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Downregulation of bone morphogenetic protein receptor 2 promotes the development of neuroblastoma

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ABSTRACT

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood. In this study, we examined the expression of bone morphogenetic protein receptor 2 (BMPR2) in primary NB and adjacent non-tumor samples (adrenal gland). BMPR2 expression was significantly downregulated in NB tissues, particularly in high-grade NB, and was inversely related to the expression of the NB differentiation markers ferritin and enolase. The significance of the downregulation was further explored in cultured NB cells. While enforced expression of BMPR2 decreased cell proliferation and colony-forming activity, shRNA-mediated knockdown of BMPR2 led to increased cell growth and clonogenicity. In mice, NB cells harboring BMPR2 shRNA showed significantly increased tumorigenicity compared with control cells. We also performed a retrospective analysis of NB patients and identified a significant positive correlation between tumor BMPR2 expression and overall survival. These findings suggest that BMPR2 may play an important role in the development of NB.

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1. Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood, and the most common type of cancer diagnosed before 1 year of age [1]. NB, which is responsible for ~15% of pediatric cancer deaths [2], is an embryonic tumor of the sympathetic nervous system that arises mostly from the adrenal medulla but may develop anywhere along the sympathetic nervous system chain from the neck to the pelvis; however, it rarely occurs in the central nervous system. NB is clearly associated with abnormal development, but its etiology is not well understood. Intriguingly, it is one of the few human malignancies to demonstrate spontaneous regression from an undifferentiated state to a completely benign cellular appearance [3]. The majority of NBs are sporadic, and the molecular events that predispose to their development are largely unknown. However, it has been reported that the primary cause of familial NB, which accounts for about 1–2% of cases, is a germline mutation in the ALK gene [4]. A number of other genetic alterations are also implicated in various forms of the tumor [5]. The treatment of NB differs for each individual and depends on the site of the primary tumor, tumor histology, and metastasis, which is present in ~70% of patients at diagnosis. The prognosis of children with NB has improved, which is largely attributable to improved treatments for patients with low-grade tumors. However, the survival rate of patients with high-risk disease (high grades) is only 20–40% [6], underscoring the need to understand the molecular pathogenesis of NB and to develop novel therapeutics.

Bone morphogenetic protein receptor (BMPR) 2 is a serine/threonine kinase receptor that forms a heterodimer with BMPR1. Upon binding of its bone morphogenetic protein (BMP) ligands, BMPR2 activates intracellular Smad proteins, which then regulate gene expression [7]. BMPs are a diverse class of molecules having profound effects on the growth and differentiation of cells and the formation of multiple tissues and organs [8]. Aberrant BMP

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signaling is associated with many human diseases and pathogenic processes, including cancer progression and metastasis [9]. Perhaps reflecting their diverse activities, BMPs have been reported to promote tumor growth in some studies and to exhibit tumor-suppressive effects in other studies [10,11]. Since NB is characterized by varied differentiation status, abnormal cell growth, and metastasis, we sought to determine whether BMPR2 affects the development of NB.

In this study, we found that BMPR2 is significantly downregulated in primary NB tissue, especially high-grade disease. Enforced expression of BMPR2 in NB cells *in vitro* inhibits their proliferation and clonogenicity, whereas knockdown of BMPR2 promotes the growth of NB cells *in vitro* and *in vivo*. A retrospective analysis revealed that high BMPR2 expression is associated with better prognosis for NB patients. Thus, BMPR2 appears to play an important role in the development of NB, and activation of the BMPR2 system may be a novel strategy for its treatment.

2. Materials and methods

2.1. Cell lines and culture

The five NB cell lines used in this study, IMR-32, KP-N-NS, SK-N-SH, SH-SY5Y, and BE(2)c, were obtained from the Tissue Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

2.2. Antibodies, plasmids, and reagents

The anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA); the BMPR2 antibody used for immunoblotting was purchased from Cell Signaling Technology (Danvers, MA, USA), and the BMPR2 antibody used for immunohistochemical staining was purchased from Abcam (Hong Kong, China). Lentiviral vectors expressing *BMPR2*-targeting and control (scrambled) small hairpin RNA (shRNA) were purchased from Open Biosystems (Thermo Fisher Scientific). Three different shRNAs were used to target *BMPR2* mRNA: V3LHS_392655: AACACTGTGGTTTCTACCT; V3LHS_392657: TCATAGTGACACTCTTGGG; and V3LHS_392658: TGTGTTGACTCACCTATCT.

2.3. Lentiviral vector construction, packaging, and infection

The entire coding sequence of the *BMPR2* cDNA was amplified and cloned into the pWPXL vector (Addgene, Guangzhou, China). The primer sequences and restriction sites are listed in Table 3.

2.4. NB specimens

Samples of 48 NB and 23 adjacent non-tumor (adrenal gland) tissues were obtained from 48 patients (Table 1). Genomic DNA was extracted from the specimens for analysis of *BMPR2* copy number by quantitative PCR (q-PCR). Correlations between *BMPR2* DNA copy number and mRNA levels in each tumor were assessed using linear Pearson's R correlation coefficients. The coefficients were interpreted using the scale provided by Salkin, in which R = 0.8–1.0 is defined as very strong, 0.6–0.8 as strong, 0.4–0.6 as moderate, 0.2–0.4 as weak, and 0.0–0.2 as very weak or no relationship [12].

2.5. Immunohistochemistry

Immunohistochemical staining was performed using a standard two-step peroxidase technique [13]. Staining intensity was scored by three experienced pathologists blinded to the experimental and clinical data. Samples were scored on a semi-quantitative scale based on the percentage of positively stained cells: 1 (0–25%), 2 (26–50%), 3 (51–75%), and 4 (76%–100%). The consensus of at least two of the pathologists was required. The scoring system was applied to both xenografts and clinical NB samples.

Table 1
Clinicopathological characteristics of NB patients.

Clinical parameters		Case(%)
Gender	Male	29(60.4)
	Female	19(39.6)
Age	<12 MTH	10(20.8)
	12-18 MTH	2(4.2)
	≥18 MTH	36(75)
INSS	I	15(31.3)
	II	4(8.3)
	III	8(16.7)
	IV	20(41.7)
	IVs	1(2.1)
MYCN	A	11(22.9)
	NA	37(77.1)
Pathology	FH	23(47.9)
	UH	25(52.1)

2.6. Real-time q-PCR

DNA copy number and mRNA expression levels were quantified using a 7900 Real-Time PCR System with SDS 2.3 software (Applied Biosystems, Waltham, MA USA). First-strand cDNA was synthesized using Reverse Transcription Reagents (TaKaRa). Quantitative PCR of genomic DNA and cDNA was performed with SYBR Green Premix with ROX (TaKaRa). Repetitive long interspersed element (LINE)-1 and β-actin were used as the endogenous controls. DNA copy number was normalized to LINE-1 (forward: 5′-AAAGCCGCT-CAACTACATGG-3′; reverse: 5′-TGCTTTGAATGCGTCCCA GAG-3′) [14], and *BMPR2* expression was normalized to that of β-actin (forward: 5′-AGTG TGACGTGGACATCCGCAAAG-3′; reverse: 5′-ATCCACATCTGCTGGAAGG TGGAC-3′).

2.7. Cell proliferation and colony-forming assays

Cell proliferation was assessed with the CCK-8 kit according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). For colony-forming assays, cells were seeded in 6-well plates at 2000 cells/well and cultured at 37 °C for 2 weeks. At the end of the incubation, the cells were fixed with 100% methanol and stained with 0.1% crystal violet. Cell colonies were counted using Image-Pro Plus 5.0 software (Media Cybernetics, Bethesda, MD, USA). Each measurement was performed in triplicate and each experiment

Table 2
Immunohistochemical analysis of BMPR2 expression.

Tissues	Cases(N)	score of BMPR2 expression				*p*
		1	2	3	4	
NB	48	17	12	19	0	0.0003*
NT	23	1	2	6	14	
INSS						
1	15	1	2	12	0	
2	4	0	1	3	0	
3	8	1	6	1	0	
4	20	15	3	2	0	
4s	1	0	0	1	0	
Grade[a]						
Low	20	1	3	16	0	<0.0001*
High	28	16	9	3	0	

*P < 0.01.
[a] Low grade refers to INSS stages 1, 2, and 4s; high grade refers to INSS stages 3 and 4.

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