Prognostic significance of immunohistochemical expression of VEGFR2 and iNOS in spinal chordoma

Reza Akhavan-Sigari · Michael Robert Gaab · Veit Rohde · Mehdi Abili · Helmut Ostertag

Received: 31 December 2013 / Revised: 10 June 2014 / Accepted: 10 June 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract

Purpose To clarify whether vascular endothelial growth factor receptor 2 (VEGFR2) and inducible nitric oxide synthase (iNOS) are involved in the angiogenesis and recurrence of spinal chordoma tissues and influence the overall survival.

Methods All patients affected by a spinal chordoma surgically treated between 1986 and 2007 were reviewed. We examined the expression of VEGFR2 and iNOS with immunohistochemistry using a tissue microarray containing 120 chordoma samples. Local recurrence and overall survival (OS) were analyzed.

Results A series of 40 chordoma patients who underwent surgery for a total of 120 lesions (including 80 recurrent lesions) were identified (sacrum 77.5 %, lumbar spine 17.5 %, cervical/thoracic spine 5 %). Surgical margins were wide in 30 (75 %), marginal in 8 (20 %) and intralesional in 2 (5 %) patients. Median follow-up was 120 months. The 5- and 10-year OS of the entire series of patients was 78.6 and 30 %, respectively. There were five primary chordomas (12.5 %) with moderate and 35 (87.5 %) with strong expression of VEGFR-2. All recurrent spinal chordomas displayed strong expression of VEGFR-2. The expression of iNOS was predominately moderate to high in primary chordomas: There were 15 tumors (37.5 %) with moderate and 25 tumors (62.5 %) with strong expression. All recurrent chordomas displayed strong expression of iNOS.

Conclusion The high expression of VEGFR-2 and iNOS affected the OS. The OS at 10 years was only 30 %.

Keywords Spinal chordoma · VEGFR-2 · iNOS · Angiogenesis

Introduction

Chordomas are rare tumors that occur along the spine and comprise 1–4 % of all bone tumors [1]. They are thought to originate from notochordal remnants.

Chordomas can occur at any age, but especially between the ages of 40 and 60 years. They arise from the sacrococcygeal region in approximately 50 % of cases, from the sphenoeoccipital area in 35 % and from the spine in 15 % of cases [2]. Locally, these tumors are highly aggressive and frequently recur locally even after wide resection [3]. There are no effective chemotherapeutic regimens
available for the treatment of chordomas [4] and surgery remains the mainstay of chordoma management. Targeted molecular therapy, such as imatinib and EGFR inhibitors, has shown promising results in the treatment of malignancies with historically poor responses to chemotherapy [5–7]. However, the expression of molecular markers in chordomas is not well understood. Thus, a better understanding of the molecular pathogenesis of this disease is required.

Vascular endothelial growth factor receptor 2 (VEGFR2) is a marker for malignant vascular tumors, biphasic synovial sarcoma epithelium and chordoma [8]. Cytotoxic chemotherapy in the treatment of chordoma is known to be inactive. Furthermore, information on VEGFR2 expression and inducible nitric oxide synthase (iNOS) expression, as a potential biological relevance in different malignant human tumors, may be useful in developing targeted oncologic therapy via VEGFR2-specific tyrosine kinase inhibitors.

In this study, we use immunohistochemistry to examine the expression patterns of VEGFR2 and iNOS in spinal chordoma, to discover potential targets for VEGFR2 and iNOS inhibitor-based targeted therapy.

Methods

Chordomas

A series of 40 chordoma patients who underwent surgery for a total of 120 lesions (including 80 recurrent lesions) at the neurosurgical departments of the Nordstadt Hospital and of the Hannover Medical School, Germany, between 1986 and 2007 were considered for the present analysis. Tumor recurrence was defined as a return of symptoms and signs with radiological verification of tumor regrowth (Table 1). All patients were included; we had no exclusion criteria other than lack of available material for immunohistochemical analysis. Clinical data were extracted from the two institutional surgical databases. The data retrieved at presentation included: sex, age at diagnosis, tumor site, tumor size, primary versus recurrent chordoma, chordoma subtype, VEGFR-2 and iNOS expression, margin status, adjuvant radiotherapy (yes/no, dose in Gy). Before surgery, all patients underwent staging with computed tomography (CT) scan (Table 2).

Cohort demographics

The patients (15 women and 25 men) ranged in age from 16 to 88 years (median age 58 years). All patients experienced recurrence and subsequent resection. Locations of the chordomas were sacral 31, lumbar spine 7 and cervical/thoracic spine 2.

Multi-tumor tissue microarray construction

A multi-tumor tissue microarray was assembled and used for comparing molecular marker expression of chordoma. Following institutional review board approval, we constructed the tissue microarray as previously described [9]. The tissue microarrays (TMA) were constructed using a tissue-arraying instrument (Beecher Instruments, Hackensack, NJ, USA). Three tissue core cylinders with a diameter of 0.6 mm were punched from each donor paraffin block in targeted areas corresponding to previously demarcated neoplastic areas on the parallel slide. These tissue cores

<table>
<thead>
<tr>
<th>Table 1</th>
<th>There were 62 lesions as first, 12 as second, and 6 as third recurrences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary spinal chordoma</td>
<td>N = 40</td>
</tr>
<tr>
<td>Recurrences</td>
<td>1st</td>
</tr>
<tr>
<td>N = 62</td>
<td>N = 12</td>
</tr>
<tr>
<td>Total</td>
<td>N = 120</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Patient characteristics at first observation with clinical and histopathological features (N = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient characteristics</td>
<td>N (%)</td>
</tr>
<tr>
<td>Total number</td>
<td>40</td>
</tr>
<tr>
<td>Parameters</td>
<td>VEGFR-2</td>
</tr>
<tr>
<td>k</td>
<td>0.3300</td>
</tr>
<tr>
<td>p</td>
<td>0.0023</td>
</tr>
<tr>
<td>iNOS</td>
<td>k</td>
</tr>
<tr>
<td>p</td>
<td>0.0887</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
</tr>
<tr>
<td>Male</td>
<td>25 (62.5)</td>
</tr>
<tr>
<td>Age (year)</td>
<td>Median 58</td>
</tr>
<tr>
<td>Range (16–88)</td>
<td>Site of primary tumor</td>
</tr>
<tr>
<td>Lumbar spine 7 (17.5)</td>
<td></td>
</tr>
<tr>
<td>Cervical/thoracic spine 2 (5)</td>
<td></td>
</tr>
<tr>
<td>Size (cm), median (IQ range) 10 (8–14)</td>
<td></td>
</tr>
<tr>
<td>Surgical margins</td>
<td>Wide 30 (75 %)</td>
</tr>
<tr>
<td>Marginal 8 (20 %)</td>
<td></td>
</tr>
<tr>
<td>Intralesional 2 (5 %)</td>
<td></td>
</tr>
</tbody>
</table>
were then deposited into a recipient “master” paraffin block. The punches were placed 1 mm apart on the x-axis and 1.5 mm apart on the y-axis.

Two microarray blocks, respectively, contained 75 and 45 punches. Sections 5-μm thick were cut from the master block, stained with H&E, and reviewed to ensure the presence of morphologically pure cores of chordoma for each case. Morphologic features of each core were confirmed by reviewing the corresponding whole tissue sections stained with H&E. We obtained tissue cores from paraffin-embedded formalin-fixed tissue blocks from the archives at the Department of Pathology, Nordstadt Medical Center, Klinikum Hannover. A pathologist (H.O.) reviewed slides from all blocks to select representative areas of invasive tumor or normal tissue to be scored. Chordoma subtype (conventional, chordoid, dedifferentiated) was indicated.

Immunohistochemistry

All slides were processed simultaneously under identical conditions using standard methods. Immunohistochemistry was performed on the 120 lesions for the following antibodies: inducible nitric oxide synthase, iNOS (1:100, Rabbit Polyclonal Antibody, Dunn, Asbach, Germany), vascular endothelial growth factor receptor 2, Flik-1/KDR/VEGFR2 (1:100, Rabbit Polyclonal Antibody, Dunn, Asbach, Germany) and CD-34 (1:30, Mouse Monoclonal Antibody, Abcam PIC, Cambridge, UK). The sections were treated with antigen retrieval. They were then treated with a primary antibody, followed by staining with an avidin–biotin–peroxidase complex (Immunotech, Marseille, France) or an alkaline phosphatase detection kit (Vector, Burlingame, CA, USA), according to standard immunohistochemical techniques [10]. All slides were run simultaneously under identical conditions and included negative control slides. Positive and negative control sections were included for each antibody and slide pretreatment, respectively. TMA slides in which incubation with primary antibody was omitted served as the negative controls for each antigen retrieval regimen.

Immunohistochemical scoring for VEGFR2, iNOS and CD-34

Immunoreactivity was evaluated independently by two pathologists who had no prior knowledge of the clinical data or other histologic findings. Immunoreactivity was scored as described previously [11, 12].

Every tumor was given a score according to the intensity of the nuclear or cytoplasmic staining (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3) and the extent of stained cells (0 = none; 1 = <25%; 2 = 25–50%; and 3 = more than 50%). We determined the sum of these two parameters to evaluate the expression of antibodies used in this study, from 0 to 6. The cells were graded as negative when there was a complete absence of staining (score 0), weak staining (score 1), moderate staining (score 2), originating from baseline expression. The scores 3–6 were graded as strongly positive.

Structures were only counted as microvessels if they stained positively with the vascular marker and appeared vascular morphologically. Expression of CD34 was used to detect microvessels.

Light microscopic study

Nuclear pleomorphism, intratumoral necrosis, and intratumoral bleeding were evaluated as positive or negative. As previously reported, mitosis [13–18] and apoptosis [16–18] were scarce. Therefore, we also assessed them as positive or negative instead of calculating mitotic or apoptotic indices.

Statistical analysis

We evaluated the correlation of marker expression by Spearman rank correlation test. All calculations and analyses were performed with SPSS 18.0 for Windows. Significance was considered to be $p < 0.05$.

Results

Histopathology and immunohistochemistry

The histologic hallmark of chordoma is large tumor cells with abundant vacuolated cytoplasm, referred to as physaliphorous cells [19]. All the chordomas in our cohort were reviewed and classified as conventional chordomas by means of morphology and immunohistochemistry. They show prominent lobules separated by fibrous septa. The tumor cells are arranged in cords or sheets or may be floating singly in the abundant myxoid matrix often present.

Expression of VEGFR-2 and iNOS showed a cytoplasmic staining pattern with diverse intensity. Tumor cells as well as endothelial cells were stained. VEGFR2 displayed intracytoplasmic and membrane-located staining. Immunoreactivity tended to concentrate at the tumor periphery, but in several cases also appeared homogeneously over a certain tumor area, whereas CD34 immunoreactivity was confined to endothelial cells and scattered single positive cells in 40% of the tumor stroma. Over 15% of lesions had a high immunoreactivity for CD34. Spinal chordomas displayed predominately strong expression of VEGFR-2.
There were 5 tumors (12.5 %) with moderate and 35 (87.5 %) with strong expression. The recurrent spinal chordoma tumors displayed in all cases strong expression of VEGFR-2. A representative micrograph of strong VEGFR-2 expression in spinal chordoma is shown in Fig. 1. Chordomas displayed mild to strong expression of CD-34. A strong expression of CD-34 was found in 20 cases of primary chordomas (50 %), whereas the recurrent lesions displayed predominately strong CD-34 expression (Fig. 2). The expression of iNOS was predominately moderate to high in primary chordomas. There were 15 tumors (37.5 %) with moderate and 25 tumors (62.5 %) with strong expression. All recurrent chordomas displayed strong expression of iNOS. A representative micrograph of strong iNOS expression in chordoma is shown in Fig. 3.

Relationships between expression of VEGFR2, iNOS, CD-34 and clinicopathological factors

A relationship between VEGFR2 expression, iNOS and CD-34 was detected. There was a significant correlation between VEGFR2 expression and iNOS expression levels ($P = 0.0023$, Spearman $\rho = 0.3300$) as well as VEGFR2 ($P = 0.0013$, Spearman $\rho = 0.3506$) and CD-34 expression (Table 2). The expression pattern of primary and recurrent spinal chordomas is summarized in Figs. 4 and 5a–c. VEGFR-2 and iNOS expression were significantly
higher in the older than in the younger patient group. Men showed higher expression of VEGFR-2 and iNOS than women; the difference for VEGFR-2 was statistically not significant between men and women. Clear VEGFR-2 and iNOS expression differences were noted between primary and recurrent spinal chordoma. No correlations were seen between site of primary tumor, age and sex. We could not find any statistically significant correlation between type of surgery and recurrence rate. Wide surgical margins were achieved in 30 patients (75 %), marginal resections in 8 (20 %), and intrallesional resections in 2 (5 %) patients (Table 2).

**Prognosis**

Detailed follow-up data, available for 35 patients, ranged from 48 to 120 months after the initial surgery. The Kaplan–Meier survival curve was used for calculating survival rates. Patients with higher levels of VEGFR-2 expression (score 4–6) were found to have a significantly poorer overall survival at 10 years than those with lower VEGFR-2 expression (score 0–3) (Fig. 6).

The overall survival at 10 years in patients with a high positive reaction (score 4–6) for iNOS expression was also associated with significantly poorer prognosis (P = 0.034) (Fig. 7).

**Discussion**

Chordoma is a slow-growing malignant neoplasm that is believed to originate from notochordal remnants located along the craniovertebral axis [20]. Chordomas typically present in adults 40 years of age or older and males are affected more often than females. Receptor tyrosine kinases (RTKs) are one of the families of proteins most studied and targeted in cancer treatment. They are the primary means by which extracellular signals such as growth factors are able to initiate transduction cascades responsible for molecular events such as activation of growth, differentiation and proliferation [21].

In the present study, we decided to focus our investigations on the differential protein expression of iNOS and VEGFR-2 in spinal chordoma. As inhibitors of iNOS and VEGF become more widely used in the clinical setting, a better understanding of the signaling pathway in vivo, their

---

**Fig. 5** Expression pattern of first (N = 62), second (N = 12), and third (N = 6) recurrences of spinal chordomas is summarized in (a–c). There was a slight increase in expression of iNOS, VEGFR-2 and CD34 among the first to the third recurrences. **A** 1st recurrence (N = 62); **B** 2nd recurrence (N = 12); **C** 3rd recurrence (N = 6).

**Fig. 6** Kaplan–Meier curve for the overall survival of patients with expression of VEGFR-2. At the 10-year survival point, patients with a higher level of VEGFR-2 expression were found to have a significantly poorer prognosis than those with lower VEGFR-2 expression (P = 0.023). **Y1** VEGFR-2, score 0–3 (N = 26). **Y2** VEGFR-2, score 4–6 (N = 9).

**Fig. 7** Survival at 10 years according to the expression of iNOS in spinal chordomas. Patients having lesions with a higher level of iNOS expression were found to have a significantly poorer prognosis than those with lower iNOS expression (P = 0.034). **Y1** iNOS, score 0–3 (N = 22). **Y2** iNOS, score 4–6 (N = 13).
pattern of expression and activation is of critical importance because it may be helpful in predicting the target tumor population that will benefit from therapies.

Inducible nitric oxide synthase

Nitric oxide is a highly reactive free-radical compound with a short half-life known to affect many cellular processes, including vasodilation, cytotoxicity in immunological responses, and neurotransmission. Nitric oxide has been shown to contribute to tumor growth by promoting neovascularization of tumor masses. The expressions of iNOS and VEGF are closely related to tumor angiogenesis [22]. Nitric oxide produced through iNOS induction may increase the vascular permeability and accelerate the nutrient supply of tumor tissue and finally promote the tumor growth [23]. In the literature, there are no comparative studies noted for chordomas. However, it was documented that a strong correlation exists between the activity of the NOS pathway, angiogenesis and metastatic behavior in head and neck cancer [24]. In this study, we found that the rate of expression of iNOS in primary spinal chordoma was variable from no expression to strong expression. In the recurrent spinal chordoma, the iNOS expression was high, which can be ascribed to the high infiltration of chordoma with monocyte/macrophages. We assume that the high infiltration of chordoma with monocyte/macrophages enhances the tumor growth through the release of iNOS. This has been shown in our previous study with skull base chordoma [25].

Vascular endothelial growth factor receptor-2

Vascular endothelial growth factor receptor-2 is exclusively expressed in endothelial cells and appears to play a pivotal role in endothelial cell differentiation and vasculogenesis [26]. Many studies using molecular techniques have provided evidence for the role of VEGFR-2 in tumor vascularization, growth, and metastasis [27]. VEGFR-2 is considered to be the main mitogenic-signaling receptor for VEGF [28]. In the present study, we found that the rate of expression of VEGFR-2 was related to the iNOS expression ($P = 0.0023$, Spearman $\rho = 0.3300$). VEGF produced by the tumor cell can be bound with the surface acceptor of vascular endothelial cell, and promote the production of nitric oxide that can transmit messages between the cells and induce tumor angiogenesis [29]. A study by Song et al. [22] showed that the expressions of iNOS and VEGF were closely related to tumor angiogenesis and might be important factors involved in gastric carcinoma angiogenesis. In the present study, we have shown that the majority of the spinal chordomas have high expression of VEGFR-2, while this was low in skull base chordoma as shown in our previous study [25]. Miettinen et al. [8] have observed that chordoma also seems to commonly contain VEGFR2-positive tumor cells, perhaps as a reflection of its distant relationship with cartilage. Zhang et al. [30] noted that regulating VEGFR-2 expression seems to have a profound effect on angiogenesis. Although numerous authors report surgical resection margins, the most important predictor of survival and local recurrence, a cure is rare and recurrence is not uncommon [31–33]. Regardless of the recurrence frequency (1st–3rd), the recurrent spinal chordoma tumors displayed in all cases a strong expression of VEGFR-2 and iNOS. An en bloc resection aiming at achieving a tumor-free resection margin must be considered the treatment of choice.

In the present study, our analysis did not control for all confounding variables via a multivariate regression model due to the relatively small number of patients. Some biases may be inherent in this type of analysis, and we acknowledge this is a limitation. The number of cases presented here does not allow for any definitive conclusions. But because chordomas are rare, and because they present a very difficult surgical challenge and are often incurable, our work strives to increase the understanding of the molecular mechanism of chordomas. Future studies should incorporate a larger number of patients.

Conclusion

It is widely believed that angiogenesis plays a key role in malignant tumor development, growth and invasion. VEGFR-2 and iNOS might act with a synergistic effect and can positively regulate the angiogenesis in spinal chordoma. Positive expression of VEGFR-2 may indicate the local recurrence of spinal chordoma and influence the overall survival. The result suggests that some specific drugs, which inhibit VEGF or their receptor (VEGFR-2) may have a good therapeutic effect for spinal chordoma. However, angiogenesis involves a variety of molecules other than VEGF and iNOS; further studies are definitely required to determine the appropriate target molecules of this therapeutic strategy. Our results nonetheless indicate that spinal chordomas may respond to RTK inhibitors such as VEGFR-2 or modulators of other downstream-signaling molecules.

Conflict of interest None.

References

学霸图书馆

www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：

图书馆首页 文献云下载 图书馆入口 外文数据库大全 疑难文献辅助工具