Int J Clin Exp Pathol 2015;8(7):8268-8275
www.ijcep.com /ISSN:1936-2625/IJCEP0010327

Original Article

Overexpression of the BMP4/SMAD signaling pathway in skull base chordomas is associated with poor prognosis

Yanjun Feng, Qiuhang Zhang, Zhenlin Wang, Bo Yan, Wei Wei, Pu Li

Department of Otolaryngology, Head and Neck Surgery, Skull Base Surgery Center, Xuanwu Hospital, Capital Medical University, Beijing 100053, P. R. China

Received May 18, 2015; Accepted June 27, 2015; Epub July 1, 2015; Published July 15, 2015

Abstract: Chordomas are rare, locally invasive tumors with characteristic expression of the T-box transcription factor Brachyury. Little is yet known of the molecular events involved in the development of these tumors. Bone morphogenesis protein 4 (BMP4) signaling, which acts upstream of Brachyury in embryonic development, has been implicated in carcinogenesis in multiple malignancies. To explore the role of the canonical BMP4/SMAD signaling pathway in the pathogenesis of chordoma, we investigated, in 40 skull base chordomas, the expression of three major components of the signaling axis: BMP4, phospho-SMAD5 and SMAD4. Immunostaining revealed positive expression in 70%, 52.5% and 90% of cases, respectively. Eighteen (45%) of patients exhibited concurrent positive expression of these markers, which we defined as "high" expression of the BMP4/SMAD signaling pathway. Interestingly, when we compared the pattern of expression with clinicopathological parameters, we found that high expression of the pathway was more often observed in larger tumors (≥ 4 cm) than smaller ones (P = 0.010), and correlated significantly with dural invasion (P = 0.024). The Kaplan-Meier log-rank test showed that the 5-year overall survival rate for patients with high expression of the pathway was significantly lower than those with low expression (71.4% vs. 90.2%, P = 0.010). In conclusion, our results demonstrate for the first time that overexpression of the BMP4/SMAD signaling pathway could predict poor clinical outcome in skull base chordomas, suggesting activation of this pathway is involved in chordoma pathogenesis.

Keywords: Chordoma, skull base, bone morphogenesis protein 4 (BMP4), SMAD, prognosis

Introduction

Skull base chordomas are rare, slow-growing and locally aggressive tumors, and radical surgical removal is the current mainstay of treatment. Unfortunately, these tumors are easy to recur after resection and are resistant to conventional radiotherapy and chemotherapy [1]. To date, the pathogenesis of chordoma remains poorly understood. Since chordomas are hypothesized to originate from remnants of the notochord, proteins involved in the control of notochord formation and embryological development have gained increasing interest [2]. One such finding was the identification of Brachyury, a mesodermal transcription factor, as a master regulator of chordoma [3].

Bone morphogenesis protein 4 (BMP4), a member of the transforming growth factor-beta (TGFβ) superfamily, is well known for its crucial role in vertebrate embryonic development [4]. Homozygous mutations of the *Bmp4* gene lead to early embryonic lethality in mice [5]. Intriguingly, BMP4 induced high levels of Brachyury in the process of BMP4-initiated mesoderm induction in human embryonic stem cells [6-8]. Recently, an immunohistochemical study has confirmed that BMP4 protein was expressed at differing levels in multiple human tissues and tumors, implying its essential role beyond embryonic development [9]. Accumulated evidence indicates that dysregulation of BMP4 signaling is involved in carcinogenesis, and functional studies demonstrate that BMP4 is able to induce cancer cell migration, invasion, and epithelial-mesenchymal transition (EMT) [10]. BMP4 exerts its effects mainly via the SMAD-dependent signaling pathway. BMP4 binds to type II and type I serine/threonine kinase recep-

tors resulting in phosphorylation of R-SMAD (SMAD1/5/8); the phosphorylated R-SMAD (phospho-SMAD1/5/8) interacts with the Co-SMAD (SMAD4) and then translocates to the nucleus to regulate target gene expression. In addition, BMP4 also functions through SMAD-independent pathways, including p38 and ERK/MAPK pathways [11].

To the best of our knowledge, BMP4 signaling in chordoma has not previously been described. In this study, we first evaluated the expression of BMP4, phospho-SMAD5 and SMAD4, which are major components of the canonical BMP4 signaling pathway, in 40 skull base chordomas by immunohistochemistry. The aim of this study was to define the clinical significance of the BMP4/SMAD signaling pathway in chordoma.

Materials and methods

Patients and tissue specimens

Between January 2007 and May 2014, 40 consecutive patients with histologically confirmed "conventional" chordoma of skull base origin in the Department of Otolaryngology, Head and Neck Surgery and Skull Base Surgery Center of Xuanwu Hospital, Capital Medical University (Beijing, China) were enrolled in this study. All of the patients underwent one or more surgeries with curative intent via endoscopic endonasal and/or transoral approaches. Clinical information was obtained from medical records and our follow-up database. Formalin-fixed paraffin-embedded tissue specimens were collected from the Department of Pathology. In each case, only the initial surgical specimen corresponding to the most representative hematoxylin and eosin (H&E)-stained slide was selected for subsequent immunohistochemical analysis. Written informed consent was obtained from all patients and the study was approved by the research ethics committee of the hospital.

Immunohistochemistry

Briefly, 4 μm sections were deparaffinized and hydrated following standard procedures. Antigen retrieval was performed in Tris-EDTA buffer (pH 9.0) in a pressure cooker. The slides were subjected to 0.3% H_2O_2 in methanol in a bath for 15 minutes to inhibit endogenous peroxidase activity, rinsed with phosphate-buffered saline (PBS), and blocked with a ready-to-use goat serum for 30 min at room temperature. The slides were incubated with the primary antibodies at 4°C overnight in a humidified chamber. Antibodies used included a rabbit anti-BMP4 monoclonal antibody (clone EPR-6211, 1:200 dilution; Abcam, Cambridge, UK), a rabbit anti-phospho-SMAD5 monoclonal antibody (clone MMC-1-104-3, 1:200 dilution; Abcam) and a mouse anti-SMAD4 monoclonal antibody (clone B-8, 1:200 dilution; Santa Cruz Biotechnology, CA, USA). Of note, the anti-phospho-SMAD5 antibody reacts with the Serine 463 and Serine 465 phosphorylated SMAD5 of human origin and it may also cross-react with SMAD1 phosphorylated at the same site. The immunocomplexes were developed using the biotin-free anti-mouse/rabbit horseradish-peroxidase-conjugated polymer (Elivision™ super IHC Kit, Maxim Biotech Inc, Fuzhou, China) following the manufacturer's instructions, and visualized using diaminobenzidine solution (DAB Kit, Maxim Biotech Inc). Finally, the slides were counterstained with hematoxylin, dehydrated, cleared and mounted. In each experiment, colon and breast carcinoma tissues with known positivity for the antibodies served as positive controls. For negative controls, the primary antibodies were replaced with PBS.

Evaluation of immunohistochemistry

For each section, at least five fields at 200 × magnification selected from representative areas were analyzed by light microscopy. Immunostaining was evaluated according to the staining intensities (nil, weak, moderate and strong) and the percentage of immunoreactive tumor cells. The following criteria were used: cases with diffuse (≥ 75% of tumor cells) moderate/strong cytoplasm staining were considered positive for BMP4 expression, and cases with moderate/strong nuclear staining in more than 5% of tumor cells were defined as positive expressions of phospho-SMAD5 and SMAD4. All samples were evaluated independently by two pathologists who were blinded to the clinical data, and consensus was reached by simultaneous review when there was a discrepancy. For statistical analysis, cases with concurrent positive expression of BMP4, phospho-SMAD5 and SMAD4 were classified as having "high" expression of the BMP4/SMAD signaling pathway (suggesting activation of the pathway); all other cases were defined as having "low" expression.

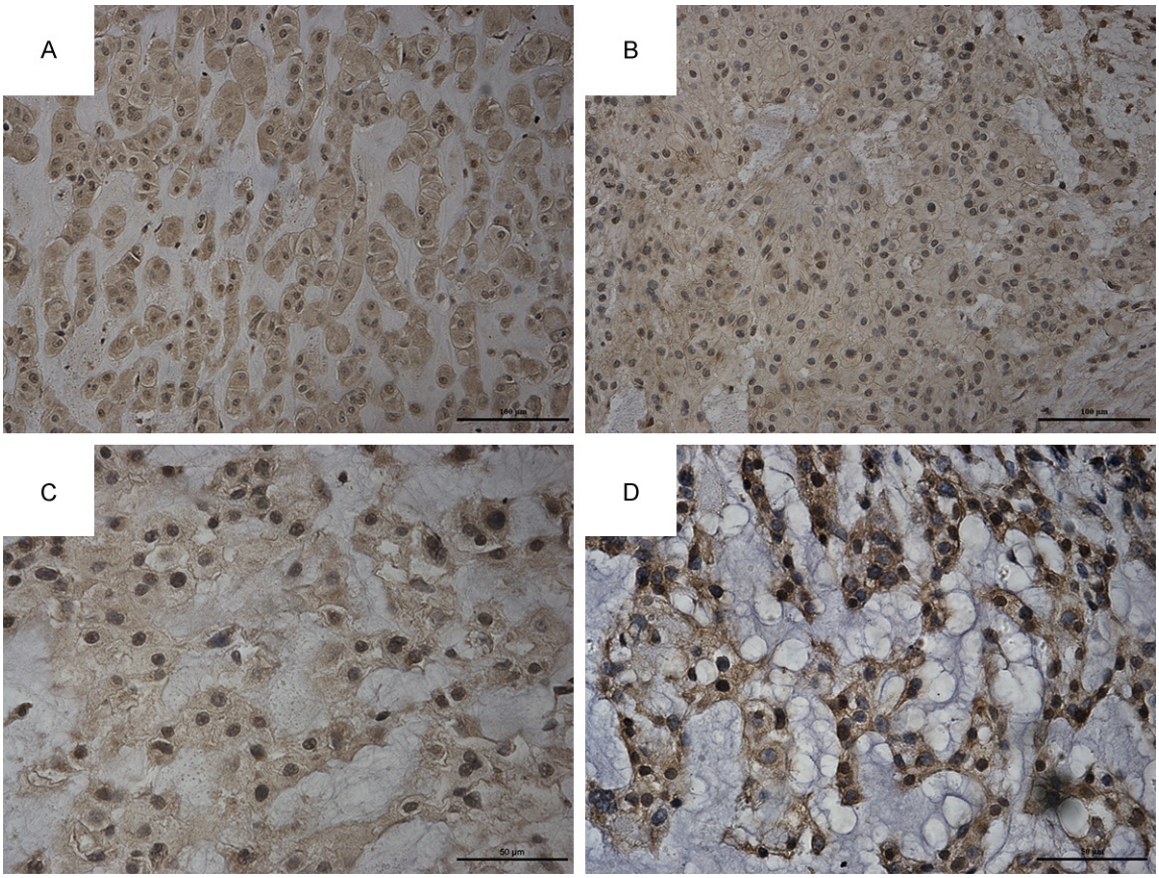

Figure 1. Representative images of BMP4 (A and B), phospho-SMAD5 (C) and SMAD4 (D) staining on chordoma tissue sections. Original magnifications 200 × (A and B), 400 × (C and D).

Statistical analyses

All statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). The Pearson's chi-square test and Fisher's exact test were applied to analyze comparisons of groups. The overall survival (OS) was defined as the period of time between the date of initial surgery and the date of death. OS was estimated using the Kaplan-Meier method, and a Log-rank test was used to evaluate the differences between survival curves. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Expression of the BMP4/SMAD signaling pathway

To evaluate whether the canonical BMP4 signaling pathway is activated in skull base chordoma, we investigated the ligand (BMP4), the phosphorylated R-SMAD (phospho-SMAD5) and the Co-SMAD (SMAD4) of the signaling axis by immunohistochemistry. In this study, immunostaining revealed that BMP4, phospho-SMAD5 and SMAD4 had positive expression in 70% (28/40), 52.5% (21/40) and 90% (36/40) chordoma specimens, respectively. As expected, BMP4 exhibited a diffuse cytoplasmic staining pattern in the chordoma. However, in contrast to a previous study, which reported predominant cytoplasmic staining in several types of tumors [9], in chordoma we also usually observed nuclear staining (**Figure 1A** and **1B**). Phospho-SMAD5 was detected both in the cytoplasm and nucleus of tumor cells with moderate immunoreactivity (**Figure 1C**). Interestingly, SMAD4 was also observed in the cytoplasm and nucleus but mainly the nucleus with a strong staining intensity (**Figure 1D**).

Correlation with clinicopathological features

To analyze the association between expression of the BMP4/SMAD signaling pathway and clinicopathological parameters, cases were dichot-

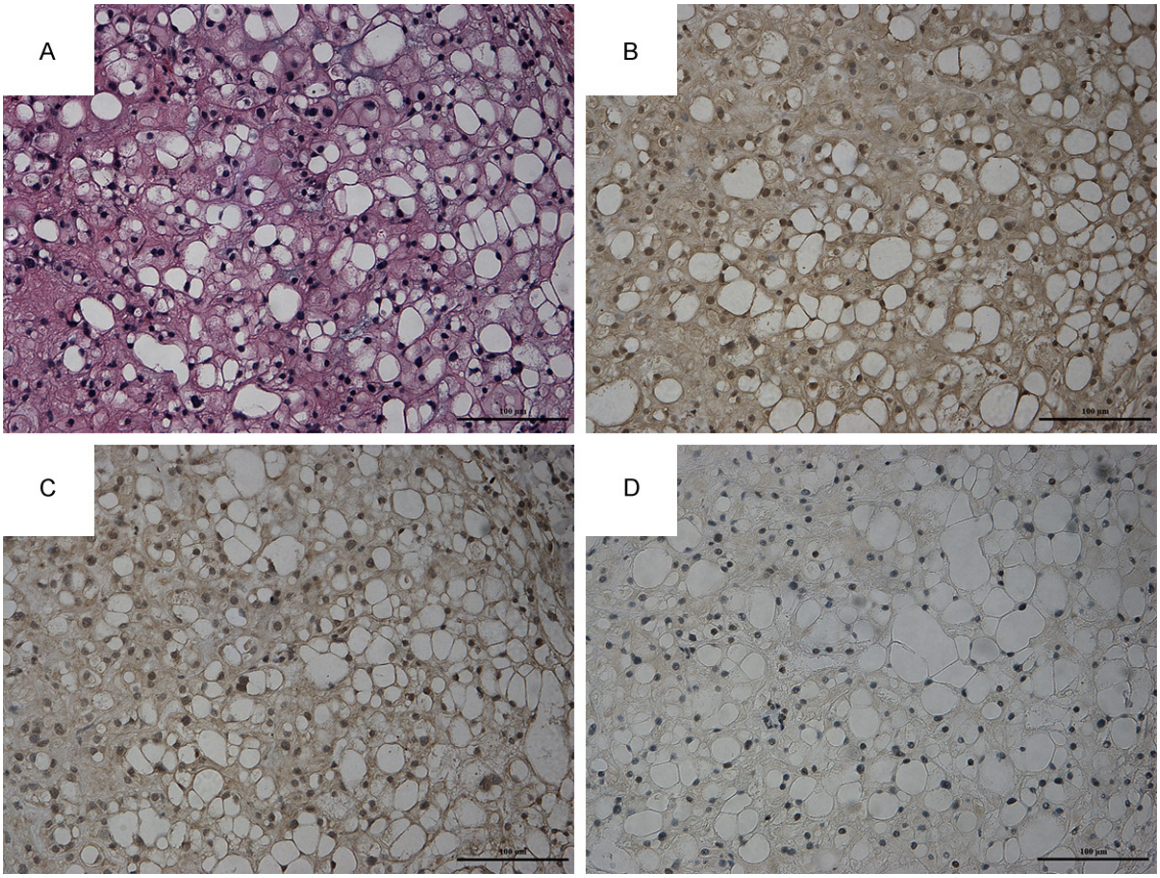

Figure 2. Representative case with "high" expression of BMP4/SMAD signaling pathway. H&E staining showing typical morphology of a "conventional" chordoma (A). This case shows concurrent immunoreactivity to anti-BMP4 (B), anti-phospho-SMAD5 (C) and anti-SMAD4 (D) antibodies. Original magnifications 200 × (A-D).

omized into "high" or "low" expression groups. "High" expression of the BMP4/SMAD signaling pathway, which reflected the activation of the pathway, was defined as co-positive expression of the 3 proteins we examined (**Figure 2**). Eighteen (45%) patients fell within the high expression group. Statistical analysis revealed that high expression of the pathway correlated significantly with dural invasion ($P = 0.024$). We also found that expression of the pathway was significantly higher in larger tumors (≥ 4 cm) than smaller ones ($P = 0.010$) (**Table 1**).

Survival analysis

To estimate the prognostic impact of expression of the BMP4/SMAD signaling pathway for skull base chordomas, we analyzed the duration of OS of the patients. Follow-up data were obtained for all of the patients enrolled in the study. In this series, there were 18 males and 22 females, and the median age was 39.5 years (range 13-69 years). The patients' survival time ranged from 5 to 204 months, and 10 patients (25%) died before the last follow-up. In patients with low expression of the pathway, only 3/22 died, whereas 7/18 patients died within the high expression group. The Kaplan-Meier survival curve showed that the 5-year OS rates were 71.4% and 90.2% for high and low expression groups, respectively. Log-rank test revealed a significant difference between the groups ($P = 0.010$; **Figure 3**), indicating that overexpression of the BMP4/SMAD signaling pathway predicted a worse prognosis.

Discussion

Skull base chordomas are low-grade malignancies but carry a poor prognosis. At present, maximal tumor resection following by adjuvant radiotherapy is the recommended treatment modality. However, total resection is not always

Table 1. Correlation between expression of BMP4/SMAD signaling pathway and clinicopathological parameters in skull base chordomas

Parameters	N	Expression of BMP4/SMAD signaling pathway		
		High (n)	Low (n)	*P*
Gender				
Male	18	8	10	1.000
Female	22	10	12	
Age (y)				
< 50	27	13	14	0.737
≥ 50	13	5	8	
Primary/Recurrent				
Primary	24	12	12	0.526
Recurrent	16	6	10	
Preoperative RT				
Yes	10	2	8	0.082
No	30	16	14	
Tumor size				
< 4 cm	21	5	16	0.010*
≥ 4 cm	19	13	6	
Dural invasion				
Yes	18	12	6	0.024*
No	22	6	16	

RT, Radiotherapy; *Statistically significant.

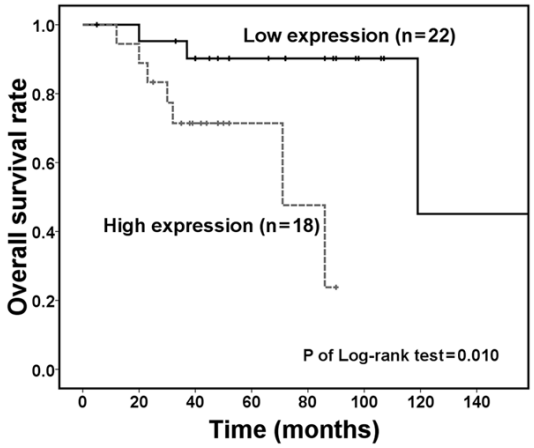

Figure 3. Kaplan-Meier curve for overall survival according to expression of BMP4/SMAD signaling pathway in skull base chordomas. Patients with a higher expression of the signaling pathway were found to have a significantly poorer prognosis than those with lower expression (Log-rank test, $P = 0.010$).

feasible because of the complexity of the local anatomy and proximity to critical neurovascular structures [1]. To date, the natural history of chordoma is still controversial, and the current knowledge of its molecular pathogenesis is limited. Therefore, identification of new biomarkers for chordoma will not only play an important role in the prediction of prognosis but also pave the way for the development of molecular targeted therapies. With this purpose, we examined the expression of BMP4, phospho-SMAD5 and SMAD4, and our findings suggest that overexpression of the BMP4/SMAD signaling pathway is associated with poor survival in skull base chordoma.

BMP4 signaling is essential in both embryogenesis and tissue homeostasis. Accumulating data indicate that dysregulation of BMP4 signaling is involved in carcinogenesis in multiple malignancies [10]. Intriguingly, BMP4 action is highly context-dependent and may play a dualistic role in cancers. In the majority of tumors, BMP4 treatment or overexpression results in reduced cell growth both *in vitro* and *in vivo* [10]. On the other hand, BMP4 promotes tumor cell migration and invasion [12, 13] and induces EMT [14, 15], which are known mechanisms contributing to tumor progression and metastasis. Many tumors show increased expression of BMP4, particularly those arising from squamous cell epithelia [9]. However, there are discrepancies on the prognostic value of BMP4 expression. BMP4 overexpression has been reported to be correlated with shorter survival time in hepatocellular carcinoma [16] and in squamous cell carcinoma of the head and neck (SCCHN) [14], while independently predicted better survival in advanced serous ovarian cancer [17]. BMP4 mainly signals via its SMAD pathway, but may crosstalk with other signaling pathways such as MAPKs, JNK and p38. Nuclear staining of phosphorylated isoforms of R-SMAD using phospho-specific antibodies has been used as an indicator of active SMAD signaling in tumor tissues [18]. To date, very few studies have examined the expression level of phospho-SMAD1/5/8, which are downstream signaling mediators of the canonical BMP4 pathway. In a previous study, nuclear phospho-SMAD1/5/8 was detected at significantly higher levels in high-grade compared with low-grade chondrosarcoma, suggesting that enhanced activation of this signaling pathway may contribute to the progression of this disease [19].

Herein, we focused our investigations on the major components of the canonical BMP4 sig-

naling axis in skull base chordoma. The expression levels of BMP4, phospho-SMAD5 and SMAD4 were assessed for deeper understanding of the BMP4/SMAD signaling pathway in chordoma. Similar to a previous study [9], our immunohistochemical results confirm BMP4 protein was mainly located in the cytoplasm of tumor cells with a diffuse staining pattern. However, the nuclear immunostaining of BMP4 we observed was in contrast to this earlier report. This might be explained by differences between the two studies in the antibody used and/or the tumor type examined. The phospho-SMAD5 protein was detected in both cytoplasm and nucleus with variable intensity, which mirrors its variable activity level in chordomas. SMAD4 has been shown to function as a tumor suppressor, and loss of SMAD4 immunostaining is commonly observed in various malignant tumors [20]. However, loss of SMAD4 expression appears to be a rare event in chordoma. As shown here, expression of SMAD4 was positive among most chordoma tissues analyzed. Taken together, our data imply that autocrine or paracrine SMAD-mediated BMP4 signaling is active in a subgroup of skull base chordomas.

In the present study, we discuss for the first time the clinical significance of the BMP4/SMAD signaling pathway in chordoma. High expression of the pathway was arbitrarily defined as concurrent positive expression of the aforementioned markers in the signaling cascade. Of interest, we here showed that patients with overexpression of the BMP4/SMAD signaling pathway had a higher rate of dura invasion. Considering that BMP4-induced EMT via the SMAD pathway could promote invasiveness in human SCCHN cell lines [14], the same mechanism might be responsible for the aggressive biology of chordomas. Chordoma displays both epithelial and mesenchymal characteristics. Loss of the expression of the epithelial marker E-cadherin and gain of expression of the mesenchymal marker N-cadherin, a phenotypic switch which typically occurs during tumor EMT, was reported to be indicative of more aggressive behavior in chordoma [21]. We suggest that BMP4/SMAD signaling might drive EMT thus enhancing the invasive ability of skull base chordoma. Higher expression of the pathway was also observed in patients with larger tumors, indicating that the activation of the pathway might be involved at the late tumor stage. Furthermore, we showed that overexpression of the pathway correlated significantly with reduced patient OS time. Recent findings have shown that Brachyury is a master regulator of oncogenesis in chordoma [3], and the expression of Brachyury was negatively correlated with patient prognosis [22]. However, what controls the expression of Brachyury still need to be elucidated. As BMP4 signaling is known to act upstream of Brachyury in development [6-8], we hypothesize that the BMP4/SMAD signaling pathway is involved in the upregulation of Brachyury expression, resulting in a worse clinical outcome. Based on these findings, we suggest that activation of the BMP4/SMAD signaling pathway may play a crucial role in chordoma invasion and progression.

Chordomas are generally resistant to cytotoxic chemotherapy. At present, no drugs are approved for the treatment of advanced chordoma [1]. As many drugs have been developed targeting TGF beta signaling [23], the BMP4/SMAD signaling pathway might provide potential targets for molecular targeted therapy in chordomas. Nevertheless, this is a single-institution study with a relatively small sample size. Further investigations in multiple centers with larger patient cohorts are needed, and a comprehensive understanding of the biological function of the signaling pathway in chordoma cell lines is warranted.

In conclusion, we show for the first time that overexpression of the BMP4/SMAD signaling pathway is predictive of poor clinical outcome in skull base chordomas. We suggest that activation of the signaling pathway is involved in chordoma pathogenesis. Our findings shed light on novel therapeutic targets for chordoma treatment, although the precise mechanisms remain to be elucidated in further studies.

Acknowledgements

The authors thank Dr. Yeqing Cui (Department of Surgical Laboratory, Xuanwu Hospital, Capital Medical University) for her valuable technical assistance during the immunohistochemical analysis. This study was funded by Key Discipline Construction Project of the Finance Bureau of Beijing (3500-114235), Specialized Project on Scientific Research within the Healthcare Circle by National Health and Family

Planning Commission of China (201202005) and National Science Foundation of China (81170894).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qiuhang Zhang, Department of Otolaryngology, Head and Neck Surgery, Skull Base Surgery Center, Xuanwu Hospital, Capital Medical University, 45, Changchunjie Street, Xicheng District, Beijing 100053, P. R. China. Tel: +86 13701267977; Fax: +86 1083198836; E-mail: zhangqiuhang330@163.com

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