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Wnt inhibitory factor-1 functions as a tumor suppressor through modulating Wnt/β-catenin signaling in neuroblastoma ☆

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ABSTRACT

Neuroblastoma is the most common extracranial solid tumor in childhood and is associated with serious morbidity and mortality. The effective treatment of neuroblastoma remains one of the major challenges in pediatric oncology. The Wnt signaling pathway has been shown to play a significant role in the pathogenesis of adult and pediatric tumors. WIF-1 has been identified as an important Wnt antagonist which inhibits Wnt/β-catenin signaling by directly binding to Wnt proteins. However, the expression and function of WIF-1 in neuroblastoma remains unknown. The present study showed that WIF-1 was downregulated with high level promoter methylation in neuroblastoma cells, and was significantly upregulated after exposure to demethylating agent. This finding suggests that downregulation of WIF-1 was associated with its promoter methylation in neuroblastoma. To further study the potential function of WIF-1 in neuroblastoma, we constructed a plasmid that over-expressed WIF-1 and transfected the plasmid into one neuroblastoma cell line SK-N-SH. We found that restoration of WIF-1 inhibited the growth and proliferation of neuroblastoma cells in vitro. Morever, Wnt/β-catenin signaling activity and target genes expression were reduced by WIF-1 restoration. These results provide support that WIF-1 is downregulated and functions as a tumor suppressor by antagonizing Wnt/β-catenin signaling in neuroblastoma, suggesting a potential role as a therapeutic target in neuroblastoma.

1. Introduction

Neuroblastoma is the most common form of pediatric cancer, accounting for approximately 30% of all infantile malignancies and 15% of all pediatric oncology deaths [1]. Patients with neuroblastoma often present with advanced disease characterized by invasive tumor masses and/or metastases to distant organs [2]. With the development of molecular biology and recognition to neuroblastoma, researchers have begun to seek specific regulators for carcinogenic signaling pathways as new therapeutic strategies.

The Wnt/β-catenin signaling pathway is a highly conserved signaling pathway during evolution. It plays an essential role in regulating embryonic development, cell proliferation, and differentiation [3]. A large body of evidence indicates that abnormal Wnt/β-catenin pathway activity is associated with many types of human tumor [4–6]. Other studies have also shown that aberrant activation of this pathway is induced by dysregulation of Wnt pathway members or by downregulation of endogenous Wnt antagonists [7–9]. Aberrant expression of Wnt/β-catenin signaling is known to play a significant role in various processes of early development

Abbreviations: WIF-1, Wnt inhibitory factor-1; MSP, methylation-specific PCR; 5-Aza-dC, 5-Aza-2′-deoxycytidine; IHC, immunohistochemistry assay; IF, immunocytofluorescence assay; ICC, immunocytochemistry assay.

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and in the pathogenesis of adult and pediatric tumors including neuroblastoma [10,11].

Constitutive activation of the Wnt/β-catenin signaling pathway can be induced by dysregulation of Wnt pathway members, such as overexpression of β-catenin [4,6]. Other workers have shown that dysregulated Wnt signaling can be experimentally induced by Wnt antagonists such as Dickkppfs and sFRPs [8,9].

Wnt inhibitory factor-1 (WIF-1) is an important antagonist involved in the Wnt/β-catenin signaling pathway. It is highly conserved between species and was initially identified from the human retina [12]. WIF-1 secretion has been shown to inhibit the upstream of Wnt/β-catenin signal transduction by directly binding to Wnt proteins in the extracellular space [13]. This prevents the activation of Wnt target genes including c-myc and cyclin D1 [14,15]. Downregulation of WIF-1 has been reported in many human malignancies [16–21]. However, the expression and functional significance of WIF-1 in neuroblastoma remains unknown.

2. Materials and methods

2.1. Tumor samples and cell lines

Tumor samples including 42 neuroblastoma tissues, 14 ganglioneuroma tissues and 11 normal adrenal gland tissues were obtained from Department of Pathology of the West China Hospital of the Sichuan University, China.

Three human neuroblastoma cell lines IMR-32, SK-N-SH, SH-SY5Y were used as experimental cell lines, and a human embryonic kidney cell line HEK293 was used as normal control. SK-N-SH and HEK293 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). IMR-32 and SH-SY5Y were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

IMR-32 cells were cultured in EMEM (Invitrogen, Carlsbuel, CA) supplemented with 10% fetal bovine serum (FBS). SK-N-SH and HEK293 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS. SH-SY5Y was cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS. All cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

2.2. Demethylation

Cultured cell lines were seeded into six-well plates at a density of 1×10^5 cells per well. Twenty-four hours later, cells were exposed to 50 μmol/L of the demethylating agent 5-Aza-dC (Sigma, St. Louis, MO) for 4 days. Fresh demethylating agent was added every 12 h. Cells not treated with 5-Aza-dC were used as controls. Genomic DNA and RNA were extracted from the cells at the end of the experiment.

2.3. Immunohistochemistry

Immunohistochemical staining (IHC) was used to determine WIF-1 expression in tissues. WIF-1 antibody (1:100; Epitomics, Burlingame, CA) and diaminobenzidine (DAB) were used for staining and visualization, respectively. Normal adrenal gland tissues were used as positive controls.

Results were independently evaluated by two pathologists in a blind fashion. IHC staining was graded on an arbitrary scale from 0 to 2: where 0 represented negative expression (0–20% positive cells), 1 represented weakly positive expression (20–50% positive cells), and 2 represented strongly positive expression (50–100% positive cells). The scale was determined according to the average number of positive cells in 10 random fields of one slide [22].

2.4. RNA extraction and real-time PCR

Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using a First Strand cDNA Synthesis kit (Fermentas, Burlington, Ontario) according to the manufacturer's protocol. Real-time quantitative RT-PCR (qRT-PCR) was performed using a relative quantification protocol on an iCycler iQ System (BioRad Laboratories, Hercules, CA). β-actin was used as an internal control. RNA extracted from HEK293 cells served as a positive control, and reagent without template served as a negative control.

The relative fold change in mRNA expression compared with control was calculated using the comparative C_t method [23]. The sequences of PCR primers are listed in Supplimental Table 1.

2.5. Protein extraction and Western blotting

Total cell proteins were isolated and purified using the Total Protein Kit (Sigma) according to the manufacturer's protocol. The protein content was determined using the BCA assay method (Pierce, Rockford, IL) with GAPDH was as an internal control. Briefly, SDS–PAGE 10% separating gels were run using 20 μg of each protein sample. After gel electrophoresis, protein extracts were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) and then blocked with 5% BSA at room temperature for 1 h. The membranes were then incubated overnight at 4 °C with antibody against WIF-1 (1:100, Epitomics), β-catenin (1:5000, Epitomics), c-myc (1:2000, Epitomics), cyclinD1 (1:10000, Epitomics) or GAPDH (1:1000, Cell Signaling, Beverly, MA). After washing, the membranes were incubated with secondary antibody (1:2000, Cell Signaling, Beverly, MA) at room temperature for 2 h.

The membranes were visualized using the Bio-Rad ChemiDoc XRS imaging system. Densitometric analyses were performed using BioRad Quantity One software. The intensity of the bands of each treatment was compared with the intensity of control.

2.6. Genomic DNA extraction and methylation-specific PCR (MSP)

Genomic DNA was extracted from neuroblastoma cell lines and HEK293 cells using Blood and Cell Culture DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Bisulfite modification of genomic DNA was performed using the EZ DNA methylation kit (Zymo Research, Orange, CA) following the manufacturer's protocol. Bisulfite-treated genomic DNA was amplified using either a methylation-specific or an non-methylation-specific primer set. Fragments of MSP and USP were 145 bp and 151 bp, respectively. The sequences of MSP primers are listed in Supplimental Table 1.

2.7. Plasmid construction and cells transfection

The full length cDNA of WIF-1 (Invitrogen) was subcloned into the HindIII-XhoI sites of pcDNA3.1 vector (Invitrogen) to generate pcDNA3.1-WIF-1 with confirmed sequence and orientation. SK-N-SH cells (1×10^5 cells) were dissociated into single cell suspension and plated in 6-well plates for transfection. One day later, cells were transiently transfected with 5 μg pcDNA3.1-WIF-1 or pcDNA3.1 (vector control) using Lipo 2000 (Invitrogen) according to the manufacturer's protocol. Forty-eight hours later, the stable transfected cells were selected for culture for 30 days with G418 (700 μg/ml). The expression of WIF-1 was assessed by Western blotting and real-time PCR. The selected stable transfected cells were cultured in DMEM containing 10% FBS and G418 (500 μg/ml).

2.8. Proliferation assay

SK-N-SH cells (5×10^3 per well) were plated in 96-well plates 24 h before transfection. After transfection with pcDNA3.1-WIF-1 or pcDNA3.1, cells were incubated for 144 h. Cell viability was determined every 24 h by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay) (Promega, Madison, WI) according to the manufacturer's protocol. Absorbance at 490 nm was measured with a microplate reader (BioRad) and used as a measure of cell number. The samples were repeated three times in triplicate.

2.9. Soft agar colony formation assay

Soft agar colony formation assay performed using 6-well plates. Each well contained 2 mL of 0.8% agar in complete medium as the bottom layer, 1 mL of 0.35% agar in complete medium with 6×10^3 stable transfected cells or vector control as the feeder layer, and 1 mL complete medium as the top layer. Cultures were maintained for 2 weeks under standard conditions. The number of colonies was determined with an inverted phase-contrast microscope at 100x magnification. Colonies containing more than 10 cells were counted.

2.10. Dual-Luciferase reporter assay

TOP flash or FOP flash plasmids were cotransfected with either pcDNA3.1-WIF-1 or empty vector, together with an internal control pRL-TK vector using Lipo 2000. Forty-eight hours later, cells were harvested and analyzed for Firefly and Renilla luciferase activity using the Dual-Luciferase reporter assay kit (Promega). Each experiment was repeated three times.

2.11. Immunocytochemistry assay

SK-N-SH cells were dissociated into a single cell suspension and plated on coverslips. One day later, cells were transfected with pcDNA3.1-WIF-1 or empty vector. Forty-eight hours later, the transfected cells were fixed with 4% paraformaldehyde solution at room temperature for 30 min. Primary antibody and DAB were for staining and visualization, respectively. Cells were counterstained with hematoxylin. The primary antibodies were WIF-1 (1:100; Epitomics, Catalog#T0809), β-catenin (1:250; Epitomics, Catalog#1247-1), c-myc (1:250; Epitomics, Catalog#1472-1) or cyclin D1 (1:100; Epitomics, Catalog#2261-1).