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von Willebrand factor expression in osteosarcoma metastasis

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A number of genes are implicated in the initiation and progression of osteosarcoma; however, cytogenetic and comparative genomic hybridization studies indicate the involvement of additional unidentified genes. An examination of gene expression profiles in 22 high-grade osteosarcoma tumor specimens from 15 patients (including paired primary and metastatic samples from five patients) indicated that von Willebrand factor (*vWF*) mRNA expression may increase during tumor progression. vWF, a large glycoprotein previously considered to be expressed exclusively by endothelial cells and megakaryocytes, is involved in platelet aggregation and adhesion to the subendothelial matrix, processes critical to hematogenous tumor cell metastasis to the lung. Analysis of paired primary and metastatic osteosarcoma tumor samples from 10 patients revealed an increase in *vWF* gene expression in metastases ($P = 0.005$). Immunohistochemistry showed that, in addition to the endothelial cells, vWF protein was also detected in osteosarcoma cells *in vivo* in 13 of 29 tumor specimens as well as in SAOS2, an osteosarcoma cell line. The tumor cell staining correlated positively with high *vWF* expression in the sample ($P = 0.006$). Although vascular endothelial cells contribute to the *vWF* mRNA detected in the tumor samples, there was neither any correlation between vascular density (VD) and *vWF* mRNA expression nor between VD and clinical outcome. These findings suggest that vWF expression is deregulated in osteosarcoma tumors, potentially contributing to metastasis.
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Osteosarcomas are tumors of mesenchymal origin that most commonly affect patients between the ages of 10 and 20 years. With the introduction of modern treatment protocols involving aggressive surgery and neoadjuvant chemotherapy, 5-year survival rates of 50–60% are common for patients presenting without metastatic disease.[1–4] However, 40–50% of patients will develop metastases and few of them will be cured. A more thorough understanding of the molecular events underlying osteosarcoma development and metastasis has the potential to lead to further advances in patient management and improved outcomes. It is generally accepted that neoplasia develops through a multistep process involving the acquisition of genetic alterations leading to malignancy and ultimately metastasis.[5] A number of genes including *p53*, *MDM2*, and *Rb* are known to be involved in osteosarcoma, but cytogenetic and comparative genomic hybridization (CGH) studies indicate that additional genes, many of which have not yet been identified, are also involved in the initiation and progression of this disease.[6–9] Given that metastases are the most common cause of death in patients with osteosarcoma and most other cancers, it is especially critical to understand the genes involved in progression from primary to metastatic tumor.[10] We therefore elected to examine multiple paired primary and metastatic tumor samples for differentially expressed genes, in order to identify additional genes which play a role in the metastatic spread of osteosarcoma.

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Using differential display analysis (DDPCR) of tumor samples, we observed that the von Willebrand factor gene (*vWF*) was more highly expressed in metastatic compared to matched primary tumor samples, suggesting that it may be involved in the metastatic cascade. vWF is a large glycoprotein that is present in blood, platelet granules and subendothelial connective matrix as a multimeric protein.[11–13] It was thought to be expressed exclusively by endothelial cells and megakaryocytes and has therefore been used as a marker for endothelial cells in tissue samples.[14,15] vWF normally functions in hemostasis, where it binds and stabilizes clotting factor VIII and allows for platelet adhesion to the subendothelial matrix.[11–13] Platelet adhesion and aggregation are two processes critical to hematogenous tumor cell metastasis.[16–18] The aggregation of tumor cells and platelets may assist in metastasis by producing a mixed platelet–tumor cell mass that supports adherence of tumor cells to the blood vessel wall and protects the tumor cells from destruction by the immune system.[16–20] In this study, we investigated the disposition of *vWF* in osteosarcoma through the analysis of messenger RNA (mRNA) and protein expression in tumor samples. Our results suggest that inappropriate expression of the *vWF* gene occurs during the metastatic spread of osteosarcoma.

Materials and methods

Tumor Samples

High-grade osteosarcoma tumor specimens were obtained at the time of surgical biopsy, resection of primary tumors and lung metastases. Following tumor removal, a specimen of viable tumor was chosen based on frozen section pathological examination. Histological diagnosis and grading were performed in the manner described by Dahlin.[21] Patients underwent chemotherapy between biopsy and resection with the exception of tumor samples 1007, 210, and 84, which received postresection chemotherapy.

RNA and DNA Extraction

Frozen tumors were crushed in a Brinkmann Retsch crusher. The RNA was extracted using the Trizol reagent method (GibcoBRL), DNase treated and quantitated by both spectrophotometric determination and quantitative reverse-transcriptase PCR (RT-PCR). In addition, RNA was extracted from five cell cultures: two osteosarcoma cell lines (KHOS, SAOS2), two breast carcinoma cell lines (MDA231, MCF7), and an osteoblast culture grown for two passages from collagenase-treated bone fragments. The osteoblast nature of these cells was confirmed by alkaline phosphatase activity and osteocalcin, CBFA1 and MSX2 gene expression.[22]

Differential Display

Differential display PCR (DDPCR) was performed essentially as described by Liang and Pardee[23] and Bauer *et al*[24] except that the PCR was performed using a range of cycles (33–36 cycles) to prevent saturation of the PCR products. A total of 27 specimens were examined using 19 DDPCR primers pairs: five progression sets (12 samples) each consisting of two or more samples from matched primary and metastatic tumor specimens from the same patients, three high-grade tumors that did not metastasize, six metastatic samples, one osteoblast culture and five cell lines (HOS, KHOS, MNNG, MG63, SAOS2). We previously showed that *CDK4* has a wide range of gene expression in osteosarcoma and therefore used *CDK4* to optimize the DDPCR conditions and confirm our ability to detect differentially expressed genes in osteosarcoma tumor samples (DDPCR primers for *CDK4*: T_{12}CC, 5′ CCTGAGATGG).[9] Bands representing potentially differentially expressed genes were excised from the DDPCR gels and reamplified before being directly sequenced using the Amersham Thermosequenase kit. Bands from DDPCR gels were chosen for analysis based on: (i) the range of expression of a band (based upon band intensity among samples), (ii) the presence of the band in multiple samples and (iii) the consistency of the expression pattern among the different tumor stages and grades. The DDPCR primers that led to identification of the *vWF* band were T_{12}GC and 5′ CCTGAGATGG.

vWF RT-PCR Quantitation

Total cellular RNA from 39 high-grade tumor samples and five cell lines (MCF7—breast, MDA231—breast, KHOS—osteosarcoma, SAOS2—osteosarcoma and a short-term primary osteoblast culture) was reversed transcribed into cDNA by reverse transcriptase (MMLVRT) and amplified for *vWF* and an internal control gene asparagine synthetase (*AS*). The cDNA was amplified using the following PCR primers: AS1 5′ACATTGAA GCACTCCGCGAC, AS4 5′CCTGAGGTTGTTCTTCA CAG, vWF F1 5′TAAGTCTGAAGTAGAGGTGG, and vWF R1 5′AGAGCAGCAGGAGCACTGGT primers. The *vWF* and *AS* RNA-specific primers were chosen to amplify a region containing at least one intron to prevent genomic DNA amplification. The *vWF* primers were chosen to avoid amplification of the *vWF* pseudogene.[25] A range of PCR cycles was examined for each sample to ensure that the reaction was in the exponential phase of amplification when the amount of product corresponds to the amount of the initial template.[9,26] The products were run on 12% polyacrylamide gels, stained with ethidium bromide and photographed for quantitative densitometry using a Molecular Dynamics densitometer. The ratio of *vWF* to *AS* was normalized against a

control sample (#511) to control for variations between PCR reactions.

Immunohistochemistry and Vessel Density Count

Formalin-fixed paraffin-embedded tissue sections were stained with anti-vWF antibody and counterstained with hematoxylin. Positive cytoplasmic tumor cell staining for vWF was assessed at low ($\times 40$) and high ($\times 400$) power. A vascular 'hot spot' was selected at low power and vascular density (VD) was determined using a Chalkley grid (0.18 mm^2 field size, $\times 250$ magnification) for vessel counting. A spot was considered positive if the grid marked a vWF positive endothelial cell or a vascular space lined by vWF-positive endothelial cells.

Immunofluorescence

SAOS2 osteosarcoma cells were plated on poly-D-lysine-coated coverslips (Becton Dickinson), washed $2\times$ with PBS, fixed for 10 minutes in 2% paraformaldehyde, washed $2\times$ with PBS, permeablized with 0.2% Triton for 5 min and washed $4\times$ with PBS. The coverslips were incubated with anti-vWF (factor VIII-related antigen/vWF Ab-1, Oncogene Research Products) in 3% BSA in PBS for 60 min, washed $2\times$ in PBS, incubated with 2 μg/ml goat anti-rabbit IgG Texas red conjugate (Molecular Probes) for 30 min, and washed $4\times$ in PBS. The coverslips were mounted with mounting media containing DAPI (Oncogene Research Products) and analyzed with a deconvolution microscope (Olympus 1X70).

Results

Detection of Differential *vWF* mRNA Expression in Osteosarcoma

Genes differentially expressed between primary and metastatic samples were identified by examining 21 high-grade primary and metastatic osteosarcoma tumor samples and six cell lines with DDPCR (Figure 1). For five of the metastatic samples, one or more matched primary samples from the same patient were included. In all, 11 primary samples and 11 metastatic samples were examined. Bands representing housekeeping genes were uniformly present in all samples. Other DDPCR products, however, were of variable intensity and represented genes with differing expression among samples. These were excised from the DDPCR gels, reamplified and sequenced. One of these bands represented *vWF* and indicated *vWF* was more highly expressed in metastases than primary tumors (Figure 1).

Quantitative *vWF* Gene Expression in Osteosarcoma Tumor Samples

Quantitative RT-PCR was used to analyze *vWF* mRNA expression in 39 high-grade tumor samples from 25 patients and from five cell lines (Figure 2a). These included the 22 high-grade DDPCR samples and 17 additional samples, with 10 sets of matched primary and metastatic tumor samples from the same patients. In the 39 samples, *vWF* was expressed at higher levels in metastases compared to primary tumors (mean 6.2 and 1.9, respectively; $P=0.006$, *t*-test). As shown in Figure 2b, this was particularly evident in the 10 patients with matched specimens where *vWF* levels were significantly higher in metastasis in nine of the 10 primary-metastasis tumor pairs (mean 8.3 in metastases and 2.3 in primary tumors; $P=0.005$, paired *t*-test). *vWF* was not detected in most cell lines (HOS, KHOS, MCF-7) or in an osteoblast culture, but low-level expression was identified in the osteosarcoma cell line SAOS2 (relative level of 0.3).

To determine whether the level of expression in the primary tumor was related to clinical outcome, *vWF* mRNA levels of tumors from patients with and without relapse were compared. *vWF* mRNA levels were considered high or low based on the median relative level of 1.0 (range 0.3–6.9) for primary osteosarcoma tumor samples. There was no significant difference in the proportion of patients alive without systemic relapse with low levels of *vWF* expression in the primary tumor (5/11) compared to those (5/10) with high *vWF* mRNA levels ($P=1.0$, Fisher's exact test).

Immunohistochemistry of vWF: VD

vWF is a commonly used marker for vascular endothelial cells in tumor samples. One possible explanation for the higher vWF expression in metastases was that there is a greater amount of vascularity in metastatic osteosarcoma. This was examined by determining VD in 29 tumor samples from 19 patients (Figure 3a, b). A wide range of VD was observed, with no difference between primary and metastatic samples (mean 4 for both groups). There was no association between the VD (value of ≥ 4 was considered high) in primary samples and patient outcome ($P=0.62$, Fisher's exact test) (Table 1). In addition, there was no correlation between VD and *vWF* mRNA expression ($P=0.45$, Fisher's exact test), suggesting that another source, possibly the osteosarcoma cells themselves, may account for the difference in mRNA expression between primary and metastatic samples.

Immunohistochemistry of vWF: Osteosarcoma Cells

The unexpected finding of *vWF* mRNA expression by RT-PCR in the osteosarcoma cell line SAOS2 indicated that it may produce vWF protein. This was confirmed by immunofluorescence (Figure 3c, d). vWF production by SAOS2 also suggested that osteosarcoma tumor cells, in addition to the endothelial cells present in the tumor samples, may

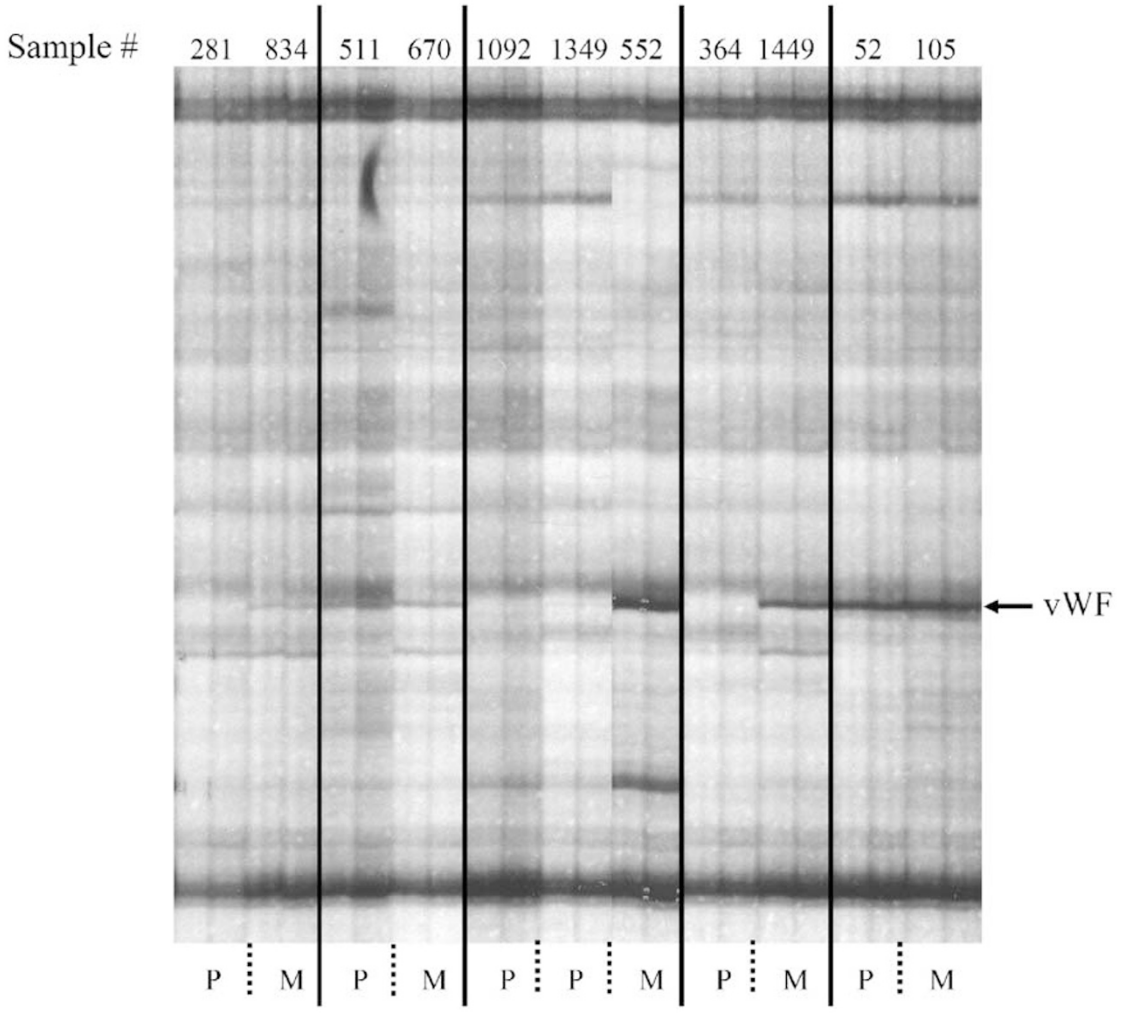

Figure 1 DDPCR of osteosarcoma tumor samples. A section of a DDPCR gel including matched primary and metastatic tumor samples. 'P' refers to primary tumor biopsy or resection samples while 'M' indicates metastatic osteosarcoma. Tumor samples from different patients are separated by a solid line. The DDPCR band identified as vWF is indicated by an arrow.

produce vWF protein. In total, 29 osteosarcoma tumor samples were examined for vWF protein expression using immunohistochemistry (IHC), and 13 samples from nine cases displayed positive vWF staining in the tumor cells themselves (Table 1, Figure 3e). In these specimens, less than 10% of the osteosarcoma cells were positive for vWF by IHC with some cases having only occasional cells that were considered positive (<1%). There was no association between positive vWF tumor cell staining and high VD ($P=1.0$, Fisher's exact test). However, *vWF* mRNA expression and vWF tumor cell staining did exhibit a significant correlation: samples expressing a high level of *vWF* mRNA were more likely to have positive vWF tumor cell staining (12/18) than samples with low *vWF* expression (1/11; $P=0.006$, Fisher's exact test). The proportion of patients alive without systemic relapse who had no primary tumor cells with detectable vWF by IHC (6/12) was higher than those with vWF immunopositive sarcoma cells in the primary tumor (2/7), although this difference was not significant ($P=0.63$, Fisher's exact test).

Discussion

In this study using DDPCR, we identified *vWF* as a gene that is differentially expressed in metastatic osteosarcoma samples compared to primary tumor samples. Furthermore, we found that in some cases osteosarcoma tumor cells themselves produced vWF. This was unexpected, given that vWF was previously thought to be expressed exclusively by endothelial cells and megakaryocytes.[11,14]

The DDPCR examination of primary and metastatic tumor specimens was successful in isolating differentially expressed genes. Perou *et al*[27] showed that paired breast primary and metastatic tumor samples are more similar to each other than to other

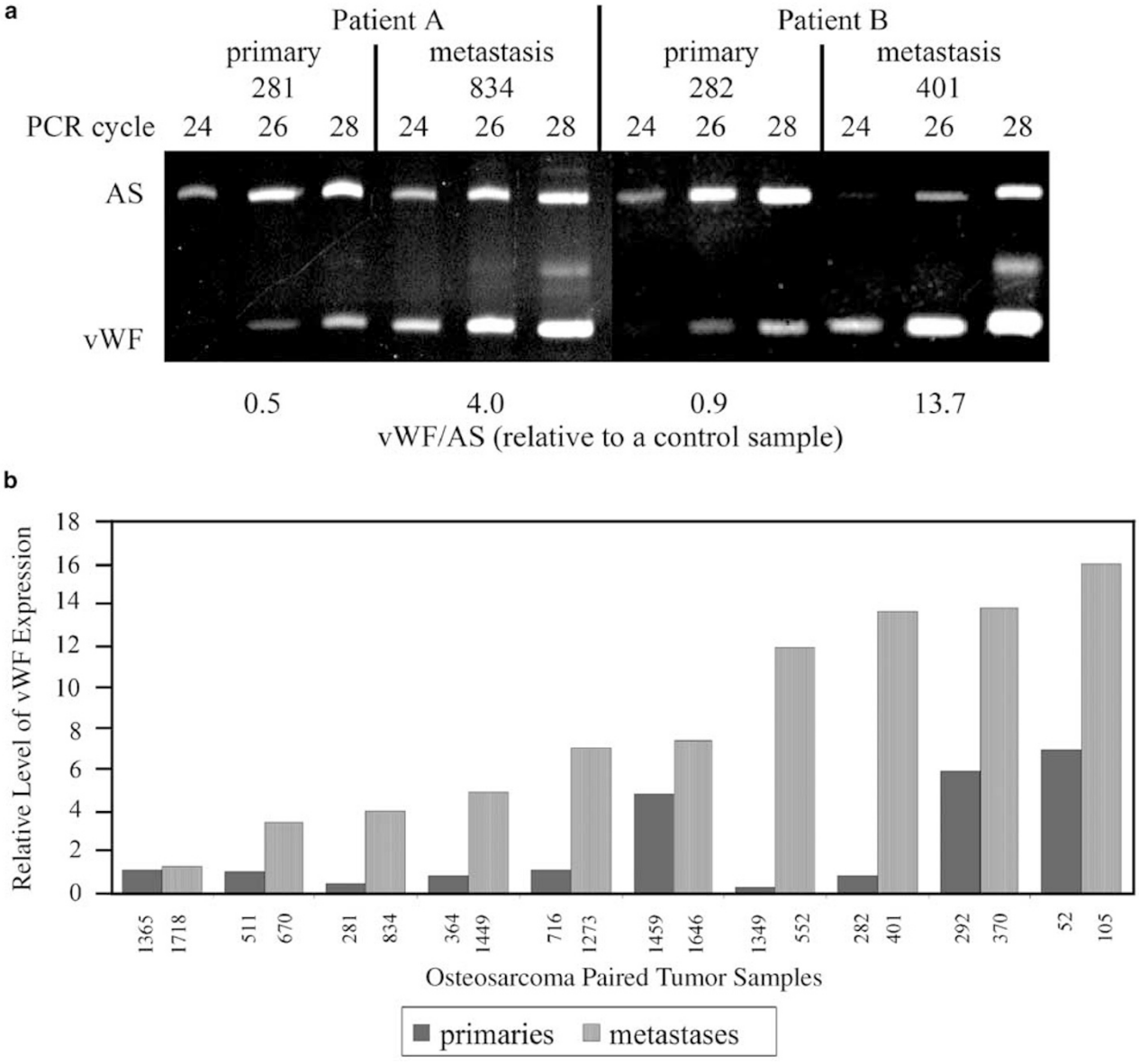

Figure 2 *vWF* mRNA expression in osteosarcoma tumor samples. (**a**) Quantitative RT-PCR analysis of *vWF* mRNA expression in human osteosarcomas. *AS* was used as an internal control gene. The relative level of *vWF* expression was normalized against tumor sample 511 as described in Materials and methods. Upper numbers indicate PCR cycles. (**b**) *vWF* mRNA expression levels in paired primary and metastatic osteosarcoma samples from 10 patients. The relative mRNA levels were determined by quantitative RT-PCR (see Materials and methods, and panel a). *vWF* expression was significantly higher in metastatic samples compared to primary samples ($P = 0.005$).

tumor samples, suggesting many of the random molecular alterations in tumors already exist in the primary tumor. The situation is likely similar in osteosarcoma. Therefore, the use of matched primary and metastatic samples from the same patient assists in the detection of metastasis-specific or primary tumor-specific genes by minimizing the background of differentially expressed genes. In this study, we used five such paired tumor sample sets for the DDPCR analysis and an additional five for the subsequent RT-PCR analysis, in addition to unrelated primary and metastatic samples. The same differential expression pattern for *vWF* observed by DDPCR was identified by quantitative RT-PCR in a larger set of tumor samples, confirming the efficacy of DDPCR analysis of sarcoma progression samples.

IHC of vWF in osteosarcoma samples was performed for two reasons: to investigate the VD of the tumors and to examine the production of vWF protein by osteosarcoma cells. The expression of *vWF* by vascular endothelial cells raised the possibility that the increase in expression as tumors metastasize might result from an increase in VD. Numerous studies have indicated that VD may be of

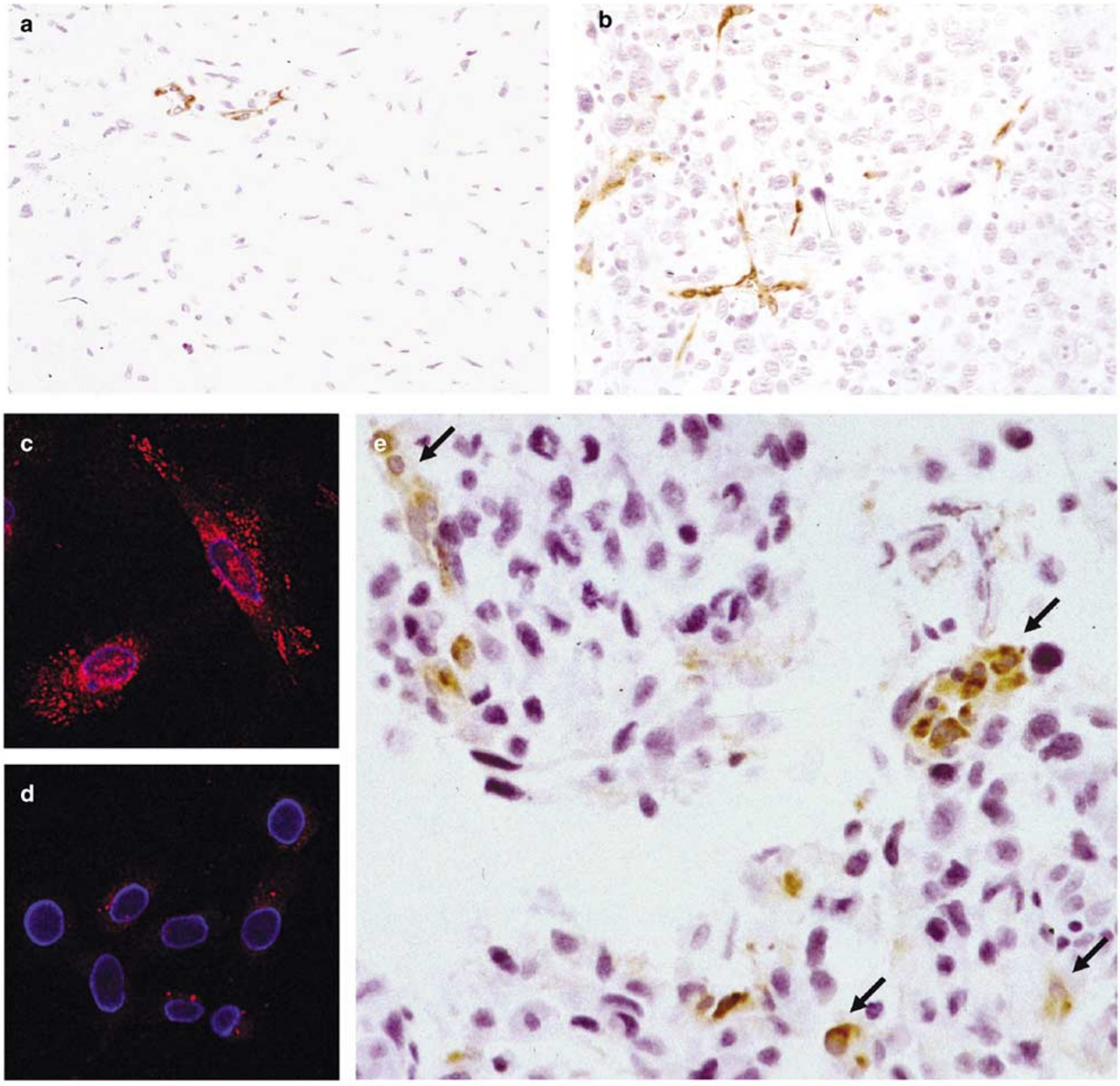

Figure 3 vWF protein and VD in osteosarcoma tumor samples and cell lines. (**a, b**) VD in high-grade osteosarcoma. Cytoplasmic vWF staining is present in endothelial cells with (**a**) showing low VD and (**b**) showing high VD (immunoperoxidase, counterstained with hematoxylin, magnification $\times 250$). (**c, d**) vWF expression in osteosarcoma cells as determined by immunofluorescent detection. Nuclei are blue and vWF protein is red. vWF protein is produced by SAOS2 cells (**c**) and not HOS cells (**d**). (**e**) Histological sections of osteosarcoma stained for vWF. High-grade osteosarcoma showing positive cytoplasmic staining for vWF in both tumor cells and endothelial cells (immunoperoxidase, counterstained with hematoxylin, magnification $\times 400$). Arrows denote osteosarcoma cells with positive vWF staining.

prognostic significance in carcinomas, with a high VD indicating a worse prognosis in numerous different tumor types (reviewed in Hlatky *et al*[28]). Unlike carcinomas, the situation is less clear for sarcomas where VD may not correlate with outcome.[29–32] However, our preliminary examination of VD in 29 osteosarcoma samples neither revealed any correlation with tumor progression (primary *vs* metastasis) nor with *vWF* expression. The lack of correlation between VD and *vWF* expression is not surprising given that *vWF* expression is affected by numerous factors including vessel type and size, tissue microenvironment, and angiogenic factors.[33–35] Kohlberger *et al*[36] showed a lack of correlation between VD and percentage of vWF-stained areas in breast carcinomas, while Zanetta *et al*[33] showed no correlation between VD and *vWF* mRNA expression in colon carcinoma.

Table 1 Clinical features of osteosarcoma cases

Case	*Primary tumor number*	*Patient outcome*[a]	*VWF tumor cell staining*[b]	*Vascular density*[c]	*Primary tumor size (cm)*	*Chemotherapy-induced tumor necrosis*[d] *(%)*
1	281	DOD	−	1	6	90
2	511	DOD	−	2	10	80
3	1349	DOD	+	4	3	60
4	52	DOD	+	3	12	50
5	716	DOD	+	5	6	80
6	282	DOD	+[e]	N/A[e]	10	70
7	292	DOD	+[e]	N/A[e]	13	70
8	1365	DOD	+	1	10	50
9	1459	DOD	+	2	10	15
10	138	DOD	−	6	12	35
11	662	AWED	−	3	9	90
12	1007	ANED	+	6	10	N/A
13	210	ANED	−	4	8	N/A
14	84	ANED	−	3	8	N/A
15	390	ANED	−	1	14	75
16	600	ANED	−	5	7	100
17	1546	ANED	−	4	11	90
18	403	ANED	−	12	17	90
19	1112	ANED	+	2	5	5

[a]ANED = alive without evidence of disease; AWED = alive with evidence of disease; DOD = dead of disease.

[b]vWF tumor cell staining in primary and/or metastatic sample.

[c]'Vascular Density' refers to the maximal density of vessels within the primary tumor.

[d]Patients received preoperative chemotherapy between biopsy and resection of the primary tumor, with the exception of cases 12, 13 and 14 that received chemotherapy following tumor resection (assessment of chemotherapy-induced necrosis was not possible).

[e]Positive vWF tumor cell staining in metastatic specimen. Primary specimen not available for determination of tumor cell staining or VD.

IHC was also performed on osteosarcoma samples in order to determine if sarcoma cells produce vWF, as suggested by *vWF* mRNA expression in the SAOS2 cell line. Surprisingly, 13 tumor samples were positive for vWF in osteosarcoma cells. Fewer than 10% of the tumor cells were implicated in these samples. These IHC-positive tumor cells may represent the highest expressing cells, while the remainder produce a lower level of *vWF* mRNA not detectable by this technique. vWF is commonly used for highlighting endothelial cells when quantitating tumor VD.[15] However, identification of vWF-producing osteosarcoma cells raises the possibility that other endothelial markers such as CD31 or CD34 may be more appropriate, especially for automated methods used to assess VD in tumors.[15,36–40]

Osteosarcomas with positive tumor cell staining tend to have high *vWF* mRNA expression ($P = 0.006$), indicating that the osteosarcoma cells may contribute a significant proportion of the *vWF* mRNA expression seen in tumors. Furthermore, patients with paired tumor samples displayed higher *vWF* expression in the metastatic samples ($P = 0.005$), suggesting that the metastatic process may select for tumor cells that express *vWF* more highly. Metastatic tumors result from the successful spread of tumor cells from the site of the primary tumor. Current models of the metastatic process indicate that tumor progression results from the clonal expansion of cells with acquired mutations that provide a growth advantage and that, eventually, cells harboring mutations that facilitate metastasis can exist in the primary tumor.[41] Therefore, in some cases, the majority of the tumor cells in the primary tumor can exhibit alterations that allow for metastasis.[41,42] Additionally, there can be a small number of cells in the primary tumor that have acquired further alterations facilitating metastasis and these are the cells that eventually successfully metastasize.[42,43] The ability of osteosarcoma cells to produce vWF themselves could provide a metastatic advantage, such that tumor cells in the metastasis may be derived from the metastatic clonal expansion of rare *vWF*-expressing cells (or those cells that express *vWF* more highly) from the primary lesion resulting in a population of cells that express a high level of *vWF* in the metastases.

vWF has been shown to be involved in platelet aggregation and adhesion, both of which are involved in hematogenous tumor cell metastasis.[16–18] Pretreatment of either tumor cells or platelets with an antibody or peptide that neutralizes vWF or blocks vWF-capable receptors (eg integrins GPIIb/IIIa and GPIb—present on numerous tumor cell lines) has been shown to inhibit tumor cell–platelet interaction *in vitro* for colon carcinoma, Walker 256 carcinosarcoma, melanoma and osteosarcoma cell lines (including SAOS2).[19,44–52] Notably, treatment with monoclonal anti-vWF antibody significantly decreased tumor cell metastases *in vivo* for colon, Lewis bladder and melanoma carcinoma cell

lines.[19,50] Gasic *et al*[18] found that tumors capable of forming platelet aggregates usually metastasize to the lung, the first subvasculature a metastatic cell would encounter in the bloodstream, while those lacking this ability have a more widespread pattern of metastasis in mice. Mehta *et al*[53] suggested that this tumor cell–platelet mechanism may be partially responsible for the common metastasis of osteosarcoma to the lung. In support of this, the osteosarcoma cell lines MG63, HOS, U2-OS, TE-85 and SAOS2 have been shown to induce platelet aggregation.[46,53–55] The vWF produced by SAOS2 may contribute to its ability to aggregate platelets. vWF expression by osteosarcoma tumor cells may contribute to tumor cell–platelet aggregation as well as tumor–subendothelium adhesion, increasing the likelihood of successful blood-borne metastasis to the lung.

The mRNA expression of *vWF* was neither statistically different in primary samples from tumors that eventually metastasized compared to those that did not ($P = 1.0$) nor was there a correlation between positive tumor cell staining and outcome ($P = 0.63$). This suggests that while *vWF* expression may be important for the metastatic process, *vWF* mRNA expression in primary tumors may not be of prognostic significance. In fact, there are no strong predictors of outcome for individual patients with osteosarcoma.[26,56] For patients with high-grade osteosarcoma who present without metastases at the time of diagnosis and undergo curative treatment, the best predictors for the subsequent development of systemic disease are the size of the primary tumor at diagnosis and the percent necrosis in the primary tumor following preoperative chemotherapy. In this study, tumor size and measures of chemotherapy induced-necrosis did not correlate with the development of metastases ($P = 0.66$ and 0.27, respectively) indicating that a greater sample size may be required to detect a correlation between *vWF* expression and patient outcome or vWF tumor cell staining and patient outcome.

In summary, an analysis of differential gene expression in various stages of osteosarcoma revealed that *vWF* expression increases during tumor progression from primary to metastatic osteosarcoma. The osteosarcoma tumor cells were unexpectedly found to be capable of producing vWF, demonstrating that *vWF* expression may be deregulated in some osteosarcomas. In addition, this suggests that care must be taken when using vWF as a marker for endothelial cells when assessing VD in osteosarcoma.

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