Prognostic Relevance of Increased Angiogenesis in Osteosarcoma

Michael Kreuter,[1] Ralf Bieker,[1] Stefan S. Bielack,[2] Tanja Auras,[1] Horst Buerger,[4] Georg Gosheger,[3] Heribert Jurgens,[2] Wolfgang E. Berdel,[1] and Rolf M. Mesters[1]

[1]Department of Medicine/Hematology and Oncology, [2]Department of Pediatric Hematology and Oncology, University Children's Hospital, [3]Department of Orthopedics, and [4]Gerhard-Domagk Institute of Pathology, University of Muenster, Muenster, Germany

ABSTRACT

Purpose: **The purpose of this work was to evaluate the prognostic relevance of microvessel density (MVD) for response to chemotherapy and long-term outcome in osteosarcoma.**

Experimental Design: **Pretherapeutic tumor biopsies of 60 patients with high-grade central osteosarcoma, who were treated according to multimodal neoadjuvant protocols of the German-Austrian-Swiss Cooperative Osteosarcoma Study Group, were evaluated for intratumoral MVD. MVD was correlated with demographic and tumor-related variables, response, and survival.**

Results: **The median intratumoral MVD was 52 microvessels per 0.26-mm^2 field area (interquartile range, 31–77 microvessels per 0.26-mm^2 field area). At a median follow-up period of 3.5 years, patients with a high (>median) MVD had significantly higher 5- and 10-year overall survival rates (84%) than patients with low (≤median) MVD (49%; $P = 0.0029$). Furthermore, increased relapse-free survival for patients with high MVD ($P = 0.0064$) was observed. In a subgroup analysis of 44 patients with primary high-grade central osteosarcoma of the extremities without primary metastases and good surgical remission, high MVD was associated with 5- and 10-year overall survival rates of 91% compared with 58% for low MVD ($P = 0.034$). Cox regression analysis revealed that MVD was an independent prognostic factor for survival. A good response to chemotherapy (histologic grading scale of Salzer-Kuntschik) correlated significantly with a high MVD ($P = 0.006$).**

Conclusions: **Increased angiogenesis is a prognostic indicator for higher survival and response rates to chemotherapy in patients with osteosarcoma. Thus, measurement of MVD might be useful in decisions selecting patients for future neoadjuvant treatment.**

INTRODUCTION

Angiogenesis, the formation of new blood vessels from preexisting ones, is involved in the growth, maintenance, and metastasis of most solid tumors (1). Several reports have demonstrated that neovascularization assessed by intratumoral microvessel density (MVD) correlates with clinicopathological factors and patient prognosis in a variety of tumors (reviewed in ref. 2).

In contrast to carcinomas derived from epithelial cells and hematologic malignancies, few data are available regarding the relevance of angiogenesis and its prognostic impact in osteosarcoma. Osteosarcoma is the most frequent primary malignant bone tumor. The inclusion of cytotoxic polychemotherapy in multimodal treatment strategies has led to dramatic prognostic improvements in patients with osteosarcoma, with survival rates reaching 50% to 80% (3–5). Recent reports have identified tumor site and size, primary metastases, response to chemotherapy, and surgical remission as independent prognostic factors in osteosarcoma (6). However, the role of angiogenesis in osteosarcoma still remains a matter of debate. Whereas one report by Wang *et al.* (7) showed evidence for decreased overall survival in osteosarcoma patients with high MVD, Mantadakis *et al.* (8) failed to demonstrate a correlation between intratumoral neovascularization and long-term outcome in patients with nonmetastatic osteosarcoma. Experimental studies on the role of tumor MVDs revealed a significant correlation between MVD and pulmonary metastasis (9). Increased pretherapeutic levels of vascular endothelial growth factor (VEGF), a well known proangiogenic factor, in patients with osteosarcoma correlated with MVD and metastasis (10). Moreover, VEGF overexpression in osteosarcoma was associated with reduced disease-free and overall survival (11). First experimental studies on the effect of anti-VEGF antibodies in osteosarcoma in a chick embryo chorioallantoic membrane model resulted in growth arrest of tumor xenografts and decreased MVD (12). Furthermore, expression of the VEGF coreceptor neuropilin-2 correlated with increased vascularity and poor prognosis in osteosarcoma patients (13).

In the present study, we examined the clinical significance of intratumoral neovascularization in patients with osteosarcoma before the initiation of polychemotherapy within a multimodal treatment strategy by correlating MVD with relapse-free and overall survival and response to chemotherapy. The aim of the study reported here was to examine the prognostic relevance of angiogenesis in chemotherapy-treated osteosarcoma. We hypothesized that increased angiogenesis, which is a predictor of shortened survival in most cancers, could favor response and

Received 5/18/04; revised 7/22/04; accepted 8/3/04.
Grant support: Grants from the Verein zur Unterstuetzung krebskranker Kinder Viersen e.V., the program "Innovative Medizinische Forschung" of the University of Muenster, and the Deutsche Forschungsgemeinschaft (Me 950/3-1), Germany.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Rolf M. Mesters, Department of Medicine/Hematology and Oncology, University Hospital Muenster, Albert Schweitzer Strasse 33, D-48129 Muenster, Germany. Phone: 49-251-8347594; Fax: 49-251-8348745; E-mail: mesters@uni-muenster.de.

survival in a chemotherapy-sensitive tumor such as osteosarcoma.

MATERIALS AND METHODS

Patients. Paraffin-embedded, pretherapeutic biopsy specimens from 60 patients with non-pretreated osteosarcoma were studied in a retrospective immunohistochemical study. There were 34 males and 26 females with a median age of 17 years (range, 5–51 years) at the time of diagnosis. These patients were treated in the University Hospital of Muenster (Muenster, Germany). Criteria for including patients in the present investigation were sufficient representative pretherapeutic biopsy material for MVD analysis and treatment within a protocol of the Cooperative German-Austrian-Swiss Osteosarcoma Study Group (COSS). Patients were excluded if written consent to the protocol and follow-up were not available.

Treatment and Follow-Up. The 60 patients were enrolled between October 1990 and December 2000 onto a neoadjuvant study of COSS and treated in the University Hospital of Muenster according to COSS protocols (6), which have been approved by the institutional review board. Informed consent was required from all patients or their legal guardians, depending on the patient's age. Preoperative and postoperative chemotherapy was to be given to all patients according to the COSS protocol active at the time of enrollment. All protocols included high-dose methotrexate (12 g/m^2) with leucovorin rescue, doxorubicin (60–90 mg/m^2), cisplatin (90–150 mg/m^2) and ifosfamide (6–10 g/m^2) each per course. Bleomycin, cyclophosphamide, and dactinomycin were used in varying combinations. The scheduled duration of chemotherapy ranged from 24 to 38 weeks. Definitive surgery was scheduled to take place between weeks 9 and 11. Follow-up was started at time of diagnosis and included systemic control for pulmonary metastasis (X-ray) and local control (X-ray, ultrasound, and, if needed, computed tomography or magnetic resonance imaging). A first basic evaluation was performed 4 weeks after termination of treatment. Time points of further controls were scheduled as follows: for local control within the first 4 years, every 3 months and afterward on suspicion; for systemic control in the 1st and 2nd years, every 4 to 8 weeks; in the 3rd and 4th year, every 8 to 12 weeks; and from the 5th year to the 10th year, every 6 months.

Assessment and Definition of Patient-, Tumor-, and Treatment-Related Variables. The following variables were evaluated for their distribution in the patient cohort and for possible correlations with outcome as described previously (6): patient age, age $>$ 40 years, patient gender, tumor site (osteosarcoma of the extremity *versus* trunk), primary metastases (at the time of diagnosis), primary or secondary osteosarcoma (patients were assessed as to whether their osteosarcoma represented the first or a subsequent malignancy), histologic subtype, response to chemotherapy [which was assessed according to the six-grade scale of Salzer-Kuntschik *et al.* (14, 15)], and surgical remission [surgical margins to the lesion according to Enneking *et al.* (16)]. A good response to chemotherapy was defined as $<$10% viable tumor cells (response grades, 1–3); a good surgical remission was defined as a radical or wide surgical margin to the lesion.

Immunohistochemical Studies. Serial sections of paraffin-embedded biopsy specimens were processed for immunohistochemical identification of microvascular endothelial cells with an anti-CD31 antibody (clone JC/70A; working dilution, 1:100; Dako, Glostrup, Denmark). Immunohistochemical localization was performed by the alkaline phosphatase/anti-alkaline phosphatase double bridge technique (Dako-APAAP kit; Dako). Before staining, tissue sections were deparaffinized in xylene, rehydrated in a graded EtOH series, and microwaved at 450 W for 7 minutes for antigen retrieval. The primary antibodies were applied overnight at 4°C. Subsequent steps were performed according to the manufacturer's instructions. The fast red substrate (Dako) was used for revelation of phosphatase activity (10 minutes at room temperature). Sections were counterstained with 0.1% (w/v) hematoxylin.

Microvessel Counting. The degree of angiogenesis was determined by the number of microvessels in defined areas of the biopsy specimen according to the method of Padró *et al.* (17) and an international consensus report (18). Microvessel counting was simultaneously assessed by two independent, experienced investigators using light microscopy. The investigators were not aware of the diagnosis and clinical characteristics of the patient before performing the microvessel counting. The entire biopsy specimen section was systematically scanned, *i.e.*, field per field, at $\times$100 magnification to find the areas showing the most intense vascularization. The decision to start counting individual microvessels was based on observing restricted areas within a field at $\times$100 magnification with an impression of a higher count of CD31 antigen-positive cells and cell clusters relative to adjacent areas of the same field and areas of the previous fields. The magnification was then changed to $\times$250 or $\times$400, and the investigators were allowed to reposition the slide until the highest number of microvessels was within the $\times$400 field. This area was defined as a hot spot after achievement of a consensus between both investigators, thus reducing the interobserver error of microvessel counting (19). Areas of vascularization adjacent to normal tissue were excluded because vascularization is not representative of neoangiogenesis in these areas. In each hot spot, both investigators performed individual microvessel counting in a $\times$400 field (0.26-mm^2 field area). In a slight modification of the method described by Weidner *et al.* (20), any red-stained endothelial cell or endothelial cell cluster, with or without a lumen, that was clearly separated from adjacent microvessels was considered as a single, countable microvessel. In each biopsy sample, microvessels were counted in at least 3 independent hot spots per section (range, 3–4 hot spots per section) and in 2 to 3 sections stained with anti-CD31 antibody. The mean value of all independent readings of the tumor biopsy specimen of a single patient was calculated, and MVD was defined as the mean count of microvessels per 0.26-mm^2 field area (*i.e.,* $\times$400 field). The median MVD of the entire group was predetermined to classify patients into two groups with high ($>$median) and low ($\leq$median) MVD according to an international consensus report (18).

Statistical Analysis. The distributions of the time-to-event variables were estimated using the Kaplan-Meier method, and comparisons were based on the log-rank test. All *P* values reported are two-sided. Potential prognostic factors for osteosarcoma (6) were tested using the Cox proportional hazards

model. A multivariate analysis included the variables MVD [high (>median) *versus* low (≤median)], primary metastasis (detectable *versus* not detectable at the time of diagnosis), and response to chemotherapy [good *versus* bad response according to Salzer-Kuntschik *et al.* (14, 15)]. A correlation analysis among age, histologic response to preoperative chemotherapy [Salzer-Kuntschik *et al.* (14, 15)], and MVD was performed by Spearman's correlation. The factors patient age (>40 years *versus* <40 years), gender (male *versus* female), tumor site (trunk *versus* extremity), primary metastases, primary and secondary osteosarcoma, histologic response to preoperative chemotherapy [good *versus* bad response according to Salzer-Kuntschik *et al.* (14, 15)], and surgical remission [good *versus* bad remission according to Enneking *et al.* (16)] compared with MVD were analyzed by the Mann-Whitney test. The comparison of MVD with histologic subtypes was analyzed by the Kruskal-Wallis test. All calculations were performed using the SPSS package (SPSS, Inc., Chicago, IL).

RESULTS

Patient Characteristics. Subjects of the present study were 60 patients with osteosarcoma (Table 1). A total of 56 primary tumors were located in an extremity, and 4 were located in the trunk. Of the 60 patients, 50 presented with apparent localized disease. Ten patients presented with distant metastases that were histologically verified or proven by progression. Osteosarcoma arose as a secondary malignancy in 3 of the 60 patients. All of the 60 osteosarcomas were high-grade central osteosarcomas. Histologic subtypes of these 60 osteosarcomas were as follows: 29 osteoblastic, 17 fibroblastic, 6 chondroblastic, 4 teleangiectatic, 3 sclerotic, and 1 small cell.

Information on histologic response to chemotherapy was available for 56 patients, 34 of whom achieved a good response [<10% viable tumor, corresponding to histologic grading scale of Salzer-Kuntschik *et al.* (14, 15), grades 1–3]. Surgical remission was available for 56 patients. Of these 56 patients, the operative margins to the lesions were, as follows: 49 were wide, 1 radical, 3 marginal, and 3 intralesional.

Microvessel Density in Osteosarcoma. The median MVD was 52 microvessels per 0.26-mm^2 field area, with an interquartile range of 31 to 77 microvessels per 0.26-mm^2 field area (Fig. 1). A representative osteosarcoma tissue specimen is shown in Fig. 2*A* for a tumor with low MVD and in Fig. 2*B* for a tumor with high MVD. Based on the median MVD of the entire group, we classified patients in two groups: those with high MVD [(MVD > median), ≥53 microvessels per 0.26-mm^2 field area] and those with low MVD [(MVD ≤ median), ≤52 microvessels per 0.26-mm^2 field area]. There was no statistically significant difference between the MVD of patients with primary metastases ($n = 10$) and the MVD of patients without primary metastases at the time of diagnosis ($n = 50$; medians, 48 and 58 microvessels per 0.26-mm^2 field area, respectively; $P = 0.215$). Regarding histologic subtypes of osteosarcoma, subtypes were evenly distributed in the high- and low-MVD groups without statistical difference (Table 2). Moreover, no statistical association of MVD with patient age of >40 years, gender, tumor site, primary or secondary osteosarcoma, and surgical remission was observed (Table 2). Furthermore, patient age did not correlate with MVD ($r = -0.163$; $P = 0.212$).

Table 1 Patient characteristics

Features	No.
Median age (range)	17 (5–51)
Gender	
Male	34
Female	26
Tumor site	
Trunk	4
Extremity	56
Primary metastases	
Absent	50
Detected	10
Primary or secondary osteosarcoma	
Primary	57
Secondary	3
Grade	
High-grade central osteosarcoma	60
Histological subtype	
Osteoblastic	29
Fibroblastic	17
Chondroblastic	6
Teleangiectatic	4
Sclerotic	3
Small cell	1
Tumor response to chemotherapy	
Good	34
Poor	22
NA	4
Response grades (refs. 14 and 15)	
Grade 1	9
Grade 2	7
Grade 3	18
Grade 4	14
Grade 5	8
Grade 6	0
NA	4
Surgical remission (surgical margins; ref. 16)	
Wide	49
Radical	1
Marginal	3
Intralesional	3
NA	4

Abbreviation: NA, data not available.

Correlation of Microvessel Density with Overall and Relapse-Free Survival. The median follow-up period was 3.5 years (range, 1 month to 11.1 years). Patients with high intratumoral MVD had a significantly longer overall survival than those with low intratumoral MVD ($P = 0.0029$). Estimated overall survival rates for patients with tumors with high MVD were 89% for 3 years and 84% for 5 and 10 years compared with overall survival rates of 55% for 3 years and 49% for 5 and 10 years for patients with low MVD (Fig. 3*A*). The estimated relapse-free survival was also significantly better in patients with tumors with high MVD as compared with patients with tumors with low MVD ($P = 0.0064$), with 3-year relapse-free survival rates of 78%, 5-year relapse-free survival rates of 73%, and 10-year relapse-free survival rates of 66% for patients with high MVD compared with 3-year relapse-free survival rates of 46% and 5- and 10-year relapse-free survival rates of 41% for patients with low MVD (Fig. 3*B*).

From 60 patients with osteosarcoma, a subgroup of 44

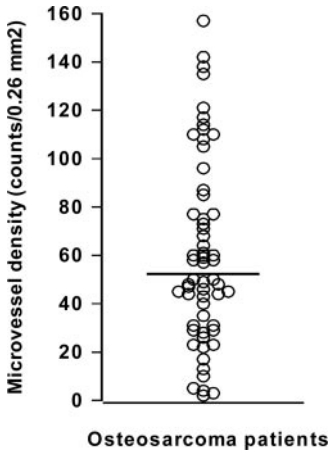

Fig. 1 Distribution of intratumoral MVD of 60 osteosarcoma patients with a median MVD of 52 microvessels per 0.26-mm^2 field area (*i.e.,* ×400 field). Microvessel quantification was performed by staining tumor slides with anti-CD31. Data are presented as individual values (○) and median MVD (*black line*).

patients with primary high-grade central osteosarcoma of the extremities without primary metastases and a good surgical remission was chosen for further subgroup analysis. This group was selected to avoid an imbalance between the two groups (low *versus* high MVD) concerning known adverse prognostic factors in osteosarcoma (6). The median follow-up for this subgroup was 3.9 years (range, 0.7–11 years). In this subgroup, we also found a significantly better overall survival for patients with high MVD than for those with low MVD ($P = 0.034$). The 3-, 5-, and 10-year estimated overall survival rates for patients in this subgroup with high MVD were 91% compared with estimated 3-year overall survival rates of 68% and 5- and 10-year survival rates of 58% for patients in this subgroup with low MVD (Fig. 4*A*). In this subgroup, estimated relapse-free survival appeared to be prolonged in patients with high MVD osteosarcoma (3-year relapse-free survival rate of 86% and 5- and 10-year relapse-free survival rates of 72%) compared with those with low MVD osteosarcoma (3-, 5-, and 10-year relapse-free survival rate of 52%). However, this was not statistically significant ($P = 0.056$; Fig. 4*B*). After exclusion of patients with chondroblastic osteosarcoma in this subgroup, we found significantly better estimated overall and relapse-free survival for patients with high MVD than for those with low MVD ($n = 41$; $P = 0.026$ and $P = 0.0361$, respectively). This exclusion was performed due to incongruent data on the prognosis of these patients (21–24). Moreover, another study excluded patients with chondroblastic osteosarcoma because cartilaginous stroma was described to be associated with hypovascularity (8).

Multivariate Cox regression analysis revealed that MVD was an independent prognostic factor for survival.

Association of Microvessel Density with Response to Chemotherapy. To study the association of tumor angiogenesis with response to chemotherapy, we evaluated the correlation between the grade of response to chemotherapy assessed by the histologic grading scale of Salzer-Kuntschik *et al.* (14, 15) and MVD. A good response to chemotherapy was defined as <10% viable tumor (response grades 1–3), whereas a bad response was defined as >10% viable tumor (response grades 4–6). Patients with a good response to chemotherapy had a significantly higher MVD than patients with a bad response ($P = 0.005$, Mann-Whitney test). Furthermore, we observed an inverse correlation between MVD and the histologic grading scale of Salzer-Kuntschik (Pearson's correlation coefficient $r = -0.364$; $P = 0.006$).

DISCUSSION

The aim of our present study was to examine the clinical significance of intratumoral neovascularization in patients with osteosarcoma before the initiation of polychemotherapy within the context of a multimodal treatment strategy. Until now, there has been a lack of clinical stratifying categories before initiating neoadjuvant chemotherapy in osteosarcoma to predict the response to chemotherapy. The present investigation demonstrates a significant association of intratumoral MVD with survival in a large group of osteosarcoma patients and a significant correlation between MVD and response to chemotherapy for patients treated within the multimodal therapy concept of COSS. Patients with a high degree of MVD within the tumor had significantly better overall and relapse-free survival than patients with low MVD.

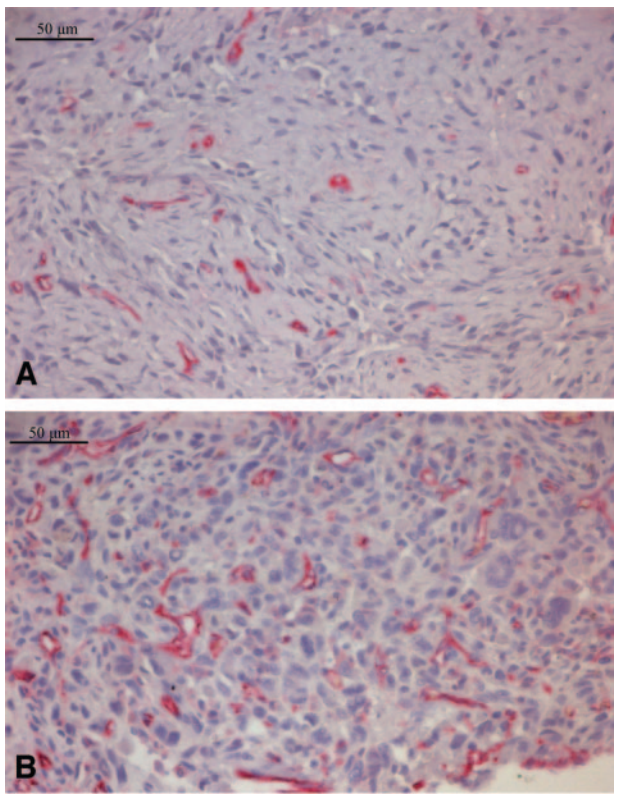

Fig. 2 Immunohistochemical staining of osteosarcoma tissue slides with anti-CD31 antibodies for quantification of MVD. *A*, section from an osteosarcoma specimen with low intratumoral MVD. *B*, section from an osteosarcoma specimen with high intratumoral MVD. Original magnification, ×200.

Table 2 Statistical analyses of patient- and tumor-related variables in relation to MVD

Variables	*n*	Median MVD (range)	*P**
Age (y)			
>40	3	31 (5–50)	0.163
≤40	57	58 (2–157)	
Gender			
Male	34	58 (2–157)	0.551
Female	26	47 (4–135)	
Tumor site			
Trunk	4	45 (5–50)	0.185
Extremity	56	58 (2–157)	
Primary metastases			
Absent	50	58 (2–157)	0.215
Detectable	10	48 (3–142)	
Osteosarcoma			
Primary	57	50 (2–157)	0.221
Secondary	3	77 (50–117)	
Histological subtype			
Osteoblastic	29	61 (3–157)	
Fibroblastic	17	50 (10–105)	
Chondroblastic	6	48 (2–60)	0.661†
Teleangiectatic	4	47 (23–114)	
Slerotic	3	47 (31–71)	
Small cell	1	58	
Tumor response to chemotherapy (refs. 14 and 15)			
Good (response grades 1–3)	34	64 (5–157)	0.005
Bad (response grades 4–6)	22	45 (2–114)	
Surgical remission (surgical margins; ref. 16)			
Good (radical/wide)	50	58 (2–157)	0.639
Bad (marginal/intralesional)	6	49 (31–71)	

NOTE. MVD is measured in microvessels per 0.26-mm^2 field area.
* Mann-Whitney-test, unless otherwise indicated.
† Kruskal-Wallis test.

The integration of intensive polychemotherapy into a multimodal treatment strategy has led to dramatic prognostic improvements in young patients with relapse-free survival rates of approximately 50% to 80% compared with <20% after surgical treatment alone (3, 6). This demonstrates that osteosarcoma is a very chemotherapy-sensitive tumor. The extent of tumor necrosis as a parameter of response to neoadjuvant chemotherapy is the strongest predictor of outcome in resectable osteosarcoma (25, 26). Extensive or complete necrosis (grades 1–3) is associated with an excellent long-term outcome, whereas patients with <90% necrosis (grades 4–6) of the primary tumor have worse prognosis (14, 15, 27). The association of the degree of MVD with response to chemotherapy described here is one of the first reports showing that a defined histologic parameter (*i.e.*, MVD) can predict response to neoadjuvant chemotherapy such as in the COSS protocol in osteosarcoma. Moreover, this relationship might explain why patients with a high degree of intratumoral MVD have a significantly longer survival than patients with low MVD. The correlation between response to chemotherapy and MVD may be due in part to improved accessibility of the chemotherapy to proliferating osteosarcoma cells. Tumor microcirculation is an important factor in drug delivery to cancer cells (28). The efficacy of drug delivery is much higher in a tumor with a high degree of microvessels than in a tumor with low MVD, especially in a chemotherapy-sensitive tumor such as osteosarcoma. Moreover, direct antiangiogenic mechanisms of cytotoxic chemotherapy by induction of endothelial cell apoptosis may contribute to the eradication of tumor cells. Indeed, direct toxic effects on endothelial cells as well as real antiangiogenic effects have been described in *in vitro* and *in vivo* models for different cytostatic agents [*e.g.*, anthracyclins, Vinca alkaloids, and paclitaxel (29–33)].

Two other studies estimating angiogenesis in osteosarcoma based on determining intratumoral MVD have been published thus far. One group (7) reported that high vascular density is associated with poor prognosis and an increased tumor proliferation index. In contrast, another group did not find any correlation between MVD and long-term outcome in nonmetastatic osteosarcoma (8). The apparent contrast of these findings to our results might be easily explained by the fact that patients in our study received a standardized treatment with intensive pre- and postoperative chemotherapy according to the COSS protocols. Therefore, increased angiogenesis might even be associated with a poor prognosis in patients who are treated exclusively by surgery or less intensive chemotherapy. Thus, the conclusion seems justified that increased angiogenesis is a predictor of prolonged survival for patients treated within a multimodal

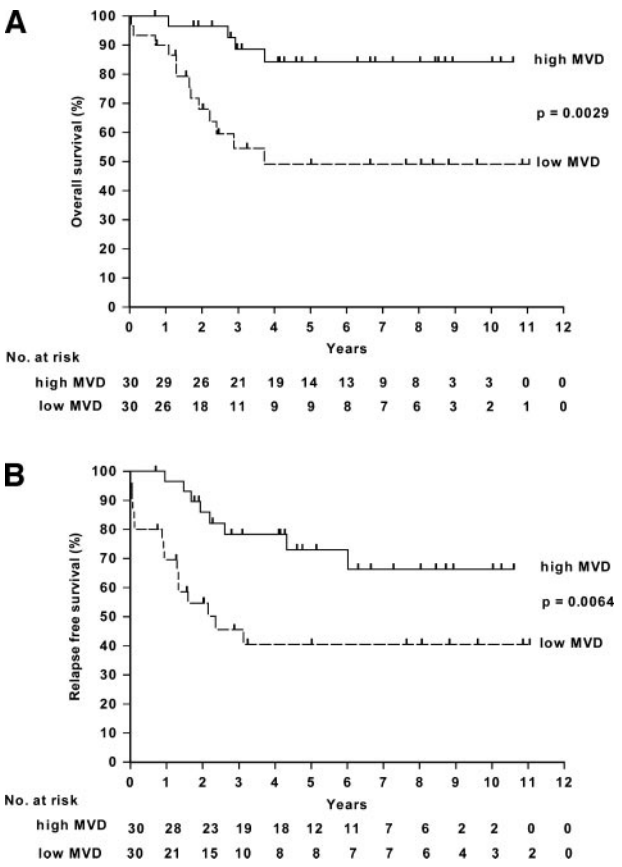

Fig. 3 Kaplan-Meier estimates of overall (*A*) and relapse-free survival (*B*) of all analyzed patients ($n = 60$). Overall and relapse-free survival were significantly longer in patients with high MVD compared with patients with low MVD after a median follow-up of 3.5 years.

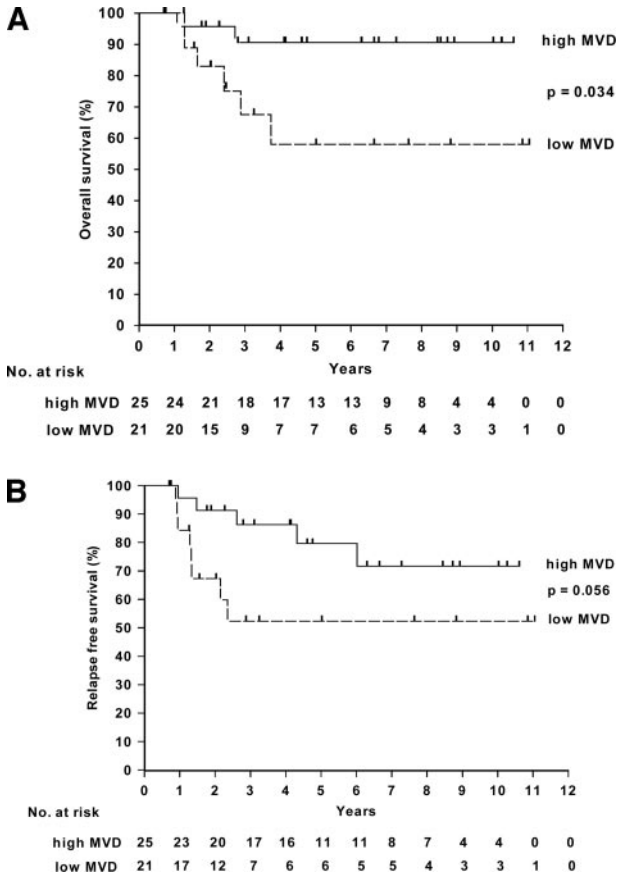

Fig. 4 Kaplan-Meier estimates of overall (*A*) and relapse-free survival (*B*) of a patient subgroup with primary high-grade central osteosarcoma of the extremities without primary metastases and a good surgical remission ($n = 44$). Overall survival was significantly prolonged in patients with high MVD.

concept such as the COSS protocols. In line with this, our study demonstrated a significant correlation between the response to chemotherapy and the degree of MVD, which was not investigated in the studies mentioned. Furthermore, differences between our results and those of the other authors can in part be explained by different study population and size and the choice of endothelial markers used for identifying MVD.

In contrast to the present study, it has been reported that high intratumoral MVD in other cancers is associated with poor prognosis and that MVD was related to invasion and distant metastasis (reviewed in ref. 34). Most of these studies, however, have been conducted in patients with epithelial cancers. Furthermore, most investigations had been performed either in advanced epithelial cancers with poor prognosis and poor response to chemotherapy or in stage I epithelial cancers treated exclusively surgically. The superior chemotherapy sensitivity of osteosarcoma compared with most of these solid tumors studied thus far and the intensive chemotherapy protocol used by the COSS investigators might be the reasons why in our investigation patients with a high MVD have a more favorable outcome than the ones with a low MVD. Moreover, most studies did not use standardized chemotherapy protocols and did not investigate the correlation between response to chemotherapy and MVD. Some of the discrepancies may also be explained by the fact that details of the methodology used to assay MVD can influence its value as a prognostic indicator [*e.g.,* the antibody (CD31, CD34, von Willebrand factor) or whether MVD is assessed at the periphery or the center of the tumor (34)]. Moreover, differences might be explained, in part, 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 metastatic spread in different malignancies.

To avoid selection bias, we performed a subgroup analysis of patients with known favorable patient characteristics (primary high-grade central osteosarcoma of the extremities without primary metastases and a good surgical remission). In this subgroup, high MVD was also significantly associated with a better overall survival. Relapse-free survival seemed to be prolonged in patients with high MVD tumors compared with those with low MVD osteosarcoma. However, this was not statistically significant ($P = 0.056$). Reasons why the relapse-free survival in this favorable subgroup was not significantly prolonged could be that the sample size in this cohort was too small to detect significant differences. Moreover, this selection could have led to imbalances of other prognostic factors such as the expression of oncogenes or growth factors and comorbidity between both groups.

Inconclusive data exist on the prognosis of patients with different histologic osteosarcoma subtypes. Especially for patients with chondroblastic tumors, it was described that prognosis might be different from that of other histotypes (21–24). In a previously reported study (8) estimating angiogenesis in osteosarcoma based on determining intratumoral MVD, chondroblastic osteosarcoma was excluded based on the association of cartilaginous stroma with hypovascularity. In our study, an association of MVD with histologic subtype was not found. Statistical evaluation of the above-described subgroup after exclusion of patients with chondroblastic osteosarcoma again showed that patients with a high degree of MVD within the tumor had significantly better overall and relapse-free survival than patients with low MVD. Therefore, our data do not suggest that histologic subtype has any influence on MVD. However, the numbers of different histologic subtypes are too low in the present study to exclude any association between MVD and histologic subtype.

Primary metastases can be detected in about 10% of osteosarcoma patients at the time of diagnosis and are detected mostly in the lung, similar to the present study (16.7%). Metastatic disease is associated with poor survival in most tumors. However, in osteosarcoma, it has been shown that patients with primary metastases have a 5-year survival of up to 30% to 50% after intensive polychemotherapy and complete resection of the metastases (35, 36). In our study, a statistical association between primary metastases (*i.e.*, at time of diagnosis) and MVD was not found, which might be explained by the favorable survival rates of osteosarcoma patients with primary metastases. Moreover, no statistical association of MVD with age, age > 40 years, gender, tumor site, primary or secondary osteosarcoma, or surgical remission was observed. However, due to the small numbers of patients in some groups [age >40 years, tumor site

(trunk), secondary osteosarcoma], additional studies in larger groups are necessary.

In conclusion, the present study provides evidence for the prognostic significance of intratumoral MVD in osteosarcoma, demonstrating higher survival rates for patients with higher intratumoral MVD treated according to intensive chemotherapy regimens such as the COSS protocols. Thus, MVD might be a potentially useful prognostic marker in osteosarcoma patients and should be further explored as a potential tool for treatment stratification of osteosarcoma patients.

ACKNOWLEDGMENTS

We are grateful to M. Kevric (Department of Pediatric Hematology and Oncology, University of Muenster) and Dr. A. Heinecke (Department of Biostatistics, University of Muenster), for their support.

REFERENCES

1. Folkman J, Watson K, Ingber D, Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature (Lond) 1989;339:58–61.

2. Hasan J, Byers R, Jayson GC. Intra-tumoural microvessel density in human solid tumours. Br J Cancer 2002;86:1566–77.

3. Link MP, Goorin MA, Miser AW, et al. The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. N Engl J Med 1986;314:1600–6.

4. Rosen G, Nirenberg A, Caparros B, et al. Osteogenic sarcoma: eight-percent, three-year, disease-free survival with combination chemotherapy (T-7). Natl Cancer Inst Monogr 1981;56:213–20.

5. Winkler K, Beron G, Delling D, et al. Neoadjuvant chemotherapy of osteosarcoma: results of a randomized cooperative trial (COSS-82) with salvage chemotherapy based on histological tumor response. J Clin Oncol 1988;6:329–39.

6. Bielack SS, Kempf-Bielack B, Delling G, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 2002;20:776–90.

7. Wang D, Chen L, Gao F. Correlation of tumor microvesseldensity with prognosis in osteogenic sarcoma. Zhonghua Bing Li Xue Za Zhi 1997;26:266–9.

8. Mantadakis E, Kim G, Reisch J, et al. Lack of prognostic significance of intratumoral angiogenesis in nonmetastatic osteosarcoma. J Pediatr Hematol Oncol 2001;23:286–9.

9. Coomber BL, Denton J, Sylvestre A, Kruth S. Blood vessel density in canine osteosarcoma. Can J Vet Res 1998;62:199–204.

10. Kaya M, Wada T, Kawaguchi S, et al. Increased pre-therapeutic serum vascular endothelial growth factor in patients with early clinical relapse of osteosarcoma. Br J Cancer 2002;86:864–9.

11. Kaya M, Wada T, Akatsuka T, et al. Vascular endothelial growth factor expression in untreated osteosarcoma is predictive of pulmonary metastasis and poor prognosis. Clin Cancer Res 2000;6:572–7.

12. Li Y, Wang D, Gao F. Experimental study of therapy effects of anti-VEGF antibody on osteosarcoma. Zhonghua Wai Ke Za Zhi 2002; 40:225–7.

13. Handa A, Tokunaga T, Tsuchida T, et al. Neuropilin-2 expression affects the increased vascularization and is a prognostic factor in osteosarcoma. Int J Oncol 2000;17:291–5.

14. Salzer-Kuntschik M, Delling G, Beron G, Sigmund R. Morphological grades of regression in osteosarcoma after polychemotherapy: study COSS-80. J Cancer Res Clin Oncol 1983;106:21–4.

15. Salzer-Kuntschik M, Brand G, Delling G. Bestimmung des morphologischen Regressionsgrades nach Chemotherapie bei malignen Knochentumoren. Pathologie 1983;4:135–41.

16. Enneking WF, Spanier SS, Goodmann MA. A system for the surgical staging of muscolo-skeletal tumors. Clin Orthop 1980;153: 106–20.

17. Padró T, Ruiz S, Bieker R, et al. Increased angiogenesis in the bone marrow of patients with acute myeloid leukemia. Blood 2000;95:2637–44.

18. Vermeulen PB, Gasparini G, Fox SB, et al. Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. Eur J Cancer 1196;32:2474–84.

19. Hansen S, Grabau DA, Rose C, Bak M, Sorensen FB. Angiogenesis in breast cancer: a comparative study of the observer variability of methods for determining microvessel density. Lab Investig 1998;78:1563–73.

20. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N Engl J Med 1991;324:1–8.

21. Petrilli AS, Gentil FC, Epelman S, et al. Increased survival, limb preservation, and prognostic factors for osteosarcoma. Cancer (Phila) 1991;68:733–7.

22. Pochanugool L, Subhadharaphandou T, Dhanachai M, et al. Prognostic factors among 130 patients with osteosarcoma. Clin Orthop 1997;345:206–14.

23. Hauben EI, Weeden S, Pringle J, van Marck EA, Hogendoorn PCW. Does the histological subtype of high-grade central osteosarcoma influence the response to treatment with chemotherapy and does it affect overall survival? A study on 570 patients with two consecutive trials of the European Osteosarcoma Intergroup. Eur J Cancer 2002;38:1218–25.

24. Bacci G, Ferrari S, Bertoni F, et al. A comment and update on Hauben et al. (23). Eur J Cancer 2003;39:548–9.

25. Davis AM, Bell RS, Goodwin PJ. Prognostic factors in osteosarcoma: a critical review. J Clin Oncol 1994;12:423–31.

26. Hudson J, Jaffe MR, Jaffe N, et al. Pediatric osteosarcoma: therapeutic strategies, results and prognostic factors derived from a 10-year experience. J Clin Oncol 1990;8:1988–97.

27. Winkler K, Beron G, Kotz R, et al. Neoadjuvant chemotherapy for osteogenic sarcoma: results of a cooperative German/Austrian study. J Clin Oncol 1984;2:617–24.

28. Hironaka S, Hasebe T, Kamijo T, et al. Biopsy specimen microvessel density is a useful prognostic marker in patients with $T_{2-4}M_0$ esophageal cancer treated with chemoradiotherapy. Clin Cancer Res 2002;8:124–30.

29. Bocci G, Nicolaou KC, Kerbel RS. Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various chemotherapeutic drugs. Cancer Res 2002;62:6938–43.

30. Hill SA, Lonergan SJ, Denekamp J, Chaplin DJ. Vinca alkaloids, antivascular effects in a murine tumour. Eur J Cancer 1993;29A: 1320–4.

31. Belotti D, Vergani V, Drudis T, et al. The microtubule-affecting drug paclitaxel has antiangiogenic activity. Clin Cancer Res 1996;2: 1843–9.

32. Maragoudakis ME, Peristeris P, Missirlis E, et al. Inhibition of angiogenesis by anthracyclins and titanocene dichloride. Ann NY Acad Sci 1994;732:280–93.

33. Schirner M, Hoffmann J, Menrad A, Schneider MR. Antiangiogenic chemotherapeutic agents: characterization in comparison to their tumor growth inhibition in human renal cell carcinoma models. Clin Cancer Res 1995;4:1331–6.

34. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. J Natl Cancer Inst (Bethesda) 2002;94:883–93.

35. Kager L, Zoubek A, Potschger U, et al. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. J Clin Oncol 2003; 21:2011–8.

36. Winkler K, Torggler S, Beron G, et al. Results of treatment in primary disseminated osteosarcoma. Analysis of the follow up of patients in the cooperative osteosarcoma studies COSS-80 and COSS-82. Onkologie 1989;12:92–6.

Clinical Cancer Research

Prognostic Relevance of Increased Angiogenesis in Osteosarcoma

Michael Kreuter, Ralf Bieker, Stefan S. Bielack, et al.

Clin Cancer Res 2004;10:8531-8537.