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MiR-483-3p regulates neuroblastoma malignant behaviors via down-regulating RIOK3

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Abstract: **The purpose of this study is to investigate the effect of microRNA-483-3P (miR-483-3P) on proliferation, invasion and migration of neuroblastoma(NB) cells, and to validate the target genes. MiRNA microarray chips were analyzed. Negative control sequences of chemically synthesized miR-483-3P inhibitor and miR-483-3P inhibitor were transfected into SH-SY5Y cell line using cationic liposome LipofectamineTM 2000. Expression of miR-483-3P in each group was detected by RT-PCR technique. Proliferation of cancer cells in each group was detected by CCK-8 assay. Invasion and migration of cancer cells in vitro detected by Transwell chamber assay. Bioinformatics software was used to predict the target gene of miR-483-3P, and luciferase reporter assay was used to test the target gene. Western-blot was used to verify the target gene. MiRNA microarray chip results showed that miR-483-3P was up-regulated in NB. The results showed that miR-483-3P inhibitor of the expression of miR-483-3P in SH-SY5Y cell line decreased. After transfection with miR-483-3P inhibitor of the proliferation of NB cells were inhibited. Ability of invasion and migration of NB decreased significantly after transfected with miR-483- 3P inhibitor. We found RIOK3 was a candidate target gene in this study. RIOK3 is one of the target genes of miR-483-3P. miR-483-3P can down-regulate RIOK3 expression and significantly promote the proliferation and migration of NB cells in vitro.**

Keywords: Neuroblastoma; MiR-483-3P; Cell Proliferation; Invasion; Target Genes

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1. Introduction

Neuroblastoma (NB) is the most common malignant tumor in children. Some cases proves that it has higher malignancy, early metastasis and higher mortality rate[1]. Therefore, searching for molecular biomarkers related to development of NB and exploring molecular mechanism of tumorigenesis is important for its treatment and prognosis. MicroRNA (miRNA) is an endogenous non-coding small molelates gene single-stranded RNA composed of 18-25 nucleotides and expression at the post-transcriptional level. In recent years, the study shows that miRNA involves in many physiological processes, including cell proliferation, differentiation and apoptosis. They are closely related to occurrence and development of tumor. In our previous study[2], miRNA microarrays screened 54 miRNAs that may be involved in NB invasion and metastasis, of which 35 miRNAs were up-regulated and 19 miRNAs down-regulated. Among them, expression of miR-483-3P was significantly up-regulated, with significant difference, which was the first place of differentially expression of miRNA. And researches have shown that miR-483 is closely related to malignancy of pancreatic cancer and gastric cancer[2]. The purpose of this study was to verify expression of miR-483-3P in NB and effect of miR-483-3P regulated target genes on biological behavior of NB. The treatment of cell tumors provides new targets and theoretical basis.

2. Materials and Methods

2.1. Materials

Nine pairs of NB and adjacent normal tissues were provided by Affiliated Hospital of Qingdao University Pathology Department and were diagnosed by pathological doctors. All specimens were approved by the ethies committee of Affiliated Hospital of Qingdao University and the parents of children were agreed. NB cells in vitro cell line ware established by our group and long-term retention. Fetal bovine serum(FBS), DMEM medium, PBS buffer, trypsin and LipofectamineTM 2000 purchased from INVITROGEN, USA; miR-483-3P inhibitor, the negative control sequence of miR-483-3P inhibitor was purchased from Guangzhou Ribobio CO., LTD; Trizol lysate, RT-PCR kit was purchased from Tiangen Biotech (Beijing) CO., LTD; CCK-8 kit purchased from Dojindo Laboratories, Japan; Transwell chamber, flow cytometry kit, containing miR-83-3P RIOK3 binding site of 3' UTR (wild-type; pmir GLO-RIOK3 WT) containing a mutation site and 3' UTR (mutant; pmirGLO-RIOK3MUT) performed by Sangon Biotech, Shanghai, China.

2.2. Method

2.2.1. Cell culture and transfection

SH-SY5Y cell lines were maintained in DMEM medium containing 10% FBS and 1% antibiotic/antimycotic solution at 37℃ in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 36h. The degree of confluence reached about 90% (2-3 days) for passage. Take logarithmic growth phase of the cells in culture frozen 6-8 tubes for follow-up experiments spare on the second day after passage. SH-SY5Y cells at 60-80% confluence were transfected with Lipofectamine TM2000 according to instructions. After 6 h of transfection, the cells were replaced with DMEM medium containing 10% for further culture. NB cells and adjacent normal tissues cells were cultured above.

2.2.2. Total RNA extraction and real-time fluorescent quantitative RT-PCR assay transfection

Total RNA was extracted at 24h, 48h and 72h after transfection in accordance with instruction of Trizol reagent. After extracted RNA, we determinated the concentration and purity of RNA with a protein nucleic acid analyzer. OD260/OD280 was between 1.8~2.2. RNA restored in -80℃ refrigerator. For miRNA reverse transcriptase polymerase chain reaction, internal reference was selected for snRNAU6, using PCR specific primers for miR-483-3P. RT-PCR reaction was the following conditions, the first step of reaction (reverse transcription cDNA): (1) Ploy (A) treatment of miRNA 3' end, 37℃ 60min; (2) Poly (A) modified miRNA reverse transcription reaction at 37℃ 60min. The second reaction (RT-PCR amplification phase): 94℃ 2min, 94℃ 20s, 60℃ 34s; then repeat 40 cycles.

2.2.3. CCK-8 detection of tumor cell proliferation

The day before transfection, adherent SH-SY5Y cells were digested and resuspended to adjust the cell density to 5×10^5/L and inoculated into 96 well culture plates in an amount of 100μL per well. Each group was provided with 5 complex holes. After overnight culture, miR-483-3P was transfected for 6 h, the cells were replaced with 100μL DMEM medium containing 10% FBS for further culture. At 0h, 48h and 72h respectively, 10μL of CCK-8 reagent was added and incubated in an incubator at 37℃, 5% CO_2 and saturated humidity for 2 h. The OD at 450nm was measured by enzyme-linked immunosorbent assay.

2.2.4. Transwell assay of tumor cell invasion and migration

SH-SY5Y cells were seeded in a 24 well culture plate in an amount of 5×10^4 cells per well on the day before transfection. When degree of cell fusion reached about 40%, gene transfection was performed according to instructions of Lipofectamine™ 2000. Six hours later, transfection into 10% FBS containing DMEM medium continued to culture. The cells were digested into cell suspension, adjusting the cell density of 3×10^5/mL, taking 100μL cell suspension added to transwell chamber. The cells in chamber are resuspended in serum-free medium, chamber was placed in a 24-well plate, and 500μL of DMEM medium containing 10% FBS was added to the 24-well plate. The cells were cultured in a 5% CO_2 incubator at 37℃ for 24h. Aspirate fluid from upper chamber of transwell and wipe the bottom surface of the upper chamber with a cotton swab. Fix with 95% ethanol for 10min, wash with 1×PBS, then stain with 0.1% crystal violet for 15min, stain with 1×PBS and counted under inverted microscope for number of cells invaded and migrated (5 wells in each well were photographed, and average number of cells per field was counted). Set two parallel holes in each group.

2.2.5. Detection of tumor cells invasion and migration ability with Transwell

Cells were seeded on 24-well plates on the first day (5×10^4/well). The next day at a cell density of 40%, transfected with Lipo-actamine ™ 2000 instructions. Then pmirGLO-RIOK3 WT or pmirGLO-RIOK3MUT and miR-483-3P hibiner were co-transfected into SH-SY5Y cells respectively. Reporter gene plasmid was 200ng/well and concentration of nucleic acid was 150nmol/L. And 48h after transfection, cell lysis was performed and luciferase activity was determined by promega fluorescence detector.

2.2.6. Effect of miR-483-3P on RIOK3 expression by Western blot

The cells were cultured in 6-well plates and transfected with the same method. RIPA lysate was added 48h after transfection to extract total protein. Protein concentration was determined by BCA method. Finally, 100μg of total protein was loaded and SDS-PAGE gel electrophoresis was performed. Membrane was blocked and added with a rabbit anti-human polyclonal antibody. After overnight at 4℃, monoclonal antibodies against goat anti-rabbit were added and incubated for 1h at room temperature. ECL imaging, Fixation and gel imaging system were used for photograph analysis.

2.3. Statistical analysis

Measurement data were expressed as mean ± standard deviation, comparison between two groups of data using nonparametric Wilcoxon test.

Difference was considered significant if p-value of two-tailed test was less than 0.05. All the analysis was finished by SPSS19.0 statistical software.

3. Results

3.1. Differential expression of miR-483-3P in NB and adjacent normal tissues

In this study, we analyzed expression of miR-483-3P using RT-PCR. The result of RT-PCR showed that the content of miR-483-3P in NB was significantly higher than that in adjacent normal tissues (t=22.52, P<0.01) (Figure 1). Our data shows that miR-483-3P was higher expressed at NB compared to adjacent normal tissues.

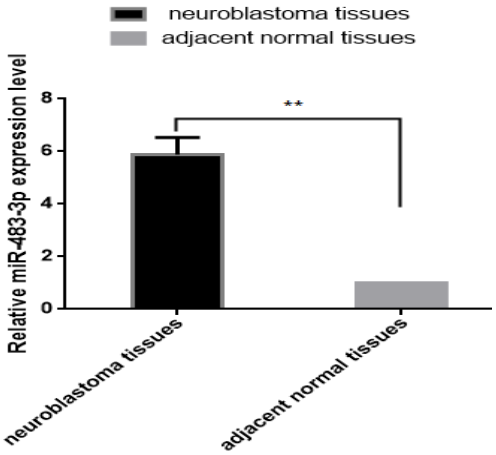

Figure 1. Compared with the cancer adjacent normal tissues, the expression of miR-483-3p protein was significantly increased following tranfection in NB tissues (P<0.01).**

3.2. Changes of miR-483-3P expression after gene transfection

After transfection of miR-483-3P inhibitor and miR-483-3P inhibitor NC into cells, total RNA was extracted at 24h, 48h and 72h after transfection. The relative expression level of miR-483-3P was detected by RT-PCR. The results were compared with the control group, miR-483-3P level decreased gradually at 24h, 48h and 72h after transfected with miR-483-3P inhibitor, especially at 48h((t=2.71~17.14, p<0.05) (Figure 2).

3.3. Effect of miR-483- 3P on Proliferation of NB Cells

The results of CCK-8 assay showed that the OD value of control group and miR-483-3P inhibitor transfected group at 0h and 24h at 450nm were 0.43±0.00, 0.43±0.00, 0.88±0.24 and 0.78±0.21, respectively. The results showed no significant difference, (t=0.94, P>0.05). The OD value of control group and miR-483-3P inhibitor transfected group at 48h and 72h at 450nm were 1.22±0.23, 1.03±0.14, 1.85±0.24 and 1.49±0.20, respectively. It also showed significant difference, (t=2.41~3.45, P<0.05). Therefore, miR-483-3P inhibitor can inhibits the proliferation of NB cells (Figure 3).

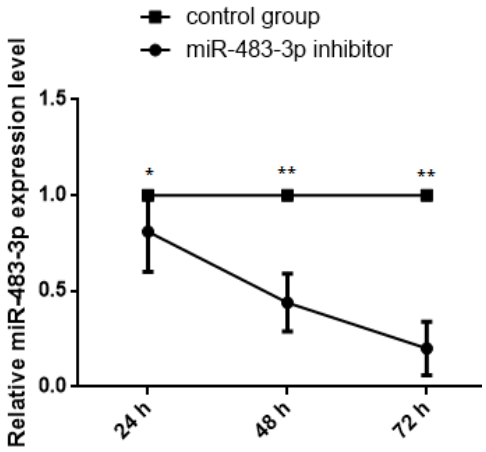

Figure 2. After gene transfection relative expression level of miR-483-3p gradually decreased after 24, 48, 72h separately (*P<0.05, **p<0.01).

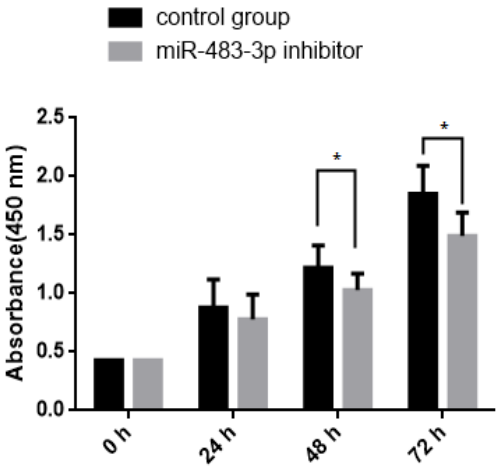

Figure 3. Transfectiom with miR-483-3p inhibitor decreased growth of NB cells (*P<0.05).

3.4. Effect of miR-483- 3P on Invasion and Migration of NB Cells

Transwell chamber experiments showed that the number of passing through the Matrigel membrane in transfected miR-483-3P inhibitor group was 24.46±3.45 and that in control group was 35.88±4.43. Migration of miR-483-3P inhibitor group decreased 31.83% by comparison with control group. Difference between two groups was significant difference (t=6.10, P<0.01). This indicates that after inhibiting the expression of miR-483-3P, the migration ability of NB cells decreased.

3.5. miR-483-3P targets regulate RIOK3 expression

Using the target can and miRNA databases, we found that the 3'UTR of RIOK3 contains the binding site of miR-483-3P. Therefore, RIOK3 was used as a candidate target gene in this study. The results of luciferase reporter assay showed pmirGLO-RIOK3 WT. The co-transfection of miR-483-3P inhibitor transfected cells significantly increased luciferase activity compared with pmirGLO-RIOK3 MUT and miR-483-3P inhibitor (t=46.58, P<0.01, Figure 4).

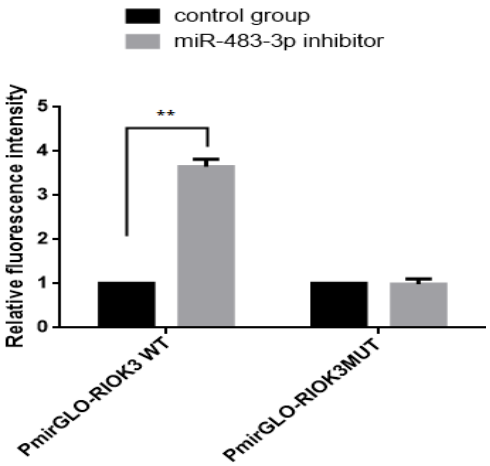

Figure 4. miR-483-3p inhibitor can increase luciferase activity (P<0.01).**

3.6. Effect of miR-483-3P on protein expression

After interfering with the expression of miR-483-3P, western blot showed that expression of RIOK3 the OD of transfected miR-483-3P inhibitor group was 167.70±15.88 and that in the control group was 210.89±15.33. It lower than the control group (t=5.09, P <0.01). Results of Western blot showed that the expression of RIOK3 in miR-483-3P cells was down-regulated, with statistical significance (Figure 5).

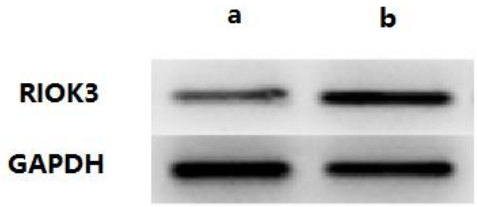

Figure 5. miR-483-3p down-regulation of RIOK's expression which a representing the result of the control group and b representing miR-483-3p group.

4. Discussion

NB is a common malignant embryogenic tumor of the sympathetic nervous system in children[3]. It is one of the most common solid tumors in children and infants with a prevalence of 1/100000, high malignancy and early metastasis, and the mortality rate is high. Therefore, it is very important to elucidate the molecular mechanism of tumor metastasis and find new treatment methods. In recent years, miRNAs have been found to play a role in tumorigenesis and progression through regulation of cell metabolism, such as cell proliferation, migration, differentiation, apoptosis and angiogenesis[4]. MiRNAs specifically or partially bind to the 3' untranslated region of a target mRNA by the way of base-pairing and thus inhibit mRNA translation or degradation of the target mRNA[5]. miRNAs have been the focus on researches for their close association with tumors in recent years. They endogenous non-coding single-stranded RNA consisting of 18-25 nucleotides which are regulated at the post-transcriptional level gene expression. miRNAs are both oncogenes and tumor suppressor genes that are involved in multiple signaling pathways in human tumors[6]. Therefore, the study of specific miRNA function provides a new direction for tumor diagnosis and treatment[7]. In addition, a large number of studies have found that miRNAs are involved in the occurrence and development of many kinds of tumors[8]. However, there were few reports on the involvement of miRNAs in the invasion and metastasis of NB[2]. The main factors affecting the prognosis of NB are invasion and metastasis of it. Therefore, the study of specific miRNA, which is closely related to the invasion and metastasis of NB.

Our group previously applied miRNA microarray analysis technique to the study of NB metastasis mechanism and screened abnormal miRNA expression profiles of NB metastases such as miR-483-3p, miR-99b, miR-145 and miR-602. The expression of miR-125a-3p and let-7f have significantly different between NB and NB primary tumors. These miRNAs may play important roles in the invasion and metastasis of NB[9]. So far, some related researches have been done, such as the related research on miR-7, miR-338, miR-338-3P, miR-92, miR-92b, miR-34a and miR-15a etc. We found that when changes in tumor cell miRNAs expression levels, cell proliferation, invasion and migration will be changed too[2]. The mechanisms that influence the biological behavior of tumor cells are caused by the changes of post-translational levels, which downstream regulatory target mRNAs[10,11]. Proteins translated by these targets such as cytokines and signaling proteins. Tumors developed when the signal pathways change[12]. Studies have shown that miR-483 is closely related to pancreatic cancer and gastric cancer, and it has a certain correlation with

the degree of malignancy. miR-483-3p plays a catalytic role in pancreatic cancer. DPC4/SMAD4 is closely related to pancreatic oncogene which is a common key protein downstream of TGF-βand BMP signaling[13]. miR-483-3p can inhibit the transcription of miRNA after transfection, and decrease the level of DPC4/SMAD4, thereby preventing the subsequent transmission of TGF-βand BMP signaling. These signaling pathways are related to cell growth, differentiation and apoptosis. Thereby promote the occurrence of tumors. It has also been reported in literature that miR-483 is located in intron 7 of the IGF2 gene and is co-expressed with the host gene IGF2[14]. The expression level of miR-483 in colorectal cancer was significantly higher than that in paracancer tissues. IGF2 gene expression was increased by a multiple in patients with colorectal cancer, both of which may become molecular markers for the diagnosis of colorectal cancer. DCL1 (HCC-1) is an important tumor suppressor gene that can reduce or delete in many malignant tumors such as liver cancer, lung cancer, prostate cancer and breast cancer. MiR-483-3P Inhibition of DCL1 transcriptional translation promotes the development of colorectal cancer[15]. In summary, the expression of miR-483-3P is elevated in many tumor cells, which is the same as that of the previous gene chip. Therefore, study of miR-483-3P in NB is particularly important. Bioinformatics revealed that miR-483-3P was closely related to RIOK3. Protein kinases are responsible for catalyzing the transfer of phosphate groups to serine, threonine and tyrosine[16] and play an important role in intracellular signal transduction and the regulation of various physiological activities in the body[17]. RIOK3 is a member of the atypical kinase RIO family. RIOK3 contains a RIO domain, but its structure is quite different from other RIO family members. Studies have shown that human protein kinase RIOK3 is very important signal molecule and it can inhibit cell proliferation and promote apoptosis. RIOK3 is localized in the cytoplasm, and proteins interacting with RIOK3 are caspase-10, Bcl-2 interacting mediator, p21-activated kinase 2 (PAK2) and cell division cycle. The role of the protein and cell apoptosis, invasion and metastasis activity are closely related. RIOK3, a member of NF-κB pathway, inhibits TNFa-induced NFB activation. RIOK3 and CASP10 can inhibit the activation of NF-κB pathway[18]. RIOK3 can reduce the expression of PAK2 at the protein level, and play an important role in the enzymatic activation of PAK2[19,20]. The function of RIOK3 in NF-κB signaling was found by studying RIOK3. RIOK3 is closely related to tumorigenesis and tumor cell migration[21].

In this study, differential expression of miR-483-3P in NB and adjacent normal tissues was verified by reverse transcriptase-polymerase chain reaction. miR-483-3P was highly expressed in NB. Furthermore, miR-483-3P inhibitor was transiently transfected into SH-SY5Y NB cell line with miR-483-3P overexpression by lipofectamine 2000 to interfere the expression of miR-483-3P.

Results showed that miR-483-3P inhibitor makes the expression of miR-483-3P in NB cell line decreased, and CCK-8 experimental results show that after transfection with miR-483-3P inhibitor the proliferation of NB cells were inhibited. Results of Transwell showed that the ability of invasion and migration of NB decreased significantly after transfected with miR-483-3P inhibitor. Next, we used a variety of bioinformatics software to predict the target of miR-483-3P, and found 3'UTR of RIOK3 has a potential binding site of miR-483-3P. Therefore, RIOK3 was used as a candidate target gene in the future. We have verified that RIOK3 is the target protein regulated by miR-483-3P. The innovative point of this study is to confirm the effects of miR-483-3P on post transcriptional transactivation of downstream target gene and RIOK3 to study the biological behaviors of NB, such as proliferation, invasion and migration. The miRNAs are associated with signal pathways. The future miRNA involved in the development of NB molecular mechanism of research and will guide the targeted biological treatment of NB.

5. Conclusion

In this study, we confirmed that miR-483-3P was highly expressed in NB. Furthermore, miR-483-3P can promote the invasion and migration of cells in NB. We also confirmed that RIOK3 is the potential target protein for miR-483-3P. After inhibiting the expression level of miR-483-3P, the expression level of RIOK3 decreased correspondingly. Therefore a conclusion was reached that MiR-483-3P down regulate RIOK3 expression and promote proliferation and migration ability of human NB cells.

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