Does the degree of intratumoural microvessel density and VEGF expression have prognostic significance in osteosarcoma?

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Abstract. The role of angiogenesis as a prognostic indicator in cancer has been extensively studied in recent times with several studies demonstrating a positive correlation for various malignant tumours. However, the role of angiogenesis in osteosarcoma remains a topic of debate. In this study, we aim to evaluate the significance of intratumoural microvessel density (MVD) and the degree of vascular epithelial growth factor (VEGF) expression as markers of angiogenesis and correlate this with disease outcome. Archival paraffin-embedded pre-treatment biopsy tissue of patients treated at St. Vincent's Hospital, Melbourne, with non-metastatic osteosarcoma at initial diagnosis was reviewed. Tissue was processed for immunofluorescent staining of the micro-vascular endothelial cells with antibodies directed against CD31 and CD34. The degree of angiogenesis was assessed, as determined by the microvessel density (MVD). Further histological examination was performed to assess the degree of VEGF expression. Histological observations were correlated with various clinicopathological factors and patient outcome in terms of recurrence, metastasis and death. Twenty-five cases were reviewed, 15 were male and 10 were female, and the median age was 26 years (range, 13-85). The mean follow-up was 21.5 months (range, 3-75 months). The median MVD was 43 microvessels/0.26mm^2 (range, 25-54) and 46 microvessels/0.26mm^2 (range, 30-58) for CD31 and CD34, respectively. Despite the moderate to high vascularity, there was no significant difference noted between the MVD and disease outcome factors for both CD31 and CD34. There was a trend towards a higher MVD in patients aged >40 years compared to those <40 years (p=0.110 for CD31 and p=0.097 for CD34). In terms of VEGF expression, 24 of 25 cases demonstrated either moderate or strong expression; however, no prognostic significance was determined. In this study, we were able to demonstrate that osteosarcoma is a relatively vascular tumour; however, the degree of MVD and VEGF expression does not provide prognostic information. It is likely that angiogenesis plays a key role in the pathogenesis of osteosarcoma and is, therefore, a potential target for novel anti-angiogenic therapies.

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Introduction

Osteosarcoma, although a relatively rare malignancy, is the most common primary tumour of the bone and the second highest cause of cancer-related death in the paediatric age group. Osteosarcoma most commonly afflicts young people in the second and early third decades of life, with a peak incidence generally corresponding to the period of rapid skeletal growth (>60% of diagnoses are made between the ages of 10-20 years). Modern treatment of this condition consists of the effective inhibition of tumour growth, with the aim of shrinking the tumour and therefore permitting safe tumour resection and reconstruction of the limb, and prevention of metastatic disease. To achieve this, aggressive and intense cure-oriented combined modality chemotherapy has been developed over the last 2-3 decades, most commonly comprising of doxorubicin, cisplatin, ifosfamide and high-dose methotrexate. Although there has been significant improvements in the long-term outcome of these patients, 25-50% of patients subsequently develop metastatic disease, which remains the major cause of death (1).

Several studies have attempted to determine possible prognostic factors that may be used to identify patients that are at risk of poorer outcome at presentation. Advanced patient age, tumour site and size, presence of primary metastases, response to chemotherapy and surgical margins have been shown to be independent prognostic indicators in osteosarcoma (2).

Much research has been directed at the possible prognostic significance of angiogenesis in cancer. It is widely believed that angiogenesis, the formation of new blood vessels from pre-existing ones, plays a key role in malignant tumour development, growth and invasion (3). Many studies have attempted to determine whether increased angiogenesis within tumours has any prognostic significance in terms of local recurrence, metastasis and death. From previous reports, it has become clear that there is a significant correlation between the density of intratumoural microvessels and outcome in many carcinomas, including breast, gastric, colorectal and

Table I. Patient and tumour characteristics.

Features	No. (%)
Gender	
Male	15 (60.0%)
Female	10 (40.0%)
Median age (range)	26 (13-85)
Tumour site	
Humerus	8 (32.0%)
Femur	7 (28.0%)
Pelvis	5 (20.0%)
Tibia	1 (4.0%)
Radius (proximal)	1 (4.0%)
Primary or secondary osteosarcoma	
Primary	21 (84.0%)
Secondary - Paget's disease	4 (16.0%)
Primary metastases	
Absent	21 (84.0%)
Present	4 (16.0%)
Histological subtype	
Osteoblastic	14 (56.0%)
Chondroblastic	9 (36.0%)
Fibroblastic	2 (8.0%)

prostate cancers (4-7). However, the role of angiogenesis in sarcomas, especially osteosarcoma, is still unclear, with the few existing reports showing varying results and conflicting conclusions (8-11).

While some authors have suggested that decreased patient survival is associated with higher MVD, a study by Mantadakis *et al* that used CD34 as an endothelial marker failed to demonstrate any correlation between intratumoural neovascularisation and long-term outcome in patients with non-metastatic osteosarcoma (8,9,11,12). In contrast, Kreuter *et al* reported that increased angiogenesis is a prognostic indicator for higher survival and favourable response rates to chemotherapy (10). Therefore, our aim in the present study was to clarify this issue and evaluate the significance of intratumoural microvessel density and the degree of VEGF expression in osteosarcoma and correlate this with clinico-pathological features and long-term outcome.

Materials and methods

Patients. The Bone and Soft Tissue Sarcoma Service at St. Vincent's Hospital and the Peter MacCallum Institute are adult tertiary referral centres for the management of musculoskeletal tumour patients in the states of Victoria and Tasmania, Australia, which service a population of approximately 4 million people. After receiving approval from the Human Ethics Committee at our institution, a retrospective analysis was performed to identify all patients that presented with osteosarcoma between 1996 and 2004 at our institution. In order to be eligible for this study, patients had to have presented with newly diagnosed osteosarcoma (either primary or secondary) and not received neoadjuvant treatment prior to the diagnostic biopsy.

Although 46 eligible patients were identified, only 25 had archival paraffin-embedded tissue available for sufficient histological and immunohistochemical analysis. There were 15 males and 10 females, and the median age was 26 years (range, 13-85 years) at the time of diagnosis. Of these patients, an analysis was performed and the following variables were evaluated: presentation of disease, clinical course and subsequent treatment, tumour histology, site, response to chemotherapy, the development of metastases and long-term survival. Histological specimens were reviewed by a senior musculoskeletal tumour pathologist, and tumours were classified according to predominant histological features and graded according to the degree of cellular differentiation and cytological atypia.

Immunohistochemical analysis of MVD and VEGF expression antibodies. To visualise tumour microvessels, monoclonal antibodies against the human endothelial markers, CD31 and CD34 (DakoCytomation, Glostrup, Denmark) were used at a 1:20 dilution. VEGF expression was localised using a polyclonal rabbit-anti-human primary antibody to VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution. The secondary antibodies included an Alexa Fluor® 594-conjugated goat-anti-mouse IgG secondary antibody (Invitrogen, Australia), Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen), Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Invitrogen) and a biotinylated goat-anti-rabbit antibody (DakoCytomation).

Immunofluorescence. Archival formalin-fixed paraffin-embedded blocks were retrieved from our institution's Pathology Department, and 5-μm sections were cut and subjected to either immunofluorescence or the immuno-peroxidase method. Briefly, sections were deparaffinised with Solv21 (United Biosciences, QLD, Australia) and rehydrated through a series of decreasing concentrations of ethanol. Antigen retrieval was performed by microwave heating in a high pH buffer consisting of 10 mM Tris and 1 mM EDTA (pH 9.0) for 12 min at low heat. Non-specific binding was blocked using 10% goat serum (VEGF) or normal rabbit serum (CD31 and CD34) for 30 min at room temperature. The primary antibodies were diluted accordingly, added to sections and incubated overnight at 4˚C. Sections were washed twice in 1X phosphate-buffered solution (PBS) for 10 min each, and the secondary antibody was applied for 30 min at room temperature in the dark.

In the case of double immunofluorescence, the sections were washed twice in 1X PBS, blocked again with the appropriate serum and the second antibody was added at the appropriate dilution and incubated overnight at 4˚C. Following this, samples were then rinsed twice in 1X PBS for 10 min each, and the appropriate secondary antibody was applied for 30 min at room temperature in the dark.

To highlight nuclear staining, sections were rinsed twice for 5 min each in 1X PBS and then incubated for 5 min with 4,6-diamidino-2-phenyl-indole dihydrochloride (DAPI).

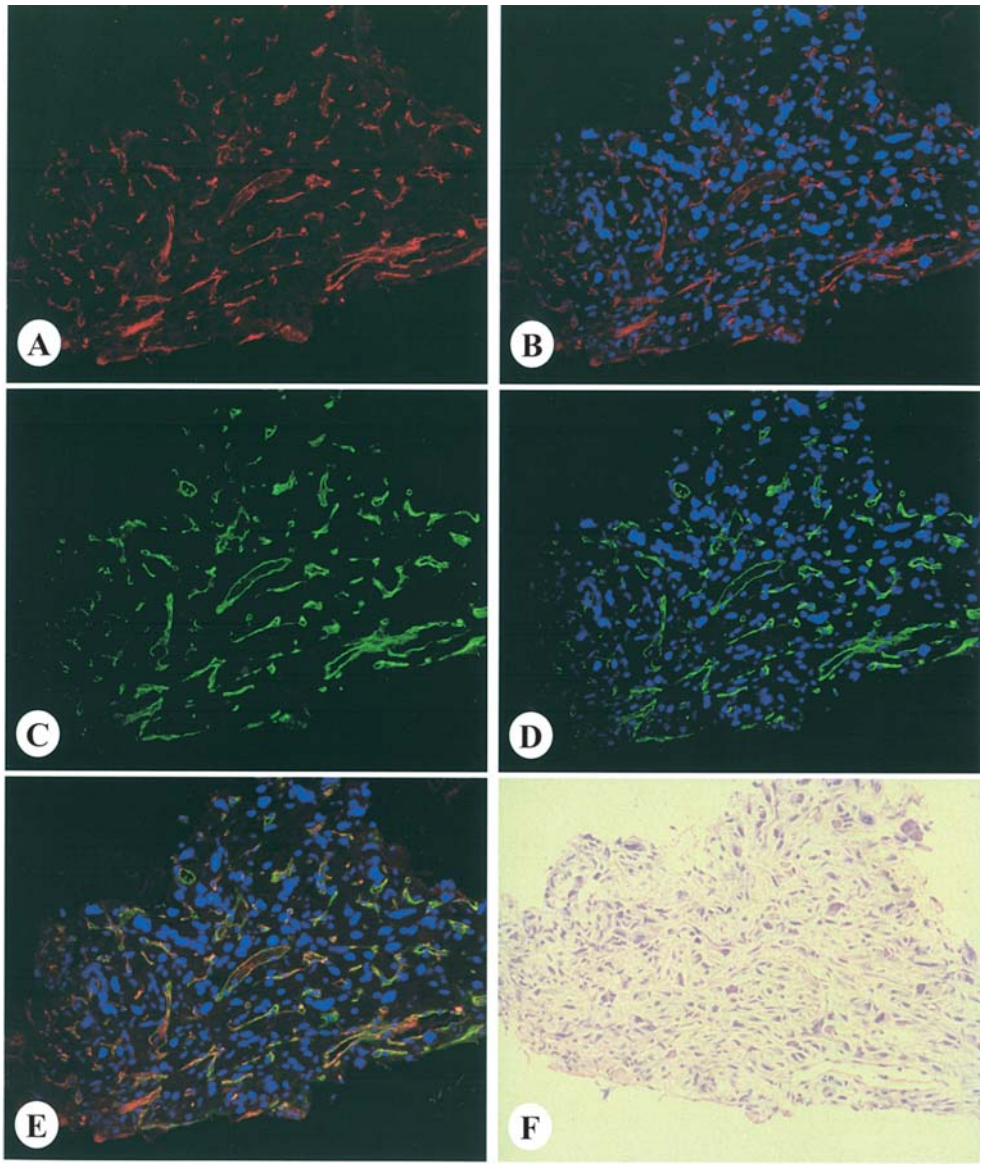

Figure 1. Immunofluorescent double staining of human osteosarcoma tissue. (A) Osteosarcoma tissue stained with anti-CD31 for the quantification of microvessel density. (C) Staining for anti-CD34. (B and D) Counterstaining of osteosarcoma nuclei in blue. (E) When both CD31 and CD34 are visualised together, there is general co-localisation of the two antigens on the endothelial cells, with the resultant staining becoming yellow. (F) A haematoxylin and eosin stain of a serial section. (A-F) Original magnification, x200.

The slides were subsequently rinsed in 1X PBS and then mounted with DakoCytomation Fluorescent mounting medium (DakoCytomation); clear nail polish was applied to the edges of the coverslips. The slides were then stored in the dark at 4˚C.

Standard indirect immunoperoxidase method. Paraffin sections were prepared as described above with the same antigen-retrieval technique. Sections were then treated for 30 min with 10% hydrogen peroxide in dH_2O to block endogenous peroxidase activity. Sections were subsequently immersed in 10% normal rabbit serum for 30 min prior to incubation with the primary antibody (VEGF), and stored overnight at 4˚C. Sections were then washed twice in 1X PBS, and the secondary antibody was added for 30 min at room temperature. Following this, the slides were washed in 1X PBS, treated with peroxidase-conjugated streptavidin for 20 min and developed in 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 5 min. Light counterstaining with Harris haematoxylin was performed prior to mounting on coverslips.

Assessment of microvessel density. Tumour microvasculature was visualised by staining the endothelial cells for CD31 and CD34, and the degree of angiogenesis was determined by calculating the microvessel density (MVD). Immunohistochemical assessment was performed using an inverted fluorescent microscope (Nikon, Eclipse TE2000-U), and images were captured using a SPOT digital camera. The MVD was assessed according to a modified version of the International Consensus Report (13), and this was conducted by two independent investigators who were blinded to the diagnosis, clinical course, management and outcome of the patients. Briefly, the entire section was systematically scanned at a x100 magnification, and the areas of most intense

Table II. Clinicopathological characteristics related to MVD.

	n	MVD-CD31	P-value	MVD-CD34	P-value
Age					
>40 years	8	58 (17-79)	NS-0.110	63 (17-79)	NS-0.097
<40 years	17	30 (0-101)		30 (0-103)	
Gender					
Male	15	46 (14-101)	NS-0.166	48 (14-103)	NS-0.134
Female	10	28 (0-61)		34 (0-64)	
Tumour site					
Trunk	5	45 (27-61)	NS-0.974	47 (30-64)	NS-0.946
Extremities	20	43 (0-101)		45 (0-103)	
Primary metastases					
Present	4	25 (17-51)	NS-0.203	27 (17-56)	NS-0.195
Absent	21	45 (0-101)		47 (0-103)	
Osteosarcoma					
Primary	21	42 (0-101)	NS-0.452	44 (0-103)	NS-0.459
Secondary	4	48 (25-79)		52 (24-84)	
Histology					
Osteoblastic	14	45 (0-81)	NS-0.177	47 (0-82)	NS-0.459
Chondroblastic	9	47 (21-101)		47 (20-103)	
Fibroblastic	2	19 (14-24)		19 (14-23)	
Response to chemotherapy					
Good (≥ 90%)	8	50 (22-74)	NS-0.408	53 (20-82)	NS-0.460
Poor (<90%)	10	37 (0-101)		42 (0-103)	

NS, not significant.

vascularisation, i.e. greatest number of CD31-antigen-positive cells, were classified as 'hot spots.' A 'hot spot' was defined as an area where the density of antigen-positive cells and cell clusters was greater relative to adjacent areas. The selected areas were viewed at a x200 magnification, the slide was repositioned over the area of the 'hot spot' with the most vessels per field, and 3 images of the same field were captured: 1) immunofluorescent staining for CD31 (wavelength 594 nm, Texus red); 2) CD34 (wavelength 488 nm, FITC green); and 3) nuclear staining with DAPI (wavelength 461 nm, blue). A positive vessel count was defined as a single endothelial cell, endothelial cell cluster, or microvessel that is clearly separated from adjacent microvessels as previously described by Weidner *et al* (5). The counting of both CD31- and CD34-stained microvessels was achieved using the image analysis software, Image Pro Plus (Media Cybernetics, MD, USA), which allowed further magnification of the captured images and subsequent labelling of each microvessel to give an accumulated count. The three 'hottest' spots were captured for each section, and the mean count of all independent readings was determined as the MVD. The MVD was assessed using CD31 and CD34 counts separately. The median vessel count was used to make a distinction between low (≤ median) and high (>median) MVD.

Evaluation of the degree of VEGF expression. Semiquantitative assessment of the degree of VEGF expression was determined using a modified version of a method described by Karavasilis *et al* (14). The intensity of cytoplasmic immunostaining was assessed using sections that were exposed with DAB, and graded as follows: 0, no staining; 1, weak; 2, moderate; and 3, strong staining. On the serial section that underwent immunofluorescent staining, the percentage of cells stained for VEGF was calculated by comparing the number of positively stained cells relative to the number of nuclei (0, no cells stained; 1, 1-25%; 2, 26-50%; and 3, >50%). By combining the two scores, the degree of VEGF expression was determined; a score of 2 represents weak expression; 3-4, moderate/multifocal; and 5-6, strong/diffuse expression.

Statistical analysis. Results were analysed using MedCalc for Windows v8.1 (Medcalc Software, Belgium). Non-parametric tests were used to allow for data that was not normally distributed. Associations between clinicopathological features (age, gender, tumour site, local recurrence, metastases, death) and MVD or the degree of VEGF expression were analysed using the Mann-Whitney U test. The comparison of MVD and VEGF expression with histological subtypes was analysed by the Kruskal-Wallis test. The correlation between MVD or

VEGF expression and patient age was determined using Spearman's rank coefficient. Disease-free and overall survival distributions were charted according to the Kaplan-Meier method, and comparisons between patients with high and low MVD were based on the log-rank test. A p-value <0.05 was considered significant.

Results

Patient and tumour characteristics. At the time of presentation, 21 patients had apparent localised disease, 4 had evidence of synchronous metastatic disease, 3 had lung metastases and 1 had a subcutaneous metastasis in the calf. Osteosarcoma arose secondarily to Paget's disease of the bone in 4 patients. Eight of the tumours were located in the humerus, 7 in the femur, 5 in the pelvis, 4 in the tibia and 1 in the proximal radius. No patient presented with evidence of a pathological fracture. In terms of the histological subtype of tumours, 14 (56%) osteosarcomas were classified as high-grade osteoblastic lesions, 9 (36%) were chondroblastic and 2 (8%) were fibroblastic (Table I).

Of the 25 patients, 24 underwent surgical resection of the tumours. Of the 24 patients, 20 had limb-sparing surgery and 3 had limb amputations (2 above the elbow and 1 below the knee). Clear margins were achieved in all cases. Nineteen patients received both neoadjuvant and adjuvant chemotherapy. The treatment regimes comprised of a combination of standard agents, which included doxorubicin, cisplatin, ifosfamide and high-dose methotrexate and were administered using current protocols. According to Bacci *et al*, 8 patients had a good response (≥ 90%) and 10 had a poor response (<90%) in terms of histological response (15). Two patients received adjuvant chemotherapy alone and 1 had a combination of adjuvant chemotherapy and radiotherapy to the lesion. In all cases, the chemotherapy was relatively well tolerated, with no notable toxicity-related complications.

The mean follow-up period was 21.5 months (range, 3-75 months). At the time of latest review, 4 patients (20%) had died of their disease, 2 of whom had not received the routine neo/adjuvant chemotherapy protocol. Fifteen patients were continuously disease-free and 3 patients were disease-free after pulmonary metastectomy. During the study, 3 patients developed local recurrence of their tumour. This was managed with chemotherapy in 2 patients and a forequarter amputation in 1 patient. Of the 21 patients that were metastasis-free at initial diagnosis, 4 (20%) developed metachronous lesions in the lung at a mean time of 13 months (range, 3-26 months). Three of these patients were surgically managed with pulmonary metastectomy.

Microvessel density in osteosarcoma. Analysis of the CD31 and CD34 immunostained sections demonstrated that the median MVD was 43 microvessels per 0.26 mm^2 (interquartile range, 25 to 54) and 46 microvessels per 0.26 mm^2 (interquartile range, 30 to 58), respectively. Not surprisingly, when both CD31 (red fluorescence) and CD34 (green fluorescence) were visualised together, there was general co-localisation of both antigens on the endothelial cells (Fig. 1). No statistical difference was observed for both CD31 and CD34 between the MVD and various clinicopathological factors such as patient age (above or below that age of 40 years), gender, tumour site, presence of primary metastases, primary or secondary osteosarcoma, and response to chemotherapy (Table II). Furthermore, there was no statistical difference in the MVD between patients with different histologic subtypes. Of interest, there was a trend towards a greater MVD in patients above the age of 40 years (n=8) compared to patients below the age of 40 years, (n=17); however, this did not reach statistical significance (p=0.110 for CD31 and p=0.097 for CD34). No correlation was demonstrated between advanced patient age and MVD (r=0.293 and p=0.151 for CD31; r=0.297, p=0.146 for CD34).

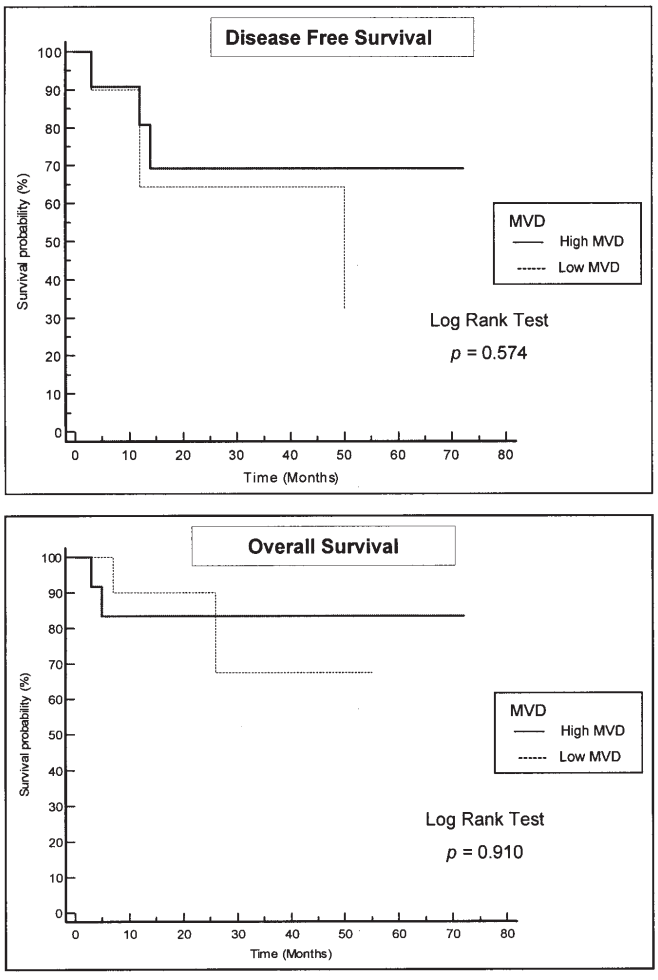

Figure 2. Kaplan-Meier estimates of disease-free (A) and overall survival (B) of osteosarcoma patients with high or low microvessel density. There was no statistically significant difference in patients with high MVD compared with patients with low MVD after a mean follow-up of 21.5 months.

Based on the median MVD, the CD31 and CD34 groups were divided into two groups of high MVD (CD31, ≥ 43 microvessels per 0.26 mm^2; CD34, ≥ 46 microvessels per 0.26 mm^2) and low MVD (CD31, <43 microvessels per 0.26 mm^2; CD34, <46 microvessels per 0.26 mm^2). Fig. 2 shows overall and disease-free survival in patients with high and low MVD. Of note, no statistical difference was found between the two groups in terms of disease-free survival (log-rank test, p=0.574) and overall survival in patients (log-rank test, p=0.910).

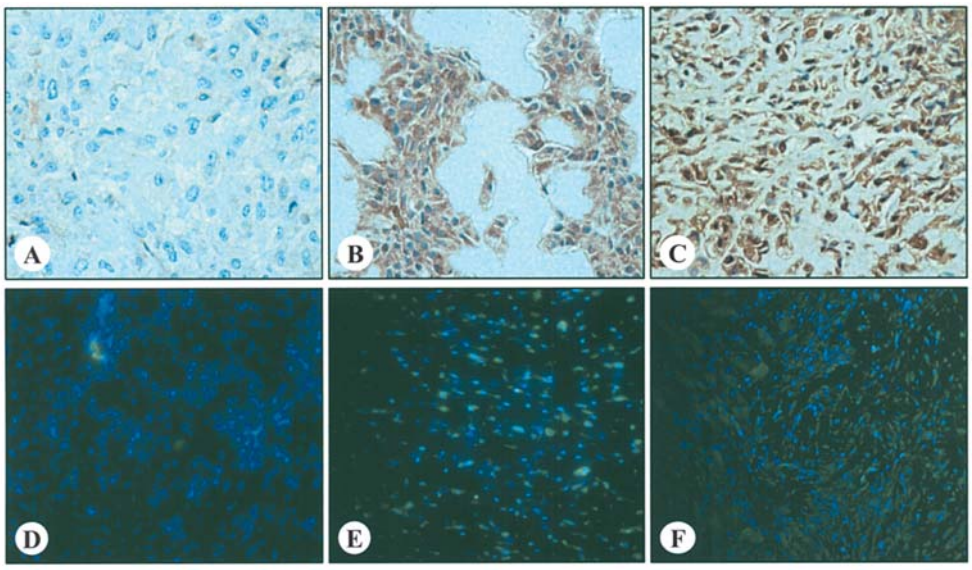

Figure 3. The degree of VEGF expression was semiquantitatively determined by assessment of the staining intensity and percentage of cells stained for VEGF. The degree of VEGF staining intensity was determined by immunoperoxidase immunohistochemistry and scored as follows: 1, weak (A); 2, moderate (B); or 3, strong (C). On a serial section, immunofluorescence was performed to determine the percentage of cells stained (green) relative to the tumour nuclei (blue). A score of 1 was given for 0-25% (D), 2 for 26-50% (E), and 3 for >50% of cells stained (F). There were also overall combined scores: 2, weak expression; 3-4, moderate/multifocal expression; and 5-6, strong/diffuse expression. (A-F) Original magnification, x200.

VEGF expression in osteosarcoma. Tumour cells in all 25 cases displayed positive cytoplasmic VEGF staining (Fig. 3). Examination of the specimens under light microscopy at a x100 magnification revealed apparent localised variation in the expression of VEGF, with areas of negative-to-weak immunostaining interspersed throughout the sections. Eleven tumours (44%) demonstrated a strong degree of VEGF expression, 13 (52%) were classified as moderate and 1 (4%) as weak. No differences were observed between the degree of VEGF expression and various factors such as patient age (>40 and <40 years), gender, development of local recurrence, metastasis or death. Furthermore, there was no correlation between VEGF expression and patient age ($r=0.175$, $p=0.391$) or the MVD ($r=0.043$, $p=0.621$).

Discussion

In the present immunohistochemical study, we were able to demonstrate that osteosarcoma is a relatively vascular tumour, evidenced by the fact that a large majority of cases displayed moderate to high levels of tumour microvessels per 0.26 mm^2. Moreover, in almost all tumour specimens, moderate or strong levels of VEGF expression were evident within the cytoplasm of the osteosarcoma cells. Although we were not able to demonstrate any correlation between the MVD and clinicopathological features or patient prognosis, this result may not be entirely surprising, as it is becoming increasingly apparent in the literature that microvessel density may not be as relevant a prognostic marker in sarcomas as it is in carcinomas (16,17). This has been further echoed in other studies that also failed to show prognostic significance of MVD in various soft tissue sarcomas (16,18,19). It is postulated that this may be due to different patterns of blood vessel distribution that exist in sarcomas compared to carcinomas, and carcinoma vessels often cluster in 'bursts,' whereas sarcoma vessels are generally more homogenous and diffusely distributed, therefore resulting in a single compartment of neoplastic cells producing uniform expression of angiogenic cytokines (16).

The quantification of tumour angiogenesis based on MVD has been used extensively as a prognostic marker to estimate the aggressiveness of a given tumour. The use of immunofluorescence staining of endothelial cells to measure MVD has not, to our knowledge, been previously described. We believe that such a technique has a higher sensitivity and provides a greater means of quantifying tumour microvessels using more modern imaging technologies. Furthermore, there is the added ability whereby more than one antigen may be stained on the same tissue section, therefore providing better localisation of endothelial cells. In the literature, both CD31 and CD34 have been used extensively as reliable endothelial markers for microvessel density assessment (13). In general, CD31 has been described as the pan-endothelial marker of choice for paraffin-embedded samples as it binds to all endothelial cells regardless of activation status (quiescent or activated or replicating) (13,19). CD34, on the other hand, has the problem of poor specificity as it stains well for lymphatic vessels and perivascular stromal cells (13,20). From immunofluorescent double staining, we observed that CD31 and CD34 are uniformly co-localised on the endothelial cells.

In our study, correlation between positive or negative expression and prognosis could not be performed, as the large majority of tumours (24/25) displayed either moderate or strong VEGF expression. Another possible explanation for the uniformly high levels of expression found in the tumour specimens is that it is routine practice for radiologists at our institution to target areas that appear be of highest metabolic activity on nuclear medicine imaging when the diagnostic biopsies are taken. Some studies have shown that areas of increased tumour cell proliferation, as determined by PCNA,

p53 or Ki-67 expression, are associated with higher levels of VEGF (21-23). Therefore, if the expression of angiogenic factors was non-uniform throughout the tumour, with some areas relatively higher than others, this may provide significant variations in sampling. In comparison to our study where all specimens displayed some degree of VEGF expression, Kaya *et al* reported positive VEGF expression in only 17 of 27 (63%) osteosarcoma specimens (11). Hence, if the expression of VEGF is used as a clinical marker, further studies are needed to determine whether there may be a variation in expression levels throughout the tumour.

In order for tumours to develop, grow and eventually metastasise, they must be able to continuously stimulate the ingrowth of new capillary blood vessels from the tumour's surroundings, one 'hallmark' of tumourigenesis (24). This is the result of an imbalance between pro-angiogenic factors (e.g. VEGF, bFGF and TGF-ß) and anti-angiogenic factors (e.g. TSP-1, angiostatin and endostatin). Folkman and Shing postulated that, as a result of the induction of the 'angiogenic switch' during tumour development, there is a threshold change in the balance between stimulatory and inhibitory influences, in the favour of angiogenesis (3). Therefore, interruption of this process could theoretically halt the progression of many tumours that are dependent on angiogenesis for further growth.

Much scientific research has been directed at determining the role of angiogenesis in tumour development, as well as characterising the several factors at play in the regulation of blood vessel growth. Despite significant improvements in chemotherapy and surgery, 25-50% of patients with initially non-metastatic osteosarcoma eventually develop metastases (1). Therefore, in light of the findings of this present study, further research is warranted into the possible role of anti-angiogenic therapy as a form of targeted tumour-specific treatment to augment the effects of chemotherapy and potentially reduce the tumour to a dormant state of low metastatic potential.

From the results of our study, intratumoural microvessel density and the degree of VEGF expression did not correlate with clinicopathological features or disease outcome in osteosarcoma. However, although angiogenesis may not be of prognostic significance, it is likely that it has a key functional role in tumour development, invasion and metastatic spread and, therefore, may be a potential target for novel anti-angiogenic therapies. Further investigation is needed to characterise the pattern of microvessel growth and expression of angiogenesis-regulating factors in mesenchymal tumours and its role in tumourigenesis.

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