Original Article

miR-26b suppresses tumor proliferation and metastasis by targeting matrix metalloproteinases 14 in neuroblastoma

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Abstract: Increasing reports suggested that microRNAs (miRNAs) act as key regulators in multiple cancers. miR-26b functions as a tumor suppressor in some malignancies. However, its role in neuroblastoma (NB) remains poorly understood. Herein, we found that miR-26b is downregulated in NB tissues. Enhanced overexpression of miR-26b significantly suppressed NB cell proliferation, migration, and invasion. Matrix metalloproteinase 14 (MMP14) was found to be a direct target of miR-26b in NB cells. Rescue experiments indicated that the suppressive effect of miR-26b on cell proliferation, migration, and invasion is partially mediated by inhibiting MMP14 expression. Collectively, the present study indicated that miR-26b might inhibit NB cell viability and motility partially by targeting MMP14. This newly identified function of miR-26b provides novel insights into neuroblastoma and may foster therapeutic applications.

Keywords: Neuroblastoma, MicroRNAs, miR-26b, MMP14

Introduction

Neuroblastoma (NB) is one of the most common extracranial childhood tumors, accounting for 7% of childhood malignancies and >15% of all childhood cancer deaths [1]. NB is characterized by heterogeneous clinical behaviors, ranging from spontaneous regression to rapid progression or resistance to multimodal treatment, such as surgery, chemoradiotherapy, and immunotherapy [2]. Despite recent therapeutic improvements in NB treatment, the overall 5-year survival rate remains poor, 73% of NB patients have already developed malignant lesions at the time of diagnosis [3, 4]. Therefore, a better understanding of the detailed mechanisms might be helpful to find new therapeutic targets and strategies for the treatment of NB.

MicroRNAs (miRNAs), a class of small non-coding RNAs with 22 to 25 nucleotides in length, are able to inhibit gene expression at the post-transcriptional or translational levels through forming base pairs with their targets, usually in the 3' untranslated region (3'-UTR) [5, 6]. miRNAs play essential roles in a variety of biological and pathological processes, including cell proliferation, migration, invasion, apoptosis, and metastasis [7]. In cancer, the function of miRNAs is dependent on their mRNA targets, so they can act as tumor suppressors or as oncogene [8]. For example, Friedman et al suggested that miR-101 acted as a tumor suppressor to modulate the cancer epigenome by repressing the polycomb group protein EZH2 [9]. Jiang et al showed that miR-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene [10]. Tsai et al indicated that miR-122 function as a tumor suppressor that regulated intrahepatic metastasis of hepatocellular carcinoma [11].

In the present study, we identified the important molecular mechanism by which miR-26b exerted its tumor suppressive effects on NB cell proliferation, migration and invasion, which involved the direct targeting the 3'-UTR of MMP14.
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Materials and methods

Patients

22 paired of NB and matched adjacent non-tumor tissues from patients were obtained from Eastern District of the First Affiliated Hospital. The diagnoses of these samples were verified by pathologists. The collection of human tissue samples was approved and supervised by the Ethics Committee of Sun Yat-sen University.

Cell culture and transfection

The human neuroblastoma cell line SHSY5Y and the HEK-293 cell lines were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). All cells were cultivated in RPMI1640 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) and maintained at 37°C in a humidified 5% CO₂ incubator. miR-26b mimics and control mimics were purchased from RiboBio (Guangzhou, China). pcDNA3-MMP14 was obtained from Applied Biosystems (Foster). Transfections of miRNA mimics or pcDNA3-MMP14 were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was extracted by TRIzol Reagent (Invitrogen). SYBR Premix Ex Taq (Takara) was used to detect gene expression on ABI Stepone plus (Applied Biosystems). miRNA was extracted using All-in-one microRNA extraction kit (GeneCopoeia). Primers for MMP14: sense, 5'-TCGGCCCAAGCAGCAGGCTTC-3' and anti-sense, 5'-CTTCATGGTGTCTGATCGACG-3'. Primers for GAPDH: sense, 5'-AACGAGTTGCTCGATTGGAATC-3', and anti-sense, 5'-GGAAGATGGTGATGGGATTGATT-3'. Primers for miR-26b and U6 were purchased from GeneCopoeia (Carlsbad). The expression of MMP14 was normalized with GAPDH, and the expression of miR-26b was normalized with U6.

Luciferase reporter assay

pGL3 plasmid encoding a luciferase reporter gene was purchased from Promega. Recombinant plasmid of pGL3-MMP14 3'-UTR (Wt) or pGL3-MMP14 3'-UTR (Mut) was constructed in our laboratory. HEK-293 cells were plated in a 24-well plate and cotransfected with either miR-26b mimics or miRNA control, either pGL3-MMP14 3'-UTR (Wt) or pGL3-MMP14 3'-UTR (Mut), and 2 ng of pRL-TK (Promega) by using Lipofectamine 2000. The pRL-TK vector was used as an internal control to correct the differences in both transfection and harvest efficiencies. HEK-293 cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega).

Cell proliferation analysis

Cell viability was measured using the MTT assay. The viability of SHSY5Y cells transfected with miR-26b or control mimics was detected every 24 h after transfection. 100 μl MTT (10 mg/ml) was added to each well of the 96-well plates and incubated for 4 h, and then the reaction was terminated by DMSO. The optical density (OD) at 490 nm was detected using a Microplate Reader (Bio-Tek).

Cell invasion and migration assays

Transfected SHSY5Y cells were seeded in the upper compartments of the 8 um Boyden chamber (BD Biosciences). Migration and invasion assays were carried out with coated Matrigel (invasion) or uncoated Matrigel (migration). After incubation for 48 h, cells migrated and invaded through the lower chamber were fixed with methanol, stained with 0.5% Giemsa, and photographed under the microscope (Olympus).
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Western blot

Transfected cells were washed twice with PBS and proteins were extracted with SDS lysis buffer (Beyotime), and separated by 10% SDS-PAGE gel. Protein samples were transferred to PVDF membrane (Millipore), and were probed with primary antibodies against MMP14 (Abcam). Membranes were incubated at 4°C overnight, followed by incubation with AP-conjugated secondary antibodies and detected by ECL (Thermo Scientific).

Statistical analysis

All data were presented as mean ± SD and analyzed by using SPSS 16.0. Two-tail Student’s t test and ANOVA were performed to determine the differences. P<0.05 was considered statistically significant.

Results

miR-26b was down-regulated in NB tissues

To investigate whether miR-26b is involved in the progression of neuroblastoma, we firstly compared the expression of miR-26b between NB tissues and adjacent non-tumor tissues. Our data showed that the expression of miR-26b was significantly decreased in NB tissues compared with matched adjacent non-tumor tissues (Figure 1, P<0.05). These data suggested that miR-26b may contribute to the progression of NB.
Ectopic expression of miR-26b inhibited NB cell viability and motility

To examine the role of miR-26b in NB cell progression, SHSY5Y cells were transfected with miR-26b mimics or miR-NC and the transfection efficiency was determined by qRT-PCR. We found that miR-26b expression was significantly increased in SHSY5Y cells after transfected with miR-26b mimics (Figure 2A, P<0.05). MTT assay revealed that over-expression of miR-26b significantly inhibited the proliferation ability of SHSY5Y cells (Figure 2B, P<0.05). In addition, transwell migration and invasion assays suggested that overexpression of miR-26b also remarkably suppressed the in vitro migration and invasion abilities of SH-SY5Y cells (Figure 2C and 2D, P<0.05). These findings indicated that miR-26b could suppress the development and progression of NB.

miR-26b directly targeted MMP14 and inhibited its expression in NB cells

To screen the function target of miR-26b in NB cells, TargetScan 6.2 was used to screen the target gene of miR-26b. MMP14 was predicted to be a target of miR-26b in NB cells (Figure 3A). We constructed luciferase reporter plasmids to contain the putative sequences (Wt) for MMP14 and their corresponding mutant sequences (Mut) as controls. Luciferase activity assay showed that miR-26b significantly inhibited the luciferase activity of the Wt 3'-UTR but not that of Mut 3'-UTR of MMP14 (Figure 3B, P<0.05). In addition, overexpression of miR-26b significantly inhibited MMP14 mRNA and protein levels (Figure 3C and 3D, P<0.05). These results suggested that MMP14 might be a direct target of miR-26b in NB cells.

MMP14 overexpression attenuated the suppressive effect of miR-26b

Further experiments were performed to explore whether overexpression of MMP14 could attenuate the suppressive effect of miR-26b. The effect of MMP14 plasmids was detected by qRT-PCR (Figure 4A, P<0.05). MTT assay revealed that supplement of MMP14 by pcDNA3-MMP14 could significantly attenuate the
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The suppressive effect of miR-26b in NB cell proliferation (Figure 4B, *P<0.05). In addition, in vitro cell migration and invasion showed that overexpression of MMP14 significantly attenuated the suppressive effect of miR-26b in NB cell motility (Figure 4C and 4D, *P<0.05). These results suggested that miR-26b might suppress NB progression by targeting MMP14 (Figure 5).

Discussion

In this study, we explored the expression of miR-26b in NB patients. Our data showed that the expression levels of miR-26b were significantly decreased in NB tissues and ectopic expression of miR-26b remarkably inhibited the proliferation, migration and invasion of NB cells. By using luciferase activity assay and Western blot, we found that MMP14 was a direct target of miR-26b. Overexpression of miR-26b suppressed cell growth and motility, whereas overexpression of MMP14 antagonized this effect. Our findings illustrated the possible role of miR-26b and MMP14 in the progression of NB.

Accumulating evidence revealed the important function of miRNAs in NB development and progression. For example, Liu et al showed that...
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Figure 5. Schematic miR-26b suppressed NB cell progression via targeting MMP14.

miR-451 inhibited NB cell proliferation, invasion and migration by targeting macrophage migration inhibitory factor [12]. Wu et al indicated that miR-362-5p inhibited proliferation and migration of NB cells by targeting phosphatidylinositol 3-kinase-C2β [13]. On the other hand, several miRNAs function as oncogenes in NB. For example, Qu et al reported that miR-558 promoted tumorigenesis and aggressiveness of NB cells through activating the transcription of heparanase [14]. Li et al found that miR-421 promoted proliferation and migration of NB cells by targeting tumor suppressor menin [15].

Recent studies showed that miR-26b acted as a tumor suppressor in some cancers. For example, Li et al suggested that miR-26b inhibited hepatocellular carcinoma cell proliferation, migration, and invasion by targeting EphA2 [16]. Li et al revealed that miR-26b suppressed the metastasis of non-small cell lung cancer by targeting MIEN1 via NF-κB/MMP-9/VEGF pathways [17]. Zheng et al showed that miR-26b inhibited osteosarcoma cell migration and invasion by down-regulating PFKFB3 expression [18]. Our study expanded the tumor suppressive role of miR-26b in NB progression.

Matrix metalloproteinases 14 (MMP14) is a “master switch” proteinase with a C-terminal sequence that acts as membrane-anchoring domain, and is a key enzyme involved in ECM degradation and invasion of tumor cells [19]. MMP14 is constitutively increased in several cancers, such as gastric cancer, tongue squamous cell carcinoma and renal cancer [20-22]. MMP14 contributes to cancer progression and was regulated by many miRNAs. For example, Chang et al found that miR-133b inhibited cell migration and invasion by targeting MMP14 in glioblastoma [23]. Li et al demonstrated that miR-150-5p could inhibit hepatoma cell migration and invasion by targeting MMP14 [24]. In this study, we found that MMP14 could also be regulated by miR-26b in NB, supporting its oncogenic role in NB progression.

In conclusion, this study identify a functional link between miR-26b and MMP14 expression in NB, indicating that miR-26b may serve as a potential therapeutic target for the treatment of NB.

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Disclosure of conflict of interest

None.

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