Regulations of LINC0196/miR-584-5p/miR-34a-5p/TRIM59 on progression of pediatric neuroblastoma

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This work was to study the regulatory mechanism of large intergenic non-coding RNA 0196 (LINC0196), miR-584-5p, miR-34a-5p, and tripartite motif 59 (TRIM59) on neuroblastoma. The interaction among the four was analyzed to provide a research basis for the clinical treatment of neuroblastoma at the molecular level. The human neuroblastoma SK-N-SH cells were collected and cultured. According to the transfection methods, the cells were divided into control group (without any treatment), si-LINC0196 group (si-LINC0196 transfection), si-LINC0196-NC group (si-LINC0196 vector transfection), miR-584-5p group (miR-584-5p mimic transfection), miR-584-5p-NC group (miR-584-5p inhibitor transfection), miR-34a-5p group (miR-34a-5p mimic transfection), and miR-34a-5p-NC group (miR-34a-5p inhibitor transfection). The proliferation, migration, and apoptosis of SK-N-SH cells in each group were compared. The effects of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 were evaluated. The expressions of LINC0196 and TRIM59 in SK-N-SH cells in si-LINC0196, miR-584-5p, and miR-34a-5p groups were up-regulated. miR-584-5p and miR-34a-5p were up-regulated in si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups decreased significantly (P < 0.05). The expression rate, migration rate, and invasiveness of SK-N-SH cells in miR-584-5p and miR-34a-5p groups were lower than those in si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups, while the apoptosis rate increased (P < 0.05). After miR-584-5p and miR-34a-5p transfections, the relative activities of WT-LINC0196 and WT-TRIM59 dual luciferase were greatly inhibited (P < 0.05). LINC0196 could regulate TRIM59 by regulating miR-584-5p and miR-34a-5p, thereby indirectly regulating cell proliferation, apoptosis, migration, and invasion of SK-N-SH cells.

INTRODUCTION

Neuroblastoma is a very common extracranial tumor in infants and young children with high malignancy, accounting for 8-10% of the incidence of malignant tumors in children (1). Benign neuroblastoma can be cured, but malignant neuroblastoma progresses rapidly and is prone to metastases, which is not easy to treat. For children with severe pathogenetic conditions, the survival rate is relatively low. According to research statistics, the deaths caused by neuroblastoma are about 15% of all malignant tumors in children (2). Therefore, it is very important to explore the analytical markers that affect the development and progression of pediatric neuroblastoma in order to provide a research basis for the clinical treatment of neuroblastoma.

Studies have found that long non-coding ribonucleic acid (RNA) (lncRNAs) are closely related to the development of various malignant tumors (3). As non-coding RNAs with lengths between 200 and 100,000 nt, lncRNAs play a certain role in regulating the cell cycle, cell differentiation, epigenetics, and other life activities (4). For example, large intergenic noncoding RNA 0196 (LINC0196) has been proven to promote the migration and invasion of gastric cancer, colon cancer, cholangiocarcinoma, and other cancer cells (5-7). When the LINC0196 gene is up-regulated, the ability of cancer cells to proliferate, invade, and migrate will be enhanced. In addition, many studies have proposed that micro-RNA (miRNA) is also involved in the differentiation process of various malignant tumor cells (8-9). miRNAs are non-coding short-chain small RNAs that can regulate gene expression after transcription. Through comprehensive analysis, some studies have discovered that miR-584-5p and miR-34a-5p can inhibit the proliferation, migration, and stemness of glioma cells and also affect the prognosis of patients (10). Both lncRNA and miRNA are involved in and are of great significance in the occurrence and development of tumor diseases. Some studies have suggested that lncRNA and miRNA are involved in the immunosuppressive process, oxidative stress response, angiogenesis, cell proliferation, metastasis, and metabolism of hepatocellular carcinoma (11). Tripartite motif 59 (TRIM 59) is a member of the TRIM protein family and has a wide range of biological functions. The roles of different species and types of TRIM are different in tumors, including promoting tumor development and inhibiting tumorigenesis (12). Studies have found that TRIM 59 can promote the proliferation of solid tumors cells of gastric cancer, bone cancer, lung cancer, and so on (13). However, TRIM 59 has different mechanisms for promoting cancer cell proliferation in different tumors.
tumor diseases, some by regulating the ubiquitination of p53 protein and some by up-regulating the expression of cell cycle-related proteins (14). LncRNA, miRNA, and messenger RNA (mRNA) have certain application value in controlling the development and treatment of tumor diseases. LncRNAs can exist as precursors of miRNAs, which can regulate the progression of tumor diseases by affecting miRNAs, and then indirectly regulate the expression of mRNAs corresponding to downstream target genes (15). However, there is a lack of current research on the effects of LINC0196, miR-584-5p, miR-34a-5p, and TRIM 59 on the biological function of pediatric neuroblastoma. Thus, further researches are needed.

To sum up, the regulatory mechanism of LINC0196, miR-584-5p, miR-34a-5p, and TRIM 59 will be explored in this work. The interaction of LINC0196, miR-584-5p, miR-34a-5p, and TRIM 59 was also analyzed to provide a research basis for the clinical treatment of neuroblastoma at the molecular level. It was expected to raise more effective treatment methods for patients clinically.

Materials and Methods

Research materials and their sources

Human neuroblastoma cell line SK-N-SH cells were purchased from the American Type Culture Collection (ATCC) Center. Minimum Essential Medium (MEM), 10% fetal bovine serum, 1% penicillin/streptomycin, and Lipofectamine 3000 transfection reagents were purchased from Invitrogen Corporation, the United States. Rabbit anti-human TRIM59 antibody and rabbit anti-human phosphorylated TRIM59 were purchased from Cell Signaling Technology, Inc., the United States. small interfering- (si-) LINC0196, pcDNA-LINC0196, miR-584-5p/miR-34a-5p, and miR-584-5p/miR-34a-5p inhibitors were from Beijing Zhong Shan -Golden Bridge Biological Technology CO., LTD. CCK-8 cell proliferation detection kits and related detection kits of real-time quantitative polymerase chain reaction (RT-qPCR) were of LightCycler 480 products, Roche Molecular Systems, Inc., Switzerland. The luciferase reporter vectors were bought from MedChemExpress LLC, the United States; the Annexin V-FITC/PI apoptosis detection kits were purchased from Jiangsu KeyGEN Biotech Co., Ltd.

Cell culture and transfection

Human umbilical vein endothelial cells (HUVECs) were placed in aMEM medium containing 10% fetal bovine serum and cultured in an incubator with 5% carbon dioxide at 37 ℃. The medium was changed 2-3 times per week, and the cells were passaged according to the ratio of 1:3. SK-N-SH cells in the logarithmic growth phase were selected and seeded in a 6-well plate, with 2.5 × 10^6 cells per well. 24 h later, si-LINC0196, miR-584-5p/miR-34a-5p, miR-584-5p/miR-34a-5p inhibitor, and their respective negative controls of SK-N-SH cells were transfected, respectively according to the instructions of Lipofectamine 3000. The cells were collected and detected 96 h after transfection.

Grouping

According to the transfection method, the human neuroblastoma cell line SK-N-SH cells were grouped. In the control group, none of the transfection as well as any other treatment was performed. In the si-LINC0196 group, si-LINC0196 transfection was performed. In the si-LINC0196-negative control (NC) group, the cells were transfected with the LINC0196 negative control vector. In the miR-584-5p group, miR-584-5p mimic transfection was made. In the miR-584-5p-NC group, miR-584-5p inhibitor negative control transfection was made. In the miR-34a-5p group, the cells were transfected with miR-34a-5p mimic. In the miR-34a-5p-NC group, the cells were transfected with miR-34a-5p inhibitor negative control. The proliferation, migration, and apoptosis of SK-N-SH cells were detected, analyzed, and compared to evaluate the effects of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59. Normal spinal cord dorsal root ganglion cells were collected (Wuhan Third Hospital) for comparative analysis.

Cell proliferation activity experiment

In this work, the CCK-8 kit was used to detect the proliferation of SK-N-SH cells. SK-N-SH cells were seeded into a 96-well plate (5 × 10^3 cells/well), and the cells were grouped after 24 h. According to CCK-8 kit instructions, the working solution was prepared. The ratio of culture medium to CCK-8 in each well was 10:1. That was, 10 μL of CCK-8 was added to 100 μL of culture medium. The working solution was added to each well. Then the cells were cultured at 37 ℃ for 1 h. The optical density (OD) of the cells in each well was detected by a microplate reader at a wavelength of 450 nm, and the proliferation activity of the cells was calculated and analyzed.

Flow cytometry detection

The Annexin V-FITC/PI apoptosis detection kit was utilized for staining SK-N-SH cells and detecting the apoptosis of cells. The SK-N-SH cultured cells were placed in a centrifuge tube, fixed with pre-cooled ethanol (75% concentration), and placed overnight at -20 ℃. 2 mL of buffer was added on the next day, then the cells were centrifuged for 5 min, and the step was repeated again. After centrifugation, SK-N-SH cells were collected (1 × 10^6 cells), and added with 500 μL buffer to resuspend the cells. Then 5 μL Annexin V-FITC and 5 μL PI staining solution were added successively to stain the cells. After mixing well, the cells were allowed to react in the dark at room temperature for 20 min. The cells were finally placed in flow cytometry to measure cell apoptosis.

Western blot experiment

The total protein in SK-N-SH cells was extracted by RIPA lysate, and the total concentration was measured by the bicinchoninic acid (BCA) method. 30 μg of protein samples were taken out and separated by 10% SDS-PAGE electrophoresis. Then the separation solution was transferred to the PVDF membrane. 5% nonfat dry milk was used for blocking overnight at room temperature of 4 ℃ with TRIM59, G1/S-specific cyclin-D1 (Cyclin D1), proliferating cell nuclear antigen (PCNA), cyclin-dependent kinase 2 (CDK2) primary antibodies. On the next day, the primary antibodies were washed off with Tris Buffered Saline Tween, and the secondary antibodies were added for incubation. After 2 h, Tris Buffered Saline Tween was utilized for rinsing. The color of the target protein bands was developed according to the instructions of the electrogenerated
MEM medium containing 10% fetal bovine serum was added to the lower chamber. The cells were incubated at room temperature of 37 °C. On the next day, the chambers were taken out and washed with buffer solution. After that, 4% paraformaldehyde was added to fix the cells, and crystal violet staining solution (0.1%) was added to stain the cells. After 10 min, the cells were washed with buffer solution. The number of transmembrane cells was counted under the microscope, and 3 fields of view were randomly selected from each group; the average value was taken.

**Dual-luciferase reporter assay**

The relationship among targets of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 was detected by dual-luciferase reporter assay. The TargetScan 7.2 database was utilized to predict the existence of binding sites among LINC0196, miR-584-5p, miR-34a-5p, and TRIM59. LINC0196 contained complementary nucleotide sequences to miR-584-5p and miR-34a-5p, and miR-584-5p and miR-34a-5p may be able to regulate TRIM59. Dual-luciferase reporter gene assay was performed to verify the results of the bioinformatics analysis. miR-584-5p, miR-34a-5p, and non-coding regions containing binding sites/mutation sites in LINC0196 and TRIM59 were amplified. These were then inserted into the pmirGLO vector to obtain wild-type (WT) plasmids WT-LINC0196, WT-miR-584-5p, WT-miR-34a-5p, and WT-TRIM59. SK-N-SH cells were seeded into a 24-well plate and co-transfected with LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 and WT or recombinant mutant vector using Lipofectamine 3000 reagent. The activity of the reporter genes was measured by the dual-luciferase reporter gene detection system at 48 h.

**Statistical methods**

The data of this work were analyzed by SPSS 22.0, and all results were expressed in the mean ± standard deviation (x̄±s). The t-test was used to compare the data between two groups, the one-way analysis of variance was used for comparing the data among multiple groups, and the SNK-q test was adopted for multiple comparisons between/among groups. P < 0.05 indicated that the difference was of statistical significance.

**Table 1. Primer information for the RT-qPCR.**

<table>
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<tr>
<th>Genes</th>
<th>Primer sequence (5’→3’)</th>
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| LINC0196 | F: 5'-CGACAAAGGGCTATGAAAGC-3’  
R: 5'-GACTATGC-CTTCCCATGTTG-3’ |
| miR-584-5p | F: 5'-TCGGGTTAATATCGGACAAC-3’  
R: 5'-CGTGCTGGGTTTGAGGTATT-3’ |
| miR-34a-5p | F: 5'-TCAAATTAATATCGGACAAC-3’  
R: 5'-CGTTTAGGGTCCGAGGTATT-3’ |
| TRIM59 | F: 5'-CTCCTCAAAAGGGCTATGAAAGC-3’  
R: 5'-GCAGATGTATTCCGGGTCAA-3’ |
| U6 | F: 5'-CGACAAAGGGCTATGAAAGC-3’  
R: 5'-CGTGCTGGGTTTGAGGTATT-3’ |
| GAPDH | F: 5'-TGACACAGCATCGGACAGA-3’  
R: 5'-GCCAGATGTATTCCGGGTCAA-3’ |
Results

Expressions of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 in normal tissues and SK-N-SH cells

With RT-qPCR, the expressions of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 in normal spinal cord dorsal root ganglion cell specimens and neuroblastoma cell line SK-N-SH cells were detected, respectively. Compared to normal spinal dorsal root ganglion tissue, the expression levels of LINC0196 and TRIM59 in neuroblastoma cell line SK-N-SH cells highly increased, while the expression levels of miR-584-5p and miR-34a-5p notably decreased ($P < 0.05$), as shown in Figure 1.

Expressions of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 in SK-N-SH cells in each group

RT-qPCR was utilized for detecting the expression levels of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 in SK-N-SH cells in the control group, si-LINC0196, si-LINC0196-NC, miR-584-5p, miR-584-5p-NC, miR-34a-5p, and miR-34a-5p-NC groups. As displayed in Figure 2, these expression levels were compared. In contrast to the control group, the levels of miR-584-5p and miR-34a-5p in the si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups were remarkably lower ($P < 0.05$); there was no significant change in LINC0196 and TRIM59 ($P > 0.05$). LINC0196 and TRIM59 in si-LINC0196, miR-584-5p, and miR-34a-5p groups were significantly decreased ($P < 0.05$); no significant change was observed in miR-584-5p and miR-34a-5p ($P > 0.05$). The comparative results among si-LINC0196, miR-584-5p, and miR-34a-5p groups were not significantly different from those of si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups ($P > 0.05$).

Compared to the control group, the levels of miR-584-5p and miR-34a-5p in the NC groups were markedly decreased ($P < 0.05$). LINC0196 and TRIM59 in si-LINC0196, miR-584-5p, and miR-34a-5p groups highly decreased ($P < 0.05$) as well.

Effects of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 on the proliferation of SK-N-SH cells

The proliferation of SK-N-SH cells in each group was detected and analyzed using CCK-8, as the specific results are shown in Figure 3. In comparison with the control group, the proliferation rates of SK-N-SH cells in si-LINC0196, miR-584-5p, and miR-34a-5p groups were greatly lower ($P < 0.05$). In contrast, the proliferation rates of SK-N-SH cells in si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups were highly increased ($P < 0.05$).

Effects of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 on apoptosis of SK-N-SH cells

The apoptosis of SK-N-SH cells in each group was...
detected by flow cytometry, and the specific results were presented in Figure 4. In contrast with the control group, there was not a significant difference in the apoptosis rate of SK-N-SH cells among si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups. While in si-LINC0196, miR-584-5p, and miR-34a-5p groups, the apoptosis of SK-N-SH cells was greatly increased ($P < 0.05$).

**Effects of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 expressions on the migration of SK-N-SH cells**

In the scratch test, the migration rate of SK-N-SH cells in each group was detected as displayed in Figure 5. Compared to the control group, none of the significant difference was shown in the migration rates of SK-N-SH cells in si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups ($P > 0.05$). The migration rates of SK-N-SH cells in si-LINC0196, miR-584-5p, and miR-34a-5p groups were remarkably decreased ($P < 0.05$).

**Effects of LINC0196, miR-584-5p, miR-34a-5p, TRIM59 expressions on SK-N-SH cell invasion**

A Transwell chamber test was adopted to detect the number of invasive cells among the SK-N-SH cells in each group, as shown in Figure 6. In contrast to the control group, the number of invasive cells in si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups increased, but these were not significantly different ($P > 0.05$). At the same time, the number of invasive cells in si-LINC0196, miR-584-5p, and miR-34a-5p groups notably decreased ($P < 0.05$).

**Effects of LINC0196, miR-584-5p, miR-34a-5p expressions on the expression of TRIM59, Cyclin D1, PCNA, and CDK2**

The protein expression levels of TRIM59, Cyclin D1, PCNA, and CDK2 in SK-N-SH cells in each group were detected by Western blot assay, which was displayed in Figure 7. In comparison with the control group, the expressions of TRIM59, Cyclin D1, PCNA, and CDK2 in SK-N-SH cells increased in si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups ($P < 0.05$). Meanwhile, the protein expression levels in SK-N-SH cells decreased in si-LINC0196, miR-584-5p, and miR-34a-5p groups ($P < 0.05$).

**Validation of the targeted relationship of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 to SK-N-SH cells**

The TargetScan 7.2 database was utilized to predict the binding sites between LINC0196 and miR-584-5p as well as miR-34a-5p and those between miR-584-5p, miR-34a-5p and TRIM59. Through dual luciferase reporter genes, the detection results of the above genes were worked out to show the relationship among them. The relative activities of WT-LINC0196 and WT-TRIM59 dual luciferases were observably inhibited after WT-LINC0196 and WT-TRIM59 were transfected with miR-584-5p and miR-34a-5p ($P < 0.05$), which was presented in Figure 8.

**Discussion**

Neuroblastoma is embryonal cancer that mainly occurs in the sympathetic nervous system. It is more common in
infants within 1 year of birth and in children under the age of 15. Neuroblastoma has serious adverse effects on children and their families, but its pathogenesis is still unclear. In recent years, some experts have proposed that lncRNAs and miRNAs play important roles in tumorigenesis (16,17). Clinical studies have also found that lncRNA can regulate mRNA expression by adsorbing miRNA, thereby regulating the development of tumor diseases (18). In this work, the relative activities of WT-LINC0196 and WT-TRIM59 dual luciferases were considerably inhibited after being transfected with miR-584-5p and miR-34a-5p, which confirmed the conclusion above. However, there are relatively fewer researches on its role in neuroblastoma.

This work was conducted to explore its role in neuroblastoma. The expression levels of LINC0196 and TRIM59 in neuroblastoma cell line SK-N-SH cells significantly increased, while the expressions of miR-584-5p and miR-34a-5p obviously decreased (P<0.05). This indicated that the occurrence of neuroblastoma was related to the increased expression of lncRNA LINC0196 and TRIM59. Wang et al. (2019) (19) found that patients with overexpression of LINC0196 in neuroblastoma had shorter overall survival than those with low expression. Therefore, LINC01296 was associated with the prognostic predictors of neuroblastoma. Chen et al. (2020) (20) concluded that overexpression of TRIM59 increased cell proliferation and promoted cancer development. In this work, the levels of miR-584-5p and miR-34a-5p in si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups were compared with those in the control group. This further supported that LINC0196 and TRIM59 had certain cancer-promoting effects, while miR-584-5p and miR-34a-5p could inhibit the development of cancer. This was consistent with the research results of Xiao et al. (2022) (21) and Kern et al. (2021) (22), suggesting that this work had a certain accuracy.

Then, CCK-8 detection, flow cytometry, scratch test, Transwell chamber, etc. were utilized to evaluate the effects of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 on proliferation, migration, apoptosis, and invasion of neuroblastoma cell line SK-N-SH cells. The results revealed that miR-584-5p and miR-34a-5p could inhibit the proliferation, migration, and invasion of SK-N-SH cells and promote the apoptosis of SK-N-SH cells. Nevertheless, the effects of LINC0196 and TRIM59 were opposite. It was suggested that miR-584-5p and miR-34a-5p could inhibit the development of cancer, while LINC0196 and TRIM59 could be used for prognosis prediction of neuroblastoma.

Later, Western Blot was also adopted to detect the expression of cell proliferation-related Cyclin D1 and PCNA, as well as the expression activity of migration and invasion-related protein CDK2. The expressions of TRIM59, Cyclin D1, PCNA, and CDK2 in SK-N-SH cells in si-LINC0196-NC, miR-584-5p-NC, and miR-34a-5p-NC groups increased (P<0.05). Such a finding indicated that up-regulated miR-584-5p and miR-34a-5p and down-regulated LINC0196 inhibited the development of neuroblastoma by inhibiting the expression of Cyclin D1, PCNA, and CDK2. Montalto and De Amicis (2020) (23) also proposed that Cyclin D1 could regulate the expression of specific miRNAs, but a related study was not conducted in this work, which required additional research. Feng et al. (2019) (24) put forward that lncRNA could regulate the proliferation of PCNA-enhanced cells. Aguennouz et al. (2021) (25) raised that miRNA could inhibit the expression of CDK2 protein and reduce the differentiation of cancer cells. The above studies all provided support for this work. It was indicated that TRIM59 could be regulated by LINC0196 via regulating miR-584-5p and miR-34a-5p, thereby indirectly regulating the expression of SK-N-SH cells.

The regulatory mechanism of LINC0196, miR-584-5p, miR-34a-5p, and TRIM59 on neuroblastoma was studied, and the interaction among them was analyzed. Thus, it was concluded that TRIM59 could be regulated by LINC0196, as miR-584-5p and miR-34a-5p were regulated by it. Thereby, the cell proliferation, apoptosis, migration, and invasion of SK-N-SH cells could be indirectly regulated. However, this work lacked clinical application research, and the clinical application value needed further exploration. It was of great significance to explore the pathogenesis of neuroblastoma and to provide a reference for finding new therapeutic targets.

References

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