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Clinical significance of Vascular Endothelial Growth Factor-A expression in Ewing's sarcoma

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ABSTRACT

The aim of our study was to gain further insight into the role of angiogenesis in Ewing's sarcoma. To this end, expression of Vascular Endothelial Growth Factor-A (VEGF-A), its receptors VEGFR-1 and -2 and microvessel density (MVD) were evaluated by quantitative immunohistochemistry in pretherapeutic biopsies of 40 patients with Ewing's sarcoma treated within standardised neoadjuvant protocols. Median expression levels were 1.5 arbitrary units (AU) for VEGF-A, 8.2 AU for VEGFR-2 and median MVD was 96/0.26 mm^2. VEGFR-1 was expressed in 12.5% of the samples, only. Ten-year relapse free and overall survival rates were significantly higher for patients with high VEGF-A expression (60% *versus* 29%, $p = 0.0216$ and 65% *versus* 25%, $p = 0.013$, respectively). Multivariate Cox regression analysis revealed that VEGF-A expression was an independent prognostic factor for survival. In conclusion, these data suggest that the angiogenic mediator VEGF plays an important prognostic role in Ewing's sarcoma.

1. Introduction

Angiogenesis – a complex, multistep process by which new microvessels are formed from the pre-existing vasculature – is involved in the growth, maintenance and metastasis of most solid tumours.[1] Numerous studies have demonstrated that intratumoural microvessel density (MVD) directly correlates with metastasis and patients' outcome in a variety of tumours.[2] Vascular Endothelial Growth Factor-A (VEGF-A) is one of the key regulators of tumour angiogenesis and evidence is emerging that VEGF-A may have an additional role in cancer through stimulation of VEGF-receptors (VEGFR) on tumour cells.[3]

In contrast to carcinomas, few data are available regarding the clinical and prognostic relevance of angiogenesis in sarcomas, especially in Ewing's Sarcoma. Ewing's Sarcoma is a primary malignant bone tumour characterised by specific translocations involving in most cases the EWS-gene on chromosome 22. The inclusion of cytotoxic polychemotherapy

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into multimodal treatment strategies has led to remarkable prognostic improvements in patients with Ewing's Sarcoma with 5-year survival rates reaching 75% for non-metastasised and 20–40% for primary metastasised Ewing's sarcoma.[4] Recent reports have identified metastatic disease at diagnosis, primary tumour site, age, tumour volume and histological response to chemotherapy as prognostic factors in Ewing's Sarcoma.[5,6]

To date, the prognostic impact of expression and secretion of VEGF-A and the role of MVD in Ewing's Sarcoma have only been explored in a limited number of studies. However, in most of these studies Ewing's sarcoma patients were only a small cohort of soft tissue sarcoma patients either demonstrating that VEGF-A-plasma levels were significantly elevated in soft tissue sarcoma patients compared with controls or that intratumoural VEGF-A expression correlated to survival[7] or tumour grade[8] in soft tissue sarcoma patients. Angiogenic factors were explored exclusively in Ewing's sarcoma patients by Fuchs and colleagues who reported that high VEGF-A expression was an independent predictor of diminished survival[9] and by Burchill and co-workers who reported that MVD is a significant prognostic indicator of survival in Ewing's sarcoma with a significant correlation of VEGF-A expression to MVD.[10,11]

In the present study we examined the clinical significance of the expression of VEGF-A and its receptors VEGFR-1 and VEGFR-2 and of MVD in patients with Ewing's sarcoma before the initiation of polychemotherapy within a multimodal treatment strategy. Aim of the study was to investigate the prognostic relevance of pretherapeutical expression of VEGF-A, its receptors and MVD in chemotherapy treated Ewing's Sarcoma patients and its correlation to established tumour and patient related variables. In accordance with our findings in osteosarcoma,[12] we hypothesised that increased angiogenesis, which is in most cancers a predictor of shortened survival, could favour response and survival in a chemotherapy sensitive tumour such as Ewing's sarcoma.

2. Materials and methods

2.1. Patients and treatment

Paraffin-embedded, pretherapeutical biopsy specimens from 40 patients with non-pretreated Ewing's Sarcoma were studied in a retrospective immunohistochemical study. Criteria for including patients were sufficient representative biopsy material and available follow-up data to which the investigators were blinded to; furthermore, treatment at the University Hospital of Muenster, Germany from 1980 until 1998 within the neoadjuvant protocols CESS 81, CESS 86, or EICESS 92[6,13,14] of the (European Intergroup) Cooperative Ewing's Sarcoma Study ([EI]CESS), which have been approved by the Institutional Review Board. Patients were excluded if written consent to the protocol and follow-up were not available.

2.2. Assessment and definition of patient-, tumour- and treatment-related variables

The following variables were evaluated for their distribution in the patient cohort and for possible correlations with outcome as described before[5,6]: age, gender, tumour site (extremity *versus* axis), primary metastases, absolute and tumour volume <100 ml *versus* ⩾100 ml, response to chemotherapy (according to Salzer-Kuntschik and colleagues[15]) and surgical remission (according to Enneking and colleagues[16]). A good response to chemotherapy was defined as less than 10% viable tumour cells (response grades 1–3), a good surgical remission as a radical or wide surgical margin to the lesion. Also, local therapy (combined surgery and radiotherapy, surgery only and radiotherapy only) was noted.

2.3. Immunohistochemical studies

Serial sections of paraffin embedded biopsy specimen were processed for MVD with an anti-CD31 antibody (clone JC/70A, Dako, working dilution 1:100), for VEGF-A with rabbit polyclonal anti-human VEGF-A (clone A-20, sc-152, Santa Cruz Biotechnology [SCB], working dilution 1:2000), for VEGFR-1 with anti-human VEGFR-1 antibody (sc-316, SCB, working dilution 1:400) and for VEGFR-2 with a mouse monoclonal anti-human VEGFR-2 antibody (sc-6251, SCB, working dilution 1:50). As described by the manufacturers and previously demonstrated,[17] the anti-VEGF-A antibody used in this study is specific for VEGF-A and does not cross-react with other known VEGF family members. The VEGFR-1 and VEGFR-2 antibodies are also specific and do not cross-react with each other or with other receptor tyrosine kinases. Controls for immunostaining using non-immune mouse IgG or rabbit IgG (sc-2025 and sc-2027, respectively, SCB) in substitution for the specific first antibodies were consistently negative. As positive control served a bone marrow specimen from an AML patient as described before[17] (data not shown).

Immunohistochemical localisation was performed by the alkaline phosphatase/anti-alkaline phosphatase double bridge technique (Dako-APAAP Kit). Tissue sections were deparaffinised in xylene, rehydrated in a graded ethanol series, and for antigen retrieval microwaved at 450 W for 7 min. The primary antibodies were applied overnight at 4 °C. Subsequent steps were performed according to the manufacturers' instructions. The fast red substrate (Dako) was employed for revelation of phosphatase activity (10 min at room temperature). Sections were counterstained with 0.1% (w/v) haematoxylin.

2.4. Evaluation of VEGF-A, VEGFR-1 and VEGFR-2 expression and microvessel density

Immunostaining and microvessel counting was simultaneously assessed by two independent experienced investigators using light microscopy. Expression of VEGF-A, VEGFR-1 and VEGFR-2 protein was semi-quantitatively assessed by scoring the proportion and intensity of stained cells as described before.[17] Results were expressed as arbitrary units (AU). In each biopsy sample, expression of VEGF-A, VEGFR-1 and VEGFR-2 was evaluated in two sections processed in independent immunostainings and the mean value was calculated. MVD was determined by the count of microvessels within three independent hot spots per section and in two sections as described before.[12] MVD was defined as the mean count of microvessels per 0.26 mm^2 field area (i.e. 400× field).

The median interobserver and intersectional variability was low (<5% and <2%, respectively). For VEGF-A, VEGFR-2 and MVD median expression levels or MVD, respectively, of the entire group were predetermined to classify patients into two groups with high (>median) and low (⩽median) expression-levels or MVD, respectively, as described before[7,12] while for VEGFR-1 expression patients were grouped according to a positive or negative expression status.

2.5. Statistical analyses

The distributions of the time-to-event variables were estimated using the Kaplan–Meier method, and comparisons were based on the log-rank test with a significance level of 0.05. All potential prognostic factors assessed, VEGF-A, VEGFR-1 and VEGFR-2 expression and MVD were tested using the Cox proportional hazards model. Spearman's correlation coefficient was calculated for MVD and for expression of VEGF-A and VEGFR-2 as a correlation analysis to age, tumour volume and to each other and the *U*-Test for VEGFR-1 expression to age and to VEGF-A, VEGFR-2 and MVD expression. The *U*-test was furthermore applied for expression of VEGF-A, VEGFR-2 and MVD and the Fisher's exact test for VEGFR-1 expression for examining group differences concerning all variables assessed as described above. In case of the association of local therapy with VEGF-A, VEGFR-2 and MVD the Kruskal–Wallis test was applied instead of the *U*-test. All *p*-values reported are two-sided. All calculations were performed using the SAS package (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Patient characteristics

Patient characteristics of the 40 patients with Ewing's Sarcoma are shown in Table 1. Systemic chemotherapy was administered to all patients.

Table 1 – Patient characteristics

Feature	Number[a]
Median age (range)	17 (5–39) years
Gender	
Male	30
Female	10
Tumour site	
Axial	24
Extremity	16
Primary metastases	
Absent	28
Detected	12
Tumour volume	
Median ($n = 34$)	213 (31–1443) ml
<100 ml	6
⩾100 ml	28
n.a.	6
Local Therapy	
Combined surgery/radiotherapy	26
Surgery alone	5
Radiotherapy alone	9
Tumour response to chemotherapy	
Good	11
Poor	5
n.a.	24
Response grades[15]	
Grade 1	8
Grade 2	1
Grade 3	2
Grade 4	4
Grade 5	1
Grade 6	0
n.a.	24
Surgical remission (surgical margins)[16]	
Wide	21
Radical	1
Marginal	4
Intralesional	3
n.a.	11

n.a., data not available.
a If not otherwise stated.

3.2. VEGF-A, VEGFR-1, VEGFR-2 expression, MVD and tumour and patient related variables

Median expression levels were 1.5 AU (interquartile range [IQR] 1–2.06) for VEGF-A, 8.2 AU (IQR 5.25–11) for VEGFR-2 and median MVD was 96/0.26 mm^2 (IQR 56–160) (Fig. 1a–c). Representative Ewing's sarcoma tissue specimens are shown in Fig. 2a with low and in Fig. 2b with high VEGF-A expression. A weak correlation between MVD and VEGF-A expression was observed without reaching statistical significance ($r = 0.28$, $p = 0.079$).

In contrast, VEGFR-1 was expressed only by 5 of 40 (12.5%) tumors. VEGFR-1 expression was significantly associated with high MVD (median MVD = 260.7 *versus* 83.6 for patients with and without VEGFR-1 expression; $p = 0.0054$).

The explorative data analyses of correlations between VEGF-A, VEGFR-1, VEGFR-2 expression, MVD and tumour and patient related variables revealed significant associations between VEGFR-1 expression and age ($p = 0.026$), and between VEGFR-2 expression and tumour volume ($p = 0.041$). Further statistically significant associations, especially for VEGF-A were not observed.

To study the association of tumour angiogenesis with response to chemotherapy we evaluated the correlation between the grade of response to chemotherapy according to Salzer-Kuntschik and colleagues[15] and VEGF-A and MVD, respectively. Patients with a good response to chemotherapy seemed to have a higher tumoural VEGF-A expression and a higher MVD than patients with a bad response ($p = 0.077$ and $p = 0.09$). However, statistical significance was not reached.

3.3. Correlation of VEGF-A, VEGFR-1 and VEGFR-2 expression and MVD with overall and relapse free survival

The median follow-up period was 7.41 years for overall and 7.37 years for relapse free survival. Median overall and relapse free survival for all patients was 3.41 (range 0.46–13.19) and 2.44 (range 0.41–13.19) years, respectively, with 10-year overall

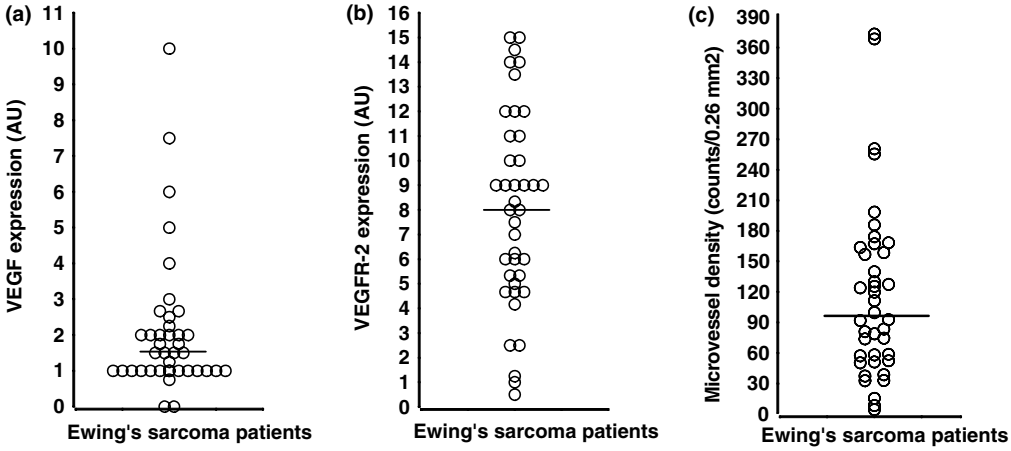

Fig. 1 – Distribution of Vascular Endothelial Growth Factor-A (VEGF-A), VEGF-receptors (VEGFR)-2 expression and microvessel density (MVD) of 40 Ewing's sarcoma patients. Median expression of VEGF-A: 1.5 arbitrary units (AU), VEGFR-2: 8.2 AU, median MVD 96/0.26 mm^2. Data are presented as individual values (open circles) and medians (solid bar).

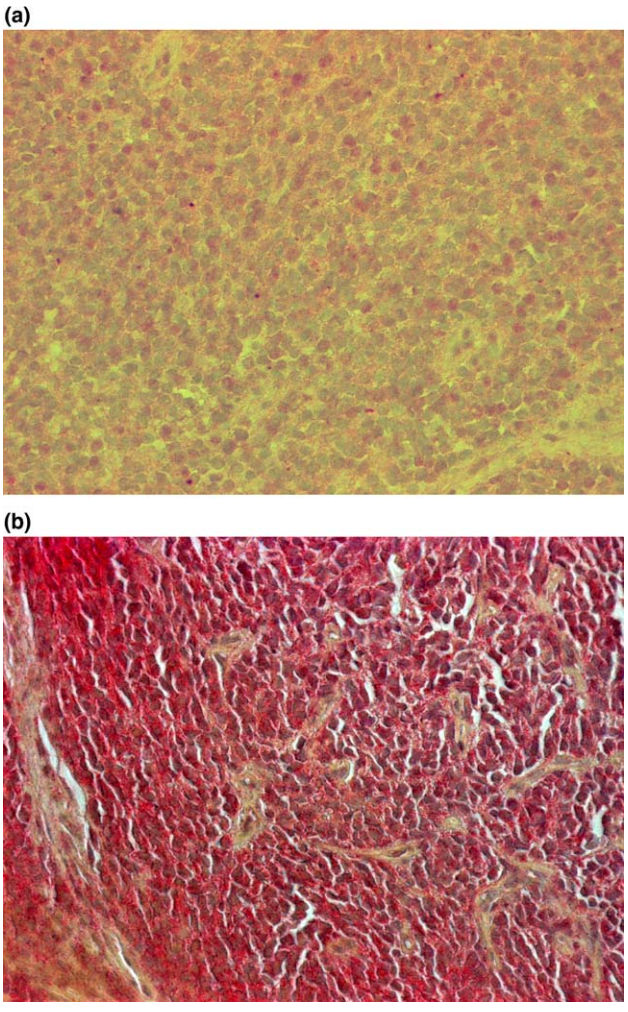

Fig. 2 – Immunhistochemical staining of Ewing's sarcoma tissue slides with anti-VEGF-A antibodies. (a) Section from an Ewing's sarcoma specimen with low VEGF-A expression. (b) Section from an Ewing's sarcoma specimen with high VEGF-A expression. Original magnification 400×.

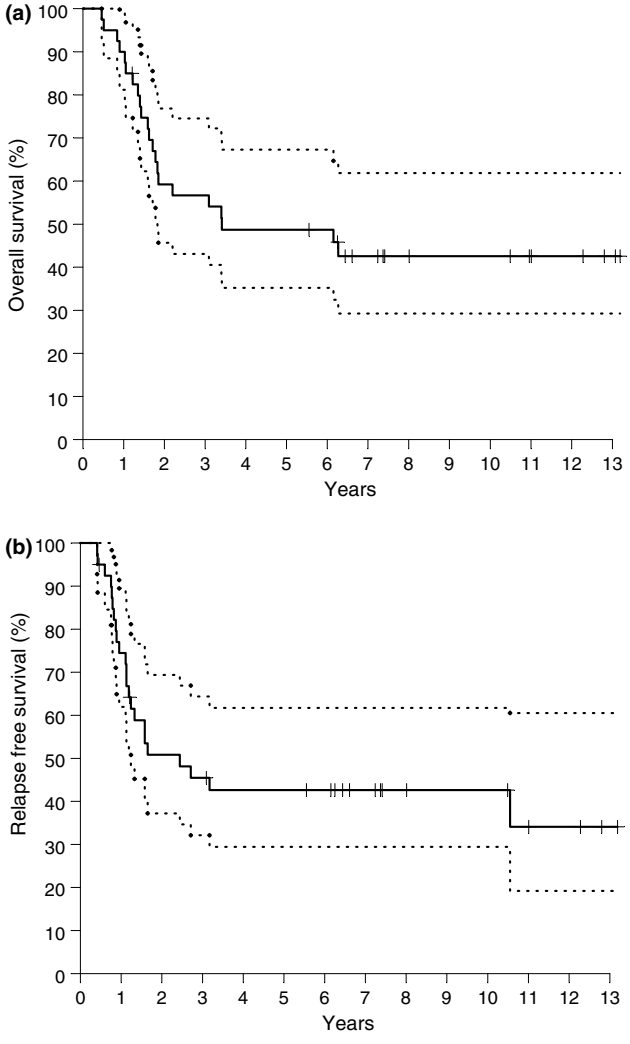

Fig. 3 – Kaplan–Meier estimates of overall (a) and relapse free survival (b) of all analysed Ewing's sarcoma patients (n = 40) at a median follow up of 7.41 and 7.37 years, respectively.

survival rates of 43% (confidence interval [CI] 29-62%) (Fig. 3a and b).

Patients with a high intratumoural VEGF-A expression had significantly longer overall and relapse free survival rates than those with a low intratumoural VEGF-A expression ($p = 0.013$ and $p = 0.0216$, respectively). Ten-year overall and relapse free survival rates for patients with high VEGF-A expressing tumours were 65% (CI 46–92%) and 60% (CI 40–88%), respectively, compared with 10-year overall and relapse free survival rates of 25% (CI 12–54%) and 29% (CI 15–56%), respectively, for patients with low VEGF-A expressing tumours (Fig. 4a and b). The distribution of patient characteristics according to the VEGF-A-expression level is shown in Table 2.

Overall and relapse free survival rates did neither differ for patients with high intratumoural MVD *versus* low MVD tumours ($p = 0.507$ and $p = 0.50$) nor for high *versus* low tumoral expression of VEGFR-2 ($p = 0.946$ and $p = 0.891$) or of VEGFR-1 *versus* non VEGFR-1 expressing tumours ($p = 0.946$ and $p = 0.644$).

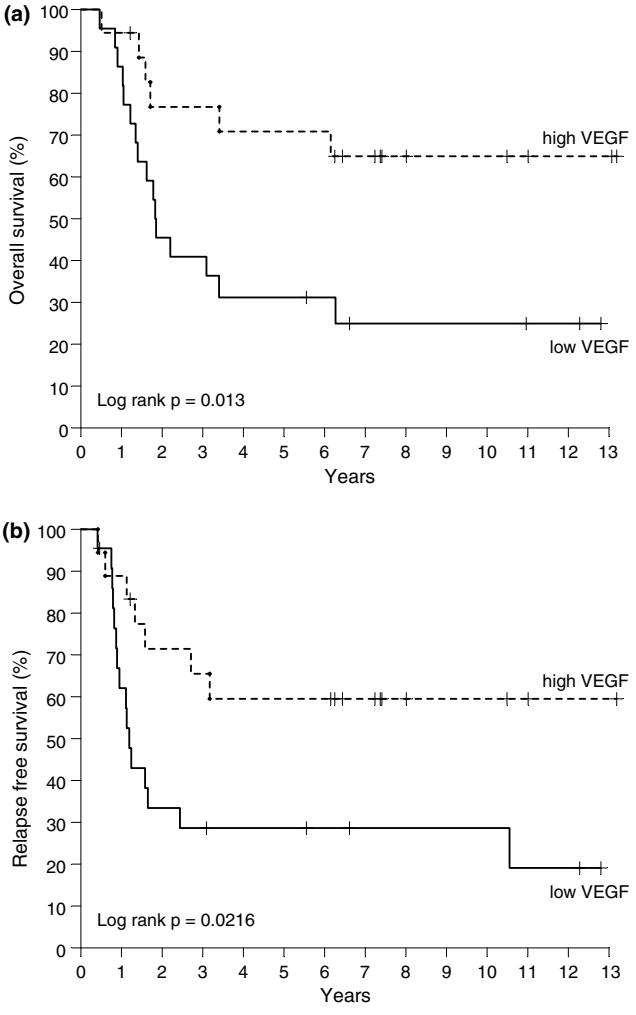

Fig. 4 – Kaplan–Meier estimates of overall (a) and relapse free survival (b) of all Ewing's sarcoma patients ($n = 40$) grouped into high and low VEGF-A tumoural expression. Overall and relapse free survival was significantly prolonged in patients with high VEGF-A expression.

Table 2 – Distribution of patient characteristics according to VEGF-A expression level

Feature	VEGF-A expression level	
	High (>median) [%][a]	Low (⩽median) [%][a]
Median age	18 years	17 years
Gender		
Male	67	81
Female	33	19
Tumour site		
Axial	50	68
Extremity	50	32
Primary metastases		
Absent	72	68
Detected	28	32
Tumour volume		
<100 ml	25	12
⩾100 ml	75	88
Tumour response to chemotherapy[15]		
Good	88	50
Poor	12	50
Surgical remission[16]		
Good	80	71
Poor	20	29

a Percent of evaluable patients, if not otherwise stated.

Multivariate Cox regression analysis revealed that VEGF-A expression and primary metastasis were independent prognostic factors for survival.

4. Discussion

The aim of our present study was to examine the clinical significance of pretherapeutical intratumoural expression of VEGF-A, its receptors VEGFR-1 and VEGFR-2 and MVD in patients with Ewing's sarcoma. The present investigation demonstrates a significant association of the expression of VEGF-A with survival in a large group of Ewing's sarcoma patients and a correlation between VEGF-A expression to the response to chemotherapy for patients treated within the multimodal therapeutic concept of the (European Intergroup) Cooperative Ewing's Sarcoma Study. Patients with a high VEGF-A expression in the biopsy specimens had significant better overall and relapse free survival than patients with a low VEGF-A expression.

The association of VEGF-A overexpression and prognosis has already been reported in several other cancers. There is evidence that VEGF-A has a dual function on tumours by inducing tumour-angiogenesis through stimulation of VEGFRs on tumour endothelium as well as a direct effect on tumour cells.[3] Besides our report, a few others have investigated the role of angiogenic factors in Ewing's sarcoma. Our finding that VEGF-A expression is an independent predictor of survival and that MVD does not correlate with prognosis is in accordance with others.[7,9] In contrast to our results, these authors

describe that VEGF-A is a negative predictor of survival. However, marked differences in patients' characteristics and especially treatment, methodology in staining and analysis might explain these divergences. In the report by Fuchs and colleagues[9] only 74% of the patients analysed had been treated by chemotherapy compared with 100% reported here. Additionally, almost half of the patients in their report already presented with metastasised disease whereas in our study only 30% of the patients had metastases at diagnosis, which is consistent with the literature.[5,18] Considering that patients without systemic treatment will relapse in almost 100% and that metastatic disease is one of the most adverse factors for survival in Ewing's sarcoma, the discrepancies between our and the report by Fuchs and colleagues[9] have to be interpreted with caution. Furthermore, in our analyses patients were grouped by the median expression level while Fuchs and colleagues[9] distinguished between expression *versus* non-expression of VEGF-A. Moreover, a negative VEGF-A expression in our patients was only observed in 5% while Fuchs and colleagues[9] reported a lack of VEGF-A expression in 45%. The use of different grading scores might explain this discrepancy. In our system a detailed graduation between high, medium, low and negative staining was applied while Fuchs and colleagues[9] graded between positive in case of intense staining and negative for absent or faint staining. Yudoh and colleagues[7] reported that concentration of VEGF-A in tumour tissue was an independent prognostic factor for patients with soft tissue sarcoma including some patients with Ewing's sarcoma (10%). However, this study did not distinguish its data between different sarcoma entities and only about 50% of the patients were treated systemically. Moreover, differences might, in part, be explained by considering that prognosis will also depend on other factors such as expression of oncogenes, adhesion molecules, growth factors, degree of apoptosis and the mode of metastastic spread.

In contrast to our report, Burchill and co-workers reported that MVD predicts survival in Ewing's sarcoma.[10,11] However, classification of patients into two groups was different from ours. While Burchill and co-workers separated patients by a predefined MVD value of 100/mm^2, median microvessel density of our entire group was predetermined to classify patients in two groups with high (>median) and low ($\leqslant$median) MVD according to an international consensus report.[19] Furthermore, the report on MVD is only published in an abstract form without giving detailed information on patient characteristics and treatment.[10] The finding that MVD correlates with VEGF-A expression[11] is consistent with ours; however, statistically significance was not entirely reached in our report ($p = 0.079$). This might be due to the small sample size.

While it is known that EWS-ETS oncoproteins upregulate VEGF-A expression[9], the exact role of VEGF-A in Ewing's sarcoma still remains unclear. At least, two probable functions are possible: an auto-/paracrine, i.e. a direct stimulatory effect on tumour growth and an induction of angiogenesis through stimulation of VEGFRs on tumour endothelium. In previous experiments an autocrine effect on Ewing's sarcoma cell lines was not be observed.[11] Treatment with VEGF receptor tyrosine kinase inhibitors had no significant effect on tumour cell proliferation. In accordance with this, we could not observe a correlation between the expression of VEGF-receptors on tumour cells to survival and VEGF-A expression which might be observable if an autocrine loop is existing. Concerning an angiogenic effect of VEGF-A, Dalal and colleagues[11] reported that conditioned medium of Ewing's sarcoma cells, containing large amounts of VEGF-A, enhanced proliferation of human umbilical vein endothelial cells *in vitro*. Also, tumour growth *in vivo* was significantly inhibited and MVD decreased following treatment with anti-VEGF treatment strategies compared with controls. In a report by Zhou and colleagues[20] VEGF-A-expression was down-regulated by adenoviral treatment with E1A gene therapy resulting in a significant reduction of tumour vessels. Targeting VEGF-A expression by a small interfering RNA expression system by Guan and colleagues[21] lead to a decrease of MVD and to tumour growth suppression *in vivo* while Ewing's sarcoma cells expressing VEGFRs were not altered *in vitro*. Taken together, these and our results suggest that the role of VEGF-A in Ewing's sarcoma may possibly be a solely angiogenic effect. Still, other functions of VEGF-A in Ewing's sarcoma can not be excluded. Furthermore, it remains debatable why MVD, a surrogate parameter for tumour angiogenesis,[19] does not correlate with survival in our and in other reports. Others proposed that MVD might not accurately represent the angiogenic capacity in sarcomas.[7] In line with this, substantial differences in the pattern of angiogenesis in sarcomas and carcinomas and a lack of prognostic significance in sarcomas have been observed.[22] Thus, we would suggest that VEGF-A expression better reflects angiogenic activity of Ewing's sarcoma than MVD.

How is this hypothesis compatible with our observation that a high degree of tumoural VEGF-A expression is associated with a significant longer survival? The integration of systemic therapy, i.e. polychemotherapy, into a multimodal treatment strategy has led to dramatic prognostic improvements in patients with Ewing's sarcoma,[4] thus underlining that Ewing's sarcoma is a very chemotherapy sensitive tumour. Through the above mentioned angiogenic effect of VEGF-A, the association between response to chemotherapy and VEGF-A expression observed in our work may be in part due to an improved accessibility of the chemotherapy to proliferating sarcoma cells as tumour microcirculation is an important factor in drug delivery to cancer cells.[23] This is in line with data which demonstrated that vascular permeability correlated with increasing VEGF-A factor expression in sarcoma patients by dynamic enhanced magnetic resonance imaging.[24] The reason why our observed association between response to chemotherapy and VEGF-A expression is only of borderline significance might be that information on histological response to chemotherapy was available only for 40% of the patients. Moreover, direct anti-angiogenic mechanisms of cytotoxic chemotherapy by induction of endothelial cell apoptosis[25–29] may contribute to the eradication of Ewing's sarcoma cells.

Still our data have to be interpreted with caution, as potential bias can not be excluded completely. Differences in gender, localisation of the tumour, rate of primary metastatic disease or surgical remission could have influenced our results (Table 2).

The role of VEGFR-expression on Ewing' sarcoma cells remains unclear. However, the observed significant correlation of a high MVD to the expression of VEGFR-1 is striking. Four

out of five patients with VEGFR-1 expression exhibited extremely high MVD. A possible explanation could be that VEGFR-1 functions as a receptor for other growth factors such as VEGF-B and Placenta growth factor (GF) with further downstream effects.[3] Yet, this remains hypothetical. In line with this, activation of VEGFR-1 by Placenta-GF resulting in transphosphorylation of VEGFR-2 or formation of VEGFR-1/VEGFR-2 heterodimers has to be taken into account.[3] However, the lack of a correlation between VEGFR-1 and VEGFR-2 expression argue against this assumption. Furthermore, the activation of VEGFR-2 by VEGF-E as a VEGF-A independent angiogenic stimulus[3] has to be discussed; still, a correlation of VEGFR-2 expression to survival or MVD was not observed. Finally, the hypothesis that the activation status of these receptors could be of a prognostic value has to be considered and to be addressed in future experiments. Additional trials have to clarify the role of VEGFRs in Ewing's sarcoma.

The VEGF-pathway is well established as one of the key regulators of pathological angiogenesis. However, other angiogenic growth factors might also play an important role in the pathogenesis of Ewing's sarcoma. On one hand can VEGF-A expression be regulated by several other growth factors and their receptors.[3] On the other hand these growth factors might have a prognostic impact in sarcomas. This issue was already addressed by other authors. Dalal and colleagues,[11] e.g. explored angiogenic growth factor expression in Ewing's sarcoma. Besides the above discussed correlation of VEGF and MVD further correlations between MVD and angiogenic growth factors were not observed. Besides VEGF, also Placenta-GF is secreted by Ewing's sarcoma tumour cells; however, the function of these angiogenic growth factors in sarcomas still remains illusive. Further studies are needed to clarify their role in sarcomas. However, similar to our observations of a possible different role of VEGF-A in Ewing's sarcoma reported here and MVD in osteosarcoma[12] compared with most other cancers, Sturla and colleagues[30] reported that basic Fibroblast-GF decreases Ewing's sarcoma growth *in vitro* and *in vivo*.

In conclusion, the present study provides evidence for the prognostic significance of tumoural VEGF-A expression in Ewing's sarcoma demonstrating higher survival rates for patients with higher VEGF-A expression when treated according to intensive chemotherapy regimens like the (EI)CESS protocols. Thus, VEGF-A expression might be a potentially useful prognostic marker in Ewing's sarcoma patients and should be further explored as a potential tool for treatment stratification of Ewing's sarcoma patients.

Conflict of interest statement

Nothing to declare.

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