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Close correlation between CXCR4 and VEGF expression and frequent CXCR7 expression in rhabdomyosarcoma

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Abstract

CXCR4 expression is reportedly correlated with both VEGF expression and poor prognosis in a variety of cancers. Its relation to CXCR7 is also noted in several malignancies, including rhabdomyosarcoma (RMS) cell lines. However, the correlations between these chemokine receptors and angiogenic factors have not yet been adequately investigated in RMS clinical specimens. By immunohistochemistry, we assessed CXCR4, CXCR7, CCR6, CCR7, and VEGF expression, microvessel density and MIB-1 Labeling index (MIB-1-LI) in 82 formalin-fixed RMS specimens, including 34 primary alveolar RMS (ARMS) and 44 primary embryonal RMS (ERMS). Twenty-six frozen samples were available for investigation by quantitative reverse transcription polymerase chain reaction to detect the mRNA expression levels of these molecules. We also evaluated their significance with respect to clinicopathological factors and patient survival rates. Primary RMS showed high expression of CXCR7 (83.1%) regardless of the histological subtype. High cytoplasmic CXCR4 and high VEGF expression revealed significant correlations in both ERMS and ARMS ($p=0.0051$, 0.0003 , respectively). By univariate analysis of ERMS cases, the tumors with high VEGF expression showed significantly poor prognoses ($p=0.0017$). High VEGF expression also was the independent adverse prognostic factor for ERMS. With the fact that CXCR4, CXCR7 and VEGF are widely expressed in RMS, the combination of these antagonists may provide a potential target for molecular therapy.

Introduction

Rhabdomyosarcoma (RMS) is the most common malignant soft tissue sarcoma in childhood and adolescence. There are two major histological subtypes in RMS: alveolar rhabdomyosarcoma (ARMS) and embryonal rhabdomyosarcoma (ERMS). The former is well known to be associated with *PAX3-FKHR* or *PAX7-FKHR* gene fusions and with significantly poor prognosis. Meanwhile, no specific fusion gene has been identified in ERMS [1].

Many kinds of chemokines display various roles in immunity, regulating angiogenesis, promoting the proliferation of tumor cells, and mediating organ-specific metastases [2]. Chemokine receptors, especially CXC chemokine receptor-4 (CXCR4) [3-7] and CXC chemokine receptor-7 (CXCR7) [8,9] have been suggested to play an important role in metastatic behavior to specific target-organs [10]. CC chemokine receptor 7 (CCR7) is primarily responsible for lymph node metastases [11,12], while CC chemokine receptor 6 (CCR6) is suggested to have relation to liver metastases [13] and tumor progression [14].

Vascular endothelial growth factor (VEGF), known as a critical mediator of angiogenesis and tumor proliferation, has revealed its overexpressed status in a variety of cancers [15]. Thus the therapeutic approaches targeting VEGF have been explored in several cancers [16-18].

CXCR4 has been believed to be the only receptor that binds stromal cell-derived factor (SDF-1). Recently, a new SDF-1 binding receptor, CXCR7, was identified through an experiment that revealed discrepancies between CXCR4 expression and SDF-1 binding on different cell lines, using cells from *CXCR4*-deficient mice [19].

In rhabdomyosarcoma cell lines, it has been reported that CXCR4 was expressed at much higher levels by highly metastatic ARMS lines, while CXCR7 was expressed in both ARMS and

ERMS, with higher expression in ERMS cell lines [20].

A significant correlation between the mRNA levels of VEGF and CXCR4 in breast cancer tumor tissue was reported in a preliminary investigation [21]. In addition, the autocrine manner of VEGF's involvement in the CXCR4/SDF-1 pathway in the invasion of breast carcinoma cells has been demonstrated [8]. Furthermore, the CXCR4/SDF-1 system was found to be involved with the PI3K/Akt pathway in a breast cancer cell line [21] and with the ERK pathway in a pancreatic cancer cell line [22].

In the present study, we immunohistochemically evaluated CXCR4, CXCR7, CCR6, CCR7, and VEGF protein expression to investigate these protein expression in a large series of rhabdomyosarcoma clinical cases, and examined the mRNA expression levels of *CXCR4*, *CXCR7*, *CCR6*, *CCR7*, and *VEGF* in frozen samples using quantitative reverse transcription polymerase chain reaction (RT-PCR). Moreover, we compared these results with clinicopathological parameters, angiogenesis factors, and prognosis in rhabdomyosarcoma.

There have been no reports on the relationship of metastasis-related chemokine receptors and angiogenesis factors in the aspects of tissue expression in large series of clinical rhabdomyosarcoma cases. The investigation for these protein expression in the clinical tumor tissue could aid in the search for new potential therapeutic targets.

Materials and Methods

Patients and Tissue Specimens

Eighty-two paraffin-embedded rhabdomyosarcoma specimens obtained from 78 patients were collected from the soft-tissue tumor file registered between 1976 and 2007 at the Department of

Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan. The 82 specimens from these 78 patients included 78 primary tumors, 3 metastatic tumors (2 in lung and 1 in thigh), and 1 local recurrent tumor. The histological diagnosis of rhabdomyosarcoma and its subtype was confirmed according to the latest World Health Organization (WHO) classification [1]. The anatomic locations of the primary tumor sites were categorized as favorable or unfavorable tumor sites according to IRS-V [23]. The patients were classified according to the pretreatment staging system by Intergroup Rhabdomyosarcoma Study Group (IRSG) [23]. Furthermore, 26 of the specimens were also snap-frozen in liquid nitrogen at the time of the surgical procedure and stored at -80°C until use. The institutional review board at Kyushu University approved this study (permission code: 25-143).

Immunohistochemistry

Immunohistochemistry was performed in 82 tumors, using formalin-fixed tissue sections in concordance with frozen material. Sections were cut at widths of 4 µm from paraffin-embedded material, dewaxed with xylene, and rehydrated through a graded series of ethanol. After the inhibition of endogenous peroxidase, sections were exposed to the primary antibodies at 4°C overnight, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). The sections were then finally reacted in 3,3'-diaminobenzidine, counterstained with hematoxylin, and mounted.

The following antibodies were used as primary antibody: anti-CXCR4 [12G5, monoclonal, 1:100 (BD PharMingen, San Diego, CA)], anti-CXCR7 [polyclonal 1:200 (GeneTex, Irvine, CA)], anti-CCR6 [11A9, monoclonal, 1:200 (BD Pharmingen)], anti-CCR7 [polyclonal, 1:250 (Capralogics, Hardwick, MA)], anti-VEGF [A-20, polyclonal 1:100 (Santa Cruz Biotechnology,

Santa Cruz, CA)], anti-CD31 [JC70A, monoclonal 1:20 (DAKO, Glostrup, Denmark)], and anti-Ki-67 [MIB-1, monoclonal 1:100 (DAKO)]. For staining with CXCR4, CXCR7, CCR6, CCR7, VEGF, and Ki-67, sections were pretreated with microwave irradiation in citrate buffer or EDTA buffer for antigen retrieval. As for CD31, sections were pretreated with trypsin for 30 minutes.

Sections from human tonsils for CXCR4, CCR6 and CCR7 and sections from human renal cell carcinoma for CXCR7 were used as positive controls [24,25].

Immunohistochemical evaluation

A consensus judgment was adopted to establish the proper immunohistochemical scores for tumors, using a scoring system in a previous report [9] that was based on the strength of cytoplasmic expression of CXCR4, CXCR7, CCR6, CCR7, and VEGF: 0 = negative; 1+ = weak staining; 2+ = moderate staining; 3+ = strong staining. The distribution of positive cells was also recorded to impart the proportion of positive cells: sporadic ($1\% \leq \text{positive cells} < 10\%$); focal ($10\% \leq \text{positive cells} < 50\%$); diffuse ($\text{positive cells} \geq 50\%$). The immunohistochemical scores were defined as follows: Score 0: no immunoreactivity; Score 1: 1+ with sporadic or focal distribution, Score 2: 1+ with diffuse distribution, or 2+ or 3+ with sporadic distribution, Score 3: 2+ with focal or diffuse distribution, Score 4: 3+ with focal or diffuse distribution. Then, we considered immunohistochemical scores 0-2 as low protein expression and scores 3 and 4 as high protein expression according to our previous study [9].

The degree of angiogenesis was determined by the number of microvessels in defined areas as previously described [9]. The CD31-positive vessels were counted in four selected hot spots in a \times 400 field (0.26-mm^2 field area). The mean of the two independent readings of each specimen was calculated, and MVD was defined as the mean number of microvessels per 0.26-mm^2 field area.

The MIB-1-labeling index (LI) was estimated by counting the number of positive cells per 1,000 tumor cells. Three independent pathologists (YO, HY, and KK), who were not aware of the clinical characteristics of the patients, judged the immunoreactivity. Then MVD and MIB-1-LI were dichotomized as high or low, based on their median value.

TaqMan PCR to detect mRNA quantities of CXCR4, CXCR7, CCR6, CCR7, and VEGF

Total RNA was extracted from frozen samples, using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Quantitative real-time RT-PCR for these chemokine receptors and for *VEGF* was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and predeveloped TaqMan assay reagents of human *CXCR4* (spanning exon 1/exon 2; ID: Hs00237052-m1), *CXCR7* (spanning exon 1/exon 2; ID: Hs00604567-m1), *CCR6* (spanning exon 1/exon 2; ID: Hs00171121-m1), *CCR7* (spanning exon 1/exon 2; ID: Hs00171054-m1), *VEGF* (spanning exon 1/exon 2; ID: Hs00173626-m1), and *GAPDH*. The PCR reaction was carried out according to the manufacturer's protocol. The standard curve was constructed with serial dilutions of the *CXCR4*, *CXCR7*, and *VEGF* cDNA samples of MCF-7, a breast cancer cell line. As for *CCR6* and *CCR7*, standard curves were constructed using inflamed human tonsils. All reactions of the samples were triplicated, and the data were averaged from the values obtained in each reaction. The obtained data were standardized by using the internal housekeeping gene, *GAPDH*. The final mRNA expression index in each sample was calculated as follows in arbitrary units (AU): mRNA expression index = *CXCR4*, *CXCR7*, *CCR6*, *CCR7*, or *VEGF* mRNA value/*GAPDH* mRNA value \times 1,000 AU.

Statistical analysis

Fisher's exact test was used to evaluate the correlation between two dichotomous variables.

The associations between immunohistochemical scores and mRNA expression were analyzed by the Mann-Whitney U-test. For univariate and multivariate analyses of overall survival, the Kaplan-Meier method with the log-rank test and Cox's proportional hazards model with stepwise procedure were used, respectively. A p-value of <0.05 was considered statistically significant.

Results

Patient characteristics

Table 1 summarizes the clinical and pathological characteristics of the 78 patients with RMS. The patients consisted of 38 males and 40 females, ranging in age from a month to 71 years. Histologically, the 82 specimens included 34 primary ARMS, 44 primary ERMS, 3 metastatic ARMS, and 1 ARMS recurrence. *PAX3/PAX7-FKHR* fusion gene transcripts were examined in 21 cases of 34 ARMS and 15 cases of 44 ERMS. *PAX3-FKHR* fusion gene transcript was identified in 14 ARMS cases, and *PAX7-FKHR* fusion gene transcript was identified in 3 ARMS cases by RT-PCR and direct sequencing. None of *PAX3/PAX7-FKHR* fusion gene transcript was detected in ERMS. Survival data were available in 76 cases, with follow-up periods ranging from 1 to 223 months (median, 17 months). Of the 78 patients, 59 received combined modality therapy including chemotherapy using some of the standardized antitumor drugs (vincristine, actinomycin D, and cyclophosphamide) and 12 patients received surgical treatment and/or radiation therapy, whereas 7 patients had no therapeutic information.

Immunohistochemistry

Table 2 summarizes the results of immunostaining for CXCR4, CXCR7, VEGF, CCR6, and CCR7. *P* value represents the statistical association of the expression of each immunostaining

factor between ERMS and ARMS. Positive staining for CXCR7, VEGF, and CCR6 was recognized mainly in the cytoplasm of the tumor cells or endothelium [26], whereas CXCR4 and CCR7 expression was seen in the nucleus as well as the cytoplasm shown in figure 1 [9,26].

CXCR4, CXCR7, and VEGF immunostaining

Of the 78 primary RMS, 38 (48.7%) showed high expression of cytoplasmic CXCR4. High CXCR4 expression was recognized in 20/34 (58.8%) in ARMS and 18/44 (40.9%) in ERMS. In the same manner, high VEGF expression was observed in 46/78 (58.9%) in all the RMS, 24/34 (70.6%) in ARMS, and 22/44 (50%) in ERMS. High CXCR7 expression was recognized in 64/77 (83.1%) in RMS, 29/33 (87.9%) in ARMS, and 35/44 (79.5%) in ERMS. RMS displayed high CXCR7 expression regardless of the histological subtype (Table 2). None of these proteins showed expression patterns that differed significantly between the histological subtypes.

Within the cases that showed high cytoplasmic CXCR4 expression, 19 out of 34 ARMS (55.9%) and 14 out of 44 ERMS (31.8%) showed high VEGF expression. This revealed a significant association between CXCR4 and VEGF expression ($p=0.0003$, 0.0051 , respectively) as shown in Figure 2. Immunohistochemical expression of CXCR7 showed no significant association with CXCR4 or VEGF expression in either ARMS or ERMS (CXCR7 vs. CXCR4; ARMS $p>0.9999$, ERMS $p=0.7161$, CXCR7 vs. VEGF; ARMS $p=0.2952$, ERMS $p>0.9999$, data not shown).

CCR6 and CCR7 immunostaining

Out of 44 cases, 15 (34.1%) showed high CCR6 expression in ERMS, while 25 out of 34 cases (73.5%) showed high expression in ARMS. High expression of CCR7 was recognized in 30/42 (71.4%) in ERMS and 16/34 (47.1%) in ARMS (Table 2). ARMS cases revealed higher

CCR6 expression than ERMS cases, with statistical significance ($p=0.0007$), while CCR7 expression showed no significant difference between the two histological types ($p=0.0967$). CCR6 and CCR7 expression levels showed no association in RMS ($p=0.6395$, data not shown). Neither CCR6 nor CCR7 showed an association with either CXCR4 or CXCR7 expression, while VEGF expression showed significant associations to both CCR6 expression and CCR7 expression ($p=0.0054$, 0.0319 , respectively) in all RMS (Figure 2).

Microvessel density

Microvessel density was assessed by immunohistochemical staining of CD31. It ranged from 5.5 to 37.25 (median, 11.00 ± 6.61). Median of MVD did not show statistical difference between histological subtypes (ERMS median, 11.13 ± 6.71 ; ARMS median, 10.50 ± 6.46 ; $p=0.996$; Table 3). No significant relationship was observed between MVD and immunohistochemical expression of CXCR4, CXCR7, VEGF, CCR6, or CCR7 ($p=0.7114$, 0.1870 , 0.3071 , 0.8337 , 0.8733 , respectively, data not shown).

MIB-1 labeling index

The MIB-1 labeling index (MIB-1-LI) ranged from 2.2 to 65.38 (median, 14.15 ± 11.29) and its median of both subtypes showed no statistical difference (ERMS median, 12.79 ± 11.85 ; ARMS median, 11.85 ± 8.41 ; $p=0.2189$; Table 3). No significant relationship was observed between MIB-1-LI and immunohistochemical expression of CXCR4, CXCR7, VEGF, CCR6, or CCR7 ($p=0.0760$, 0.9458 , 0.7761 , 0.4564 , 0.8985 , respectively, data not shown).

Quantitative mRNA expression of CXCR4, CXCR7, VEGF, CCR6, and CCR7 and their immunohistochemical expression

In comparison with the results of immunohistochemistry and quantitative real-time RT-PCR,

a statistical association was found between immunohistochemical scores and mRNA expression levels in *CXCR4* and *VEGF* ($p=0.0041$, 0.0235 , respectively; Figure 3), whereas *CXCR7*, *CCR6*, and *CCR7* revealed no association ($p=0.2706$, 0.5067 , and 0.1998 , respectively).

Survival analysis

Table 4 and 5 summarizes the results of survival analysis in all RMS cases and RMS groups separated by subtypes. By univariate analysis of ERMS cases, a poorer likelihood of survival has been revealed in the groups with high VEGF expression compared to the groups with low expression (Figure 4, $p=0.0017$). Especially about receptors expression, VEGF expression and CCR6 expression appeared to be an independent prognostic factor for ERMS (Table 5, $p=0.0008$, 0.042 , respectively). Only the VEGF expression came up as independent prognostic factors in ARMS ($p=0.0353$).

MVD did not affect survival in all RMS, whereas low MIB-1 LI in all RMS correlated significantly with poor survival in univariate analysis ($p=0.048$, Table 4).

Discussion

CXCR4, a receptor for its sole ligand, stromal cell-derived factor-1 (SDF-1/CXCL12), is known to be related to chemotaxis and homing, which are important steps in tumor metastasis [3,4]. Previous retrospective studies show that CXCR4 is highly expressed in various cancers and that its overexpression is closely correlated with lung, liver, and bone marrow metastasis as well as poor prognosis in several kinds of malignant solid tumors [4-7,9].

The important role of the CXCR4/SDF-1 pathway in tumor spread and metastasis has been demonstrated in rhabdomyosarcoma cell lines [6,7]. Especially, the cell lines derived from alveolar

1 RMS expressed higher levels of CXCR4 than cell lines derived from embryonal RMS. A recent
2 study found a significant correlation between high immunohistochemical expression of CXCR4 and
3 poor prognosis in a clinical series of 40 rhabdomyosarcoma cases [27]. Those authors also noted
4 significantly higher levels of CXCR4 expression in alveolar histology.

5 In the present study, there was no significant difference in the immunohistochemical
6 expression of CXCR4 between the two histological subtypes. However, almost half of
7 rhabdomyosarcoma showed high CXCR4 expression. It is demonstrated that CXCR4 antagonists
8 inhibit the primary tumor and metastasis in animal models of melanoma and osteosarcoma [5].
9 Therefore, CXCR4 could be a candidate for molecular target therapy in RMS with high CXCR4
10 expression.

11 We have also revealed significantly higher CCR6 expression in ARMS, and the present study
12 is the first to investigate the CCR6 immunohistochemical expression status with rhabdomyosarcoma
13 histology.

14 Recently, CXCR7 was identified as a receptor for SDF-1, and the SDF-1/CXCR7 axis was
15 reported to regulate the metastatic potential of human RMS cells, similarly to SDF-1/CXCR4 [10].
16 Among RMS cells, ERMS cells express CXCR7 highly and express very low levels of CXCR4,
17 while ARMS-like cells express CXCR4 highly and downregulate CXCR7 expression [28]. In our
18 study, no significant difference in the expression rate of CXCR7 was revealed, although CXCR7
19 showed consistently high expression in both histological subtypes (ARMS: 87.9%, ERMS: 79.5%).
20 It is reported that in hepatocellular carcinoma cell line, down-regulation of CXCR7 inhibits the
21 growth and invasion of tumor cells, which indicates that CXCR7 may be a potential target for
22 molecular targeted therapy [29]. Possibility for application of the CXCR7 antagonists to

1 rhabdomyosarcoma which widely expresses CXCR7 could be worth pursuing in the future.

2 Overexpression of VEGF has been reported in various epithelial malignancies and is thought to
3 be a potent regulator of angiogenesis. Gee MF *et al.* have shown that the VEGF and VEGF family
4 receptor mRNAs were expressed in RMS cell lines [30]. But we could not find any investigations
5 into VEGF expression in large series of clinical RMS specimens. We demonstrated significantly
6 more frequent VEGF expression in ARMS than in ERMS. Considering the statistical difference in
7 prognosis between the subtypes, VEGF could still be a potential therapeutic target in ARMS which
8 is destined for poorer prognosis,

9 Bachelder et al. demonstrated that VEGF regulates CXCR4 expression in breast carcinoma
10 cells [8]. They also demonstrated that CXCR4 mediates the migration of breast cancer cells,
11 depending on autocrine VEGF. Since then, a close correlation between CXCR4 and VEGF
12 expression has been demonstrated in several types of malignancies [21] and in osteosarcoma [31] *in*
13 *vitro* and *in vivo*. In our previous study, we have investigated the CXCR4 and VEGF expression in
14 soft-tissue sarcoma. Nine primary RMS were included in category of malignant round cell tumors,
15 and revealed higher mRNA levels of *CXCR4* and *VEGF* than the control skeletal muscle tissue.
16 However we could not detect significant association between mRNA expression levels for *CXCR4*
17 and *VEGF* in small number of malignant round-cell tumor. In the present study, we confirmed the
18 significant positive correlation between immunohistochemical CXCR4 and VEGF expression. To our
19 knowledge, no other report refers to the correlation between VEGF and CXCR4 in a large series of
20 clinical rhabdomyosarcomas.

21 The correlation between MVD and VEGF expression level and prognosis has been
22 controversial, as some reports have failed to reveal a correlation in a number of solid tumors [17] or

1 in soft tissue sarcomas [32], while other reports have questioned it in soft tissue sarcomas [33,34]. In
2 the present study, we could not find correlation between MVD and VEGF or any other
3 immunohistochemical factors in RMS. Moreover, MVD did not correlate with outcome. A similar
4 result was reported in a study with soft tissue sarcomas that compared MVD and tissue VEGF
5 concentration [33].

6 Other chemokine receptors, CCR6 and CCR7, have been revealed to bind to CCL20 and
7 CCL19/21 with involvement in liver metastases and lymph node metastases in gastrointestinal
8 carcinoma [12,13]. CCL20 was originally identified in the liver and was the only chemokine known
9 to interact with CCR6. Thus, the receptor-ligand pair CCR6-CCL20 plays an important role in the
10 chemoattraction of T cells to the liver [35]. In our series of rhabdomyosarcoma, none of the patients
11 developed liver metastasis, but ARMS showed frequently higher CCR6 expression than ERMS. The
12 importance of chemokine receptors in metastasis is mostly related to CXCR4 and CCR7, aside from
13 the correlation between CXCR4 and organ-specific metastases such as those of lung, liver, and bone
14 marrow, CCR7 expression generally correlates with increased lymph node metastases [11,12]. In
15 our study, high CCR7 expression showed no relation with other immunohistochemical factors.

16 In conclusion, both alveolar and embryonal rhabdomyosarcomas displayed association
17 between VEGF and CXCR4 expression. In addition, our results suggest that high VEGF expression
18 may be predictive prognostic factors in rhabdomyosarcoma. Considering the fact that RMS widely
19 expresses CXCR4 and CXCR7, these chemokine receptors and VEGF may provide potential targets
20 of molecular therapy as part of combined modality therapy in rhabdomyosarcomas.

Acknowledgements

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Figure legends

Fig. 1: Results of immunohistochemical expression of CXCR4 (A,B,C), CXCR7 (D,E), CCR6 (F,G), CCR7 (H,I), and VEGF (J,K,L) in primary site of rhabdomyosarcoma. (A) Alveolar and (B) embryonal rhabdomyosarcoma showing diffuse and strong immunoreactivity for CXCR4, evaluated as score 4. (C) Negative staining of CXCR4. (D) Diffuse and strong staining of CXCR7 in alveolar rhabdomyosarcoma. (E) Negative staining of CXCR7. (F) Focal and strong cytoplasmic expression of CCR6 in embryonal rhabdomyosarcoma. (G) Negative staining of CCR6. (H) Alveolar patterned tumor cells revealed diffuse and moderate CCR7 cytoplasmic immunostaining. (I) Negative staining of CCR7. (J) Cytoplasmic diffuse and strong, (K) diffuse and moderate immunoreactivity for VEGF in (J) alveolar and (K) embryonal rhabdomyosarcoma. (L) Negative staining of VEGF. (Original magnification $\times 200$)

Fig. 2: Association between immunohistochemical score of CXCR4 and VEGF. There were significant positive associations in both alveolar and embryonal rhabdomyosarcomas. There also are significant associations between VEGF and CCR6, as well as between VEGF and CCR7 in all RMS cases.

Fig. 3: Association between immunohistochemical expression status of *CXCR4* and *VEGF* and its mRNA protein expression. The immunohistochemical status was associated with the corresponding mRNA expression.

Fig. 4: Difference in overall survival between histological subtypes of rhabdomyosarcoma. A high VEGF expression in embryonal rhabdomyosarcoma (A) showed significantly poorer prognosis, but not in alveolar rhabdomyosarcoma (B).