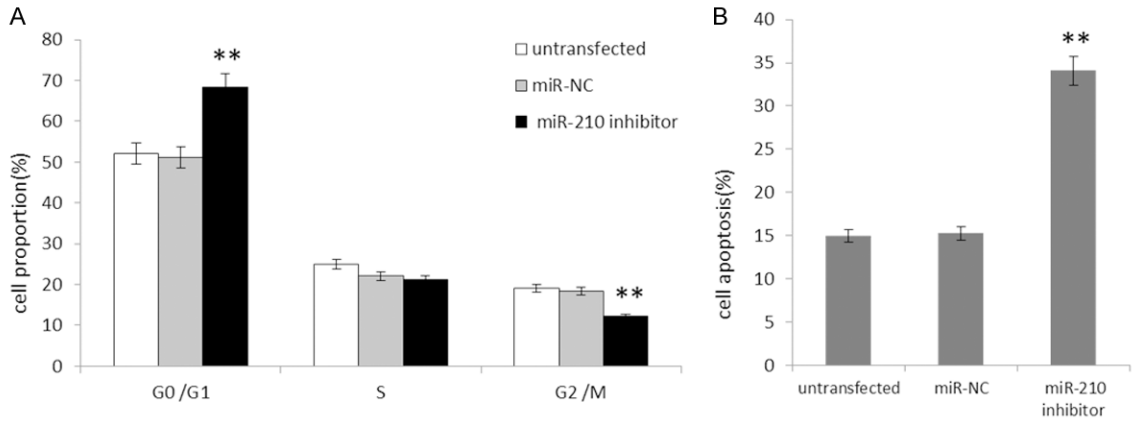

Figure 3. Effects of cell cycle (A) and cell apoptosis (B) after transfecting miR-210 inhibitor into A2780 cells. **P<0.05 vs miR-NC group and untransfected group.

((21.15 ± 2.76)% vs (22.09 ± 2.12)%, P>0.05) and the number of cells at G2/M phase decreased compared with that in the miR-NC transfection group ((12.18 ± 0.61)% vs (18. 33 ± 0.65)%, P<0.05) (**Figure 3A**). These results suggested that miR-210 inhibitor could arrest A2780 cells at G0/G1 phase and thus inhibit their proliferation.

The apoptosis rate in the miR-210 inhibitor transfection group was significantly higher than that in the miR-NC transfection group ((34.07 ± 3.51)% vs (15.25 ± 0.88)%, P<0.01), while no significant difference was found between the non-transfected group and the miR-NC transfection group (P>0.05) (**Figure 3B**). These results suggested that miR-210 inhibitor could promote the apoptosis of A2780 cells.

Migration and invasion of A2780 cells inhibited after transfecting miR-210 inhibitor into A2780 cells

Results of migration assay (**Figure 4A**) showed that the number of emigrated cells in the miR-210 inhibitor transfection group decreased significantly compared with that in the miR-NC transfection group ((342.00 ± 43.64) vs (1239.22 ± 91.68), P<0. 01), while no significant difference was found between the non-transfected group and the miR-NC transfection group (P>0.05). Besides, results of invasion assay (**Figure 4B** and **4C**) showed that matrix degradation and the number of emigrated cells in the miR-210 inhibitor transfection group (112.63 ± 26.44) decreased significantly compared with that in the miR-NC

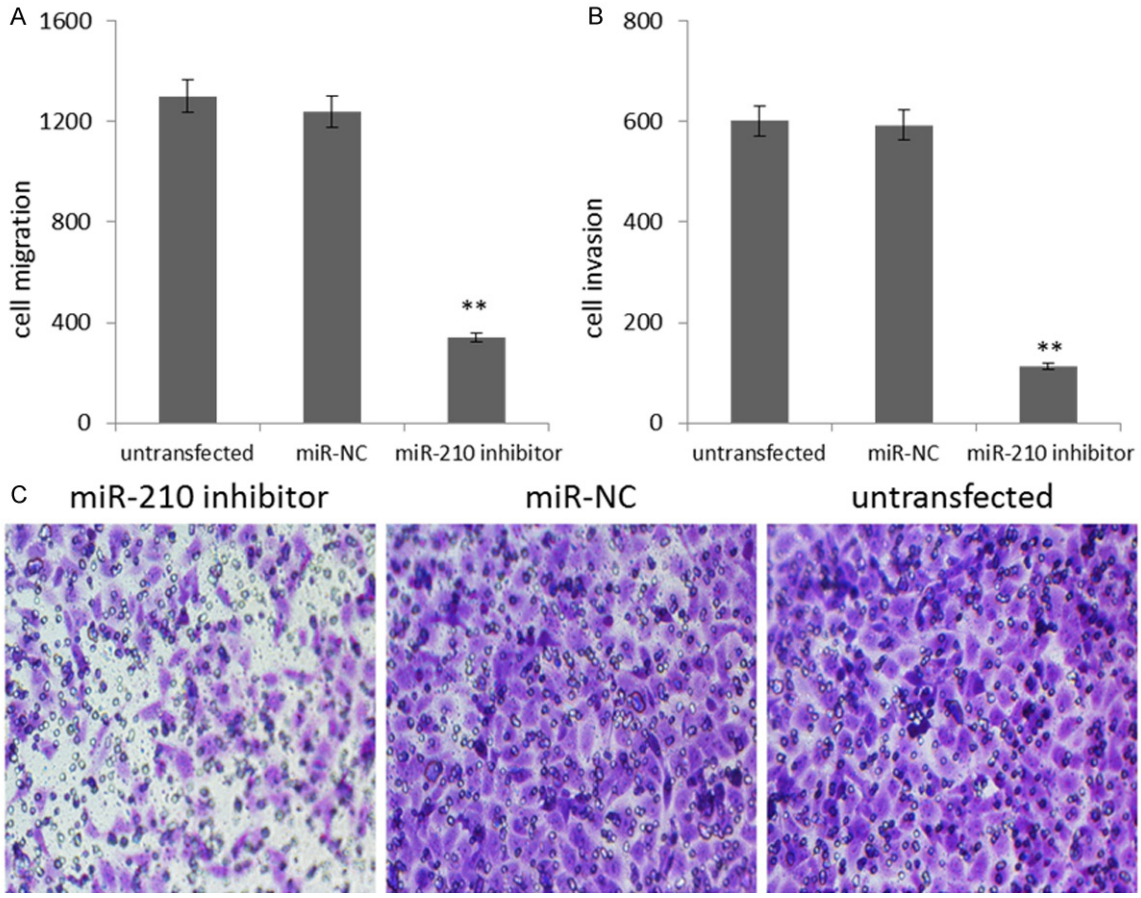

Figure 4. miR-210 inhibitor transfection inhibited the migration (A) and invasion (B and C) of A2780 cells (×100). **P<0.05 vs miR-NC group and untransfected group.

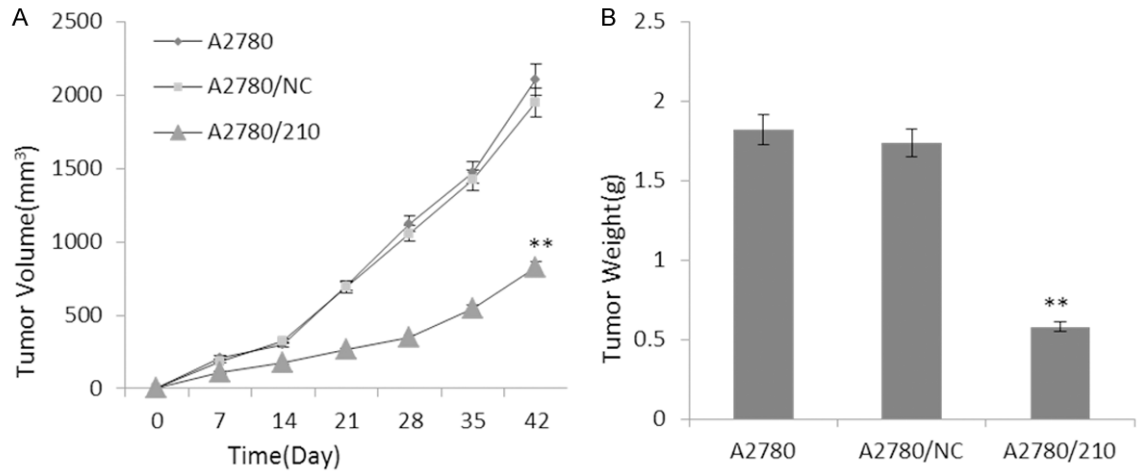

Figure 5. Effects of miR-210 silence on the in vivo tumor growth rate for the A2780 cells. A. Growth of tumor. B. At 42 days after inoculation, the mice were sacrificed and dissected, with the weight of tumor measured. Weight of tumor is expressed in mean ± SD (n = 4), **P<0.01.

transfection group (593.18 ± 21.20). The difference was of statistical significance (P<0.01). However, there was no significant difference between the non-transfected group and the

miR-NC transfection group (P>0.05). These results suggested that the migration and invasion of A2780 cells decreased significantly after the expression of miR-210 was inhibited.

The effect of transfection of miR-210 inhibitor on the tumor growth of A2780 cells

In order to detect the effect of transfection of miR-210 inhibitor on the tumor growth of A2780 cells, a subcutaneous tumor model of A2780 cells was established in BAL B/C nude mice and tumor formation and growth of A2780, A2780/NC and A2780/210 cells were observed. Results indicated that (**Figure 5A**) ovarian tumors could be detected subcutaneously in nude mice about 14 days after subcutaneous inoculation. Thereafter, tumors grew slowly. 42 days after inoculation, the tumor volume in A2780/210 group reached up to (822.3 ± 115.4) mm^3, which was smaller than that of A2780 group (2104.5 ± 131.5 mm^3) and A2780/NC group (1949.4 ± 157.9 mm^3). Results of statistical analysis showed that the difference of tumor growth rate between A2780 group and A2780/NC group was not statistically significant (P>0.05), while that of A2780/210 group was significantly slower compared with A2780 group (P<0.01) and A2780/NC group (P<0.01). After mice were killed, tumor within their body was weighed. Results showed (**Figure 5B**) that the tumor mass of A2780/210 group was (0.58 ± 0.12) g, which was significantly lower than that of A2780 group (1.82 ± 0.25) g and A2780/NC group (1.74 ± 0.21) g. The differences were both of statistical significance (P<0.01).

Discussion

Ovarian cancer is currently the most common malignant tumor found in women and its morbidity in China is increasing year by year [11, 12]. So far, there is no precise and effective treatment methods for ovarian cancer. As molecular targeting treatment has become an emerging therapeutic method in recent years, looking for an effective therapeutic target has become a hotspot. Under this circumstance, the appearance of miRNA provided a new direction for exploring the treatment and diagnosis of ovarian cancer. Studies showed [13-15] that over a half of miRNA was located at tumor-related genomic regions and fragile sites, regions of loss of heterozygosity, amplification regions or regions of break point, which suggested that miRNA could act as an oncogene or cancer suppressor gene. miRNAs regulate post-transcriptional gene-expression regulation and mainly acted as a negative regulator for gene expression [16, 17]. A role similar to cancer suppressor genes could be played when the expression of miRNA was down-regulated [18-20]. It was found that, in ovarian cancer and other tumors, miRNA often had abnormal expression, which played different roles in tumor cell migration, invasion and angiogenesis and other phases [21-23]. In this study, through quantitative detection of the expression of miR-210 in specimens taken from patients with ovarian cancer and ovarian cancer cells, it was found that the expression of miR-210 in ovarian cancer tissues and cells was higher than that of normal ovarian tissues and cells. After inhibiting miR-210 expression with miR-210 inhibitor, the proliferation and clonality of ovarian cancer cells decreased and cells were arrested at G0/G1 phase. It was also shown that transfection with miR-210 inhibitor increased the apoptosis ratio of ovarian cancer cells. Besides, when the expression of miR-210 was successfully inhibited, the invasion and migration of A2780 cells decreased significantly. It suggested that miR-210 played an important role in the invasion and migration of ovarian cancer cells.

A study [24] showed that the miRNA expression in the peripheral blood was associated with the miRNA expression in corresponding tissues and was closely related to clinical tumor stage, lymphatic metastasis and prognosis. It indicated that miRNA was a potential diagnostic and prognostic marker for ovarian cancer [25-27]. Furthermore, since serum miRNA detection has advantages such as non-invasion, good stability and high sensitivity, miRNA isqualified to become an ideal tumor marker [28]. In this study, as a highly expressed miRNA in ovarian cancer, miR-210 could become a new generation of biomarkers for the diagnosis of ovarian. In addition to the diagnostic value, miRNA could become a new targeting therapy. One miRNA could regulate the expression of multiple oncogenes or cancer suppressor genes. So it would be more effective to take miRNA as a therapeutic target rather than a single oncogene or cancer

suppressor gene. Currently, drugs associated with small interfering RNA (siRNA) whose function is similar to that of miRNA have entered into the clinical trial stage. It is reported that transfecting miRNA inhibitor into cells can specifically silence the expression of target genes which promotes tumor migration and prevent the formation of metastatic sites [29].

Conclusion

miR-210 inhibitor could inhibit proliferation, invasion and migration of ovarian cancer, which indicates silencing miR-210 is potentially promising for the treatment of ovarian cancer. Therefore, miR-210 can become a new target for the treatment of ovarian cancer and miR-210 down-regulated expression combining (combinating) with conventional therapies is expected to improve the survival rate of patients with ovarian cancer.

Disclosure of conflict of interest

None.

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