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Correlation between vascular endothelial growth factor (VEGF) expression with histopathological findings in osteosarcoma

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ABSTRACT

Vascular endothelial growth factor (VEGF) expression is associated with malignancy progression, metastasis, and poor prognosis in many malignancies, including osteosarcoma. However, studies concerning correlations between VEGF expression and histopathological prognostic factors of osteosarcoma are limited. This study aimed to evaluate the correlations between VEGF expression and histopathological findings in osteosarcoma's patients. This was a cross-sectional study using formalin-fixed paraffin embedded (FFPE) samples of 32 osteosarcoma's patients from Dr. Sardjito General Hospital, Yogyakarta. Histopathological findings of specimens were re-evaluated by two independent observers, recorded for the subtypes, invasiveness, grading, mitotic counts, and tumor infiltrating lymphocytes (TIL). Expression of VEGF was determined based on immunostaining and evaluated using immunoreactivity score (IRS). Chi-square and Spearman correlation test were used to analyze the association between variables. Range of VEGF expression score was 0 to 11, with mean 5.09. Significant negative correlation between the VEGF expression and TIL was observed (p=0.046). However, there was no significant correlations between the VEGF expression and osteosarcomas subtypes, invasion, grading or mitotic counts (p> 0.05). In conclusion, the VEGF expression is associated with TIL. Further study is needed to evaluate the roles of VEGF and lymphocytes in osteosarcoma development dan progression in order to better understand of the role of VEGF in immunotherapy of osteosarcoma.

ABSTRAK

Ekspresi *vascular endothelial growth factor* (VEGF) dikaitkan dengan progresivitas, metastasis, dan prognosis yang buruk pada keganasan, termasuk osteosarkoma. Namun demikian, penelitian tentang hubungan antara ekspresi VEGF dengan faktor prognosis histopatologi pada osteosarkoma belum banyak dilakukan. Penelitian ini bertujuan mengkaji hubungan antara ekspresi VEGF dengan gambaran histopatologi pada osteosarkoma. Penelitian ini merupakan penelitian potong lintang menggunakan sampel *formalin-fixed parafin embedded* (FFPE) dari 32 kasus osteosarkoma di Rumah Sakit Umum Pusat Dr. Sardjito, Yogyakarta. Gambaran histopatologi berupa sub tipe, invasi limfovaskular, grading, jumlah mitosis, dan *tumor infiltrating lymphocytes* (TIL) dievaluasi oleh dua penilai independen. Ekspresi protein VEGF ditentukan berdasarkan pengecatan imunohistokimia dan dievaluasi dengan *immunoreactive score* (IRS). Uji korelasi Spearman dan uji Chi-square digunakan untuk menganalisa hubungan antar variabel. Skor ekspresi VEGF bervariasi dari 0-11, dengan skor rata-rata 5,09. Terdapat hubungan signifikan secara negative antara ekspresi VEGF terhadap TIL (p=0,046; r: -0,355). Namun demikian, tidak ada hubungan signifikan antara ekspresi VEGF terhadap sub tipe, invasi limfovaskular, grading atau jumlah mitosis (p>0,05). Dapat disimpulkan, ekspresi VEGF berhubungan dengan TIL. Penelitian lebih lanjut diperlukan untuk mengkaji peran VEGF dan limfosit dalam progresivitas dan perkembangan osteosarkoma untuk lebih memahami peran VEGF dalam imunoterapi osteosarkoma.

Keywords:
histopathological findings;
osteosarcoma;
VEGF;
TIL;
malignancies;

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INTRODUCTION

Osteosarcoma is the most frequent primary bone malignancy in adolescents and young adults, and is derived from bone-forming mesenchymal cells. Although it is not a frequent type of tumor, it has a devastating effect on patients. Survival has not improved significantly over the past three decades, and osteosarcoma with metastasis is usually incurable and requires palliation.[1] Surgery and chemotherapy are the current standard treatments for osteosarcoma. Despite multimodal therapies, over 40% of patients fail to be treated within 5 years of diagnosis, generally due to distant spread of tumor.[2]

New vascular formation is essential in the growth and spreading of the tumor. The most important protein involved in tumor angiogenesis is vascular endothelial growth factor (VEGF) because it acts by increasing all steps in angiogenesis.[3] The VEGF expression is associated with cancer progression, metastasis, and worse prognosis in many tumors. Overexpression of the VEGF is significantly correlated with tumor growth and prognosis of cancer patients.[3] Enzymes of VEGF stimulate tumor growth and metastatic processes by its interactions with metalloproteinase.[4] In contrast, VEGF inhibition leads to suppression of cancer proliferation, stimulating apoptosis, reducing angiogenesis[5] and decreasing vascular density in malignant tumors while increasing the efficacy of adoptive T-cell therapy in certain cancers.[6]

Some histopathologic features of osteosarcoma have served as prognostic factors, including tumor subtype, nuclear pleomorphism, mitotic rate, lymph-vascular invasion, tumor fibrosis, and tumor viability after chemotherapy.[7] Tumor infiltrating lymphocytes (TIL) are also an important prognostic factor in many tumors and the presence of TIL in sarcoma positively correlates with a good prognosis.[8] Several studies regarding VEGF expression and the prognosis of osteosarcoma patients have been performed previously, however, there are contradictive results among studies.[9] Thus, there were various results among studies investigating association of VEGF expression with survival and other prognostic factors in osteosarcoma.

This study analyzed the correlation of VEGF expression with histopathological findings in osteosarcoma patients, including histopathologic subtype, grading, lymph vascular invasion, mitotic count, and TIL. To the best of our knowledge, this study is the first to correlate VEGF expression with TIL, mitotic count, and lymph vascular invasion in osteosarcoma obtained from human tissue. This study aimed to evaluate correlations between VEGF expression and histopathological findings in osteosarcoma to achieve better understanding of its pathogenesis and behavior which can lead to better clinical management.

MATERIALS AND METHODS

Subject recruitment

This cross sectional study was conducted at Dr. Sardjito General Hospital, Yogyakarta. This study has been approved by the Medical and Health Research Ethics Committee at the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta (Ref: KE/FK/1107/EC/2018). The subjects of this study were 32 patients who were diagnosed with osteosarcoma based on clinical, radiological, histopathological examinations and confirmed by osteocalcin immunostaining. Samples were recruited by total sampling technique, using formalin-fixed paraffin embedded (FFPE) tissue blocks archived from between 2012 to 2018. Data were analyzed based on the histopathological and immunohistochemistry (IHC) examinations performed at the Laboratory of Anatomic Pathology, Dr. Sardjito General Hospital, Yogyakarta.

Histopathological finding assessment

The slides were reviewed by two independent observers with standard hematoxylin-eosin (HE) staining to identify histopathologic subtypes, grading, mitotic count, lymph vascular invasion, and TIL. Kappa value of inter-observer agreement was measured. Discordancy, if any, were reconciled through group discussion. Histopathological subtypes and grade were divided based on the World Health Organization (WHO) Classification of Tumors of Soft Tissue and Bone 2013.[10] Mitotic count and lymph vascular invasion were analyzed as dichotomous variables. Mitotic count was divided into low mitotic count (<20/10 high power field (HPF)) and high mitotic count (≥20/10 HPF).[6] Tumor infiltrating lymphocytes in this study was defined as the percentage of peritumoral stromal area occupied by mononuclear cells including lymphocytes and plasma cells (areas of necrosis and fibrosis were not included; granulocytes and polymorphonuclear leukocytes were excluded), and divided into minimal (0-10%), intermediate (10-40%), and high (more than 40%).[11] The categoric scale of the data was used in the statistical analysis.

IHC staining assessment

The VEGF expression was evaluated based on immunostaining technique. FFPE samples were sliced 3um, and then incubated with primary antibody against VEGF (1:100 dilution; Finetest, Fine Biotech). Subsequently, the sections were incubated with Ultra Tek Anti-Polyvalent, followed by Ultra Tek HRP, which formed a complex. Diaminobenzidine was used as the final chromogen, and hematoxylin was used as the nuclear counterstain. The VEGF expressions were evaluated based on staining intensity and percentage of positive cells using the immuno reactivity score (IRS) scoring as described by Fedchenko study.[12] Positive staining was shown in cell membranes and cytoplasm. Score of positive cells percentage was multiplied by score of staining intensity, then divided in categoric scale into: negative, mild positive, moderately positive, or strongly positive (TABLE 1).

TABLE 1. IRS score for assessing VEGF expression

A (percentage of positive cells)	B (intensity of staining)	IRS score (multiplication of A and B)
0 = no positive cells	0 = no color reaction	0-1= negative
1= <10% positive cells	1 = mild reaction	2-3 = mild positive
2 = 10-50% positive cells	2 = moderate reaction	4-8 = moderately positive
3 = 51-80% positive cells	3 = intense reaction	9-12 = strongly positive

Statistical analysis

Statistical analysis was performed using SPSS Statistics version 25 (IBM Corp., Chicago). Kappa value was used to measure agreement between two independent observers of histopathological findings in osteosarcoma. Correlation between VEGF expression and histopathological findings were analyzed statistically using Spearman correlation test if both variables were ordinal, otherwise Chi-square test was used to analyze association between nominal and ordinal variables. A p-value of 0.05 or less was considered as statistically significant.

RESULTS

Tumor subtype

Among 32 FFPE samples evaluated, 25 samples (78%) were conventional osteosarcoma, including 10 samples (31.3%) of chondroblastic variant, 7

samples (21.9%) of osteoblastic variant, while 7 samples (21.9%) were fibroblastic variant, 4 samples (12.5%) were giant cell rich variant, and 1 sample (3.1%) was epithelioid variant. Other subtypes were observed including parosteal, periosteal, and telangiectatic variant, with 1 sample each (FIGURE 1).

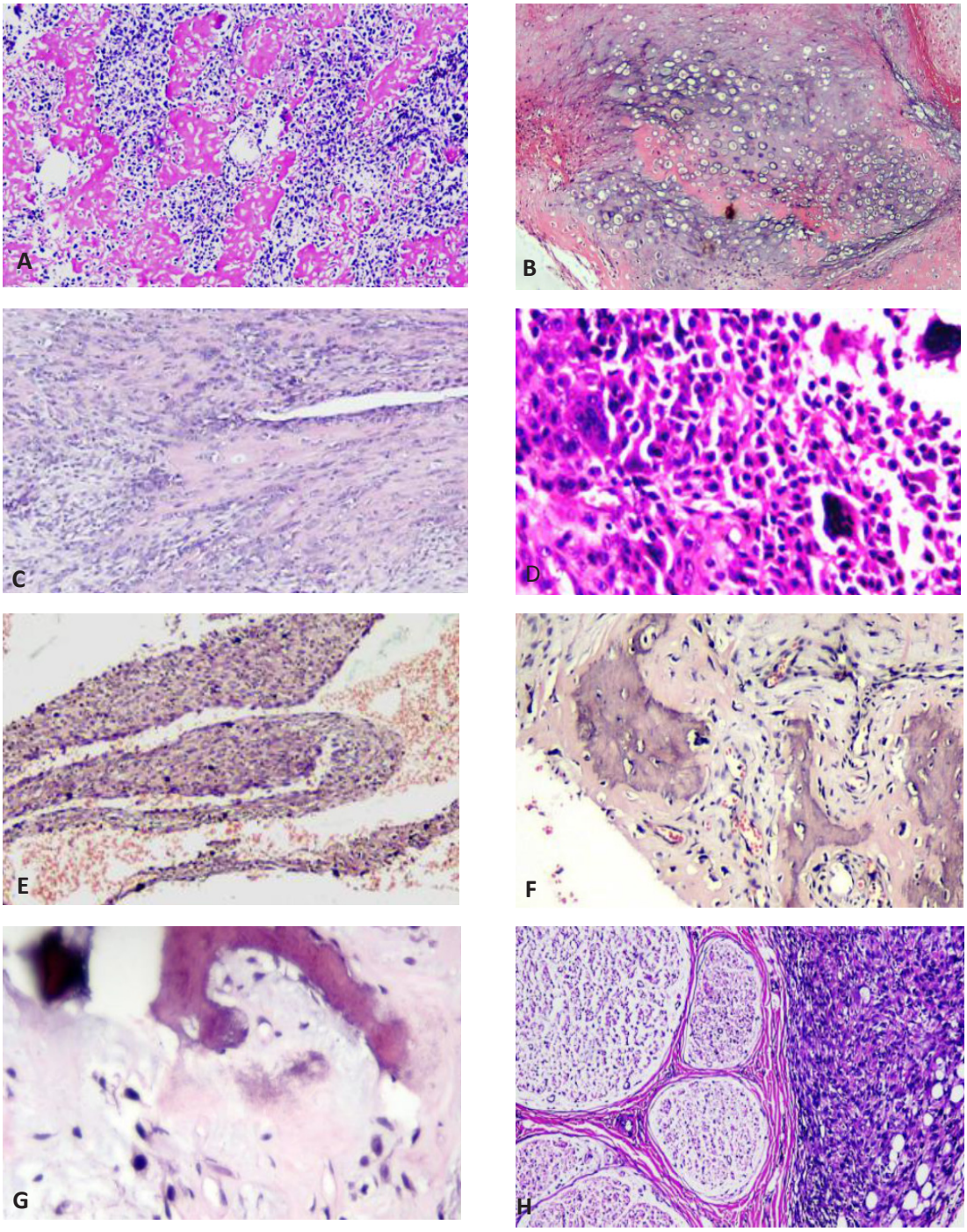

FIGURE 1. Osteosarcoma variant found in this study: (A). Osteoblastic, with dominance of osteoid matrix. (B). Chondroblastic, with a lot of chondroid matrix. (C). Fibroblastic, consist of spindle cell tumor. (D). Giant cell rich, with a lot of osteoclastic type giant cells. (E). Telangiectatic type, with dilated vascular space. (F). Parosteal, low grade osteosarcoma with mild polymorphism of tumor cells. (G). Periosteal, intermediate grade osteosarcoma with moderate polymorphism of tumor cells. (H). Epithelioid, high grade osteosarcoma with epithelioid morphology, in this case there is perineural invasion.

Histopathological finding and VEGF expression

Kappa value measurement between two independent observers of histopathological findings in osteosarcoma yielded the following results: subtype (0.843), grade (1.000), invasion (0.776), mitotic count (0.834), and TIL (0.824). Invasion to blood or lymph vessels was found in 23 samples (71.9%). Nine samples were without blood or lymph vessels invasion. High grade tumors were found in 26 (81.3%) samples, moderate grade in 4 (12.5%) samples, and low grade in 2 (6.3%) samples. Among all samples, mitotic count was analyzed and ranged from 1/10 high power field (HPF) to 40/10 HPF. There were 25 samples (78.1%) with low mitotic counts (<20/10HPF) and 7 samples (21.9%) with high mitotic counts. As for TIL, 26 samples (81.3%) showed minimal TIL, 5 samples (15.6%) were with intermediate TIL, and 1 sample (3.1%) had high TIL (TABLE 2, FIGURE 2).

TABLE 2. Histopathological findings in osteosarcoma

Tumor invasion		Mitotic count		TIL		
With invasion	Without invasion	Low (<20/10HPF)	High (≥20/10 HPF)	Minimal	Intermediate	High
23 (71.9%)	9 (28.1%)	25 (78.1%)	7 (21.9%)	26(78.1%)	5 (15.6%)	1 (3.1%)

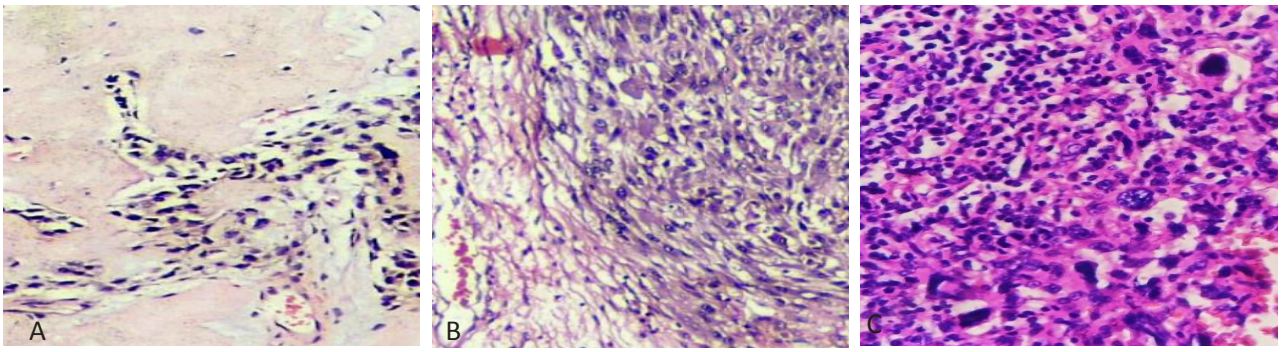

FIGURE 2. Tumor Infiltrating Lymphocytes (TIL) in osteosarcoma (A). Mild TIL with lymphocyte infiltration 0-10%, (B). Intermediate TIL with lymphocyte infiltration 10-40%, (C). High TIL with lymphocyte infiltration more than 40%

Vascular endothelial growth factor expressions were assessed using IRS score. The VEGF expression in 32 samples showed IRS from 0 to 11 (maximum IRS score: 12), with mean 5.09. Four samples (12.5%) were negatively stained with anti-VEGF antibody, 10 samples (31.3%) were mildly positive, 13 samples (40.6%) were moderately positive, and 5 samples (15.6%) were strongly positive (FIGURE 3).

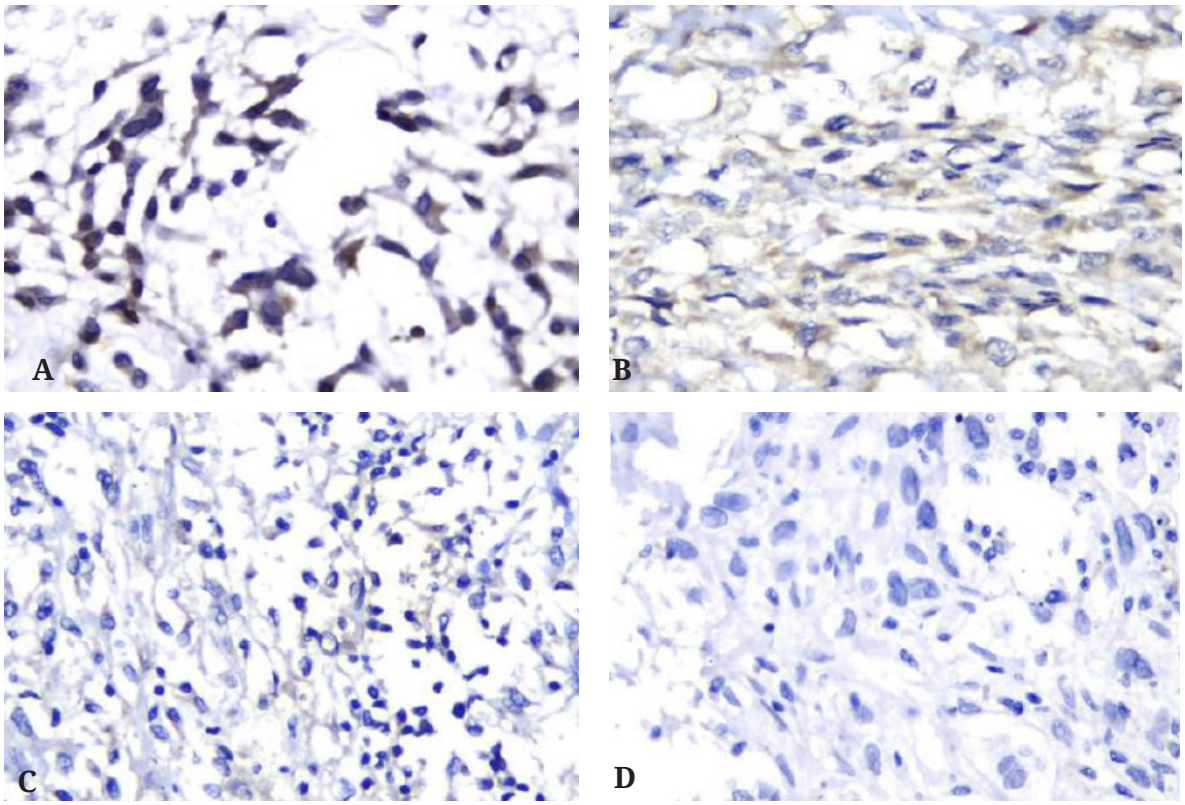

FIGURE 3. Representative images of immunohistochemistry staining in different level of VEGF expression. The degree of expression was determined by intensity of staining and percentage of positively stained cells (IRS score, as described in TABLE 1); (A) Strongly positive, (B) Moderately positive, (C) Mildly positive, (D) Negative. White bar: 200 μm.

Vascular endothelial growth factor expression and TIL showed statistically significant correlation (p=0.046), with correlation coefficient: -0.355, indicating negative correlation between VEGF and TIL. Thus, tumors with high expression of VEGF tended to have lower TIL. Analysis on histopathologic subtype, invasion, grading, and mitotic count showed no significant correlation with the VEGF expression (TABLE 3 and 4).

TABLE 3. Correlation between VEGF expression and histopathological findings on osteosarcoma's patients.

Histopathological finding	VEGF Expression				r	p
	Negative	Mild	Moderate	Strong		
Grade						
• Low	1	1	0	0		
• Moderate	0	0	1	0	0.042	0.819
• High	4	9	12	5		
Mitotic count						
• <20/10HPF	3	6	13	3	-0.085	0.480
• ≥20/10HPF	1	4	0	2		
TIL						
• Minimal	3	6	12	5		
• Intermediate	0	4	1	0	-0.355	0.046
• High	1	0	0	0		

TABLE 4. Association between VEGF expression and histopathological findings

Histopathologicalfinding	VEGF Expression				p
	Negative	Mild	Moderate	Strong	
Subtypes					
• Osteoblastic	0	3	3	1	
• Chondroblastic	1	4	3	2	
• Fibroblastic	2	1	3	1	
• Parosteal	0	1	0	0	0.808
• Periosteal	0	0	1	0	
• Teleangiectatic	0	0	0	1	
• Giant cell rich	0	1	3	0	
Lymph vascular invasion					
• Epithelioid	1	0	0	0	
• Negative (-)	1	3	3	2	0.812
• Positive (+)	3	7	10	3	

DISCUSSION

Based on histopathological type, conventional osteosarcoma comprised 78% of the tumors in this study. Based on further morphological observation on subtypes, the most common type of the conventional osteosarcoma was chondroblastic variant (31.3%), followed by osteoblastic and fibroblastic variants (each 21.9%), giant cell rich variant (12.5%) and epithelioid variant (3.1%). However, these findings are remarkably different with the report in the WHO Classification of Bone and Soft Tissue Tumorbook as it stated that osteoblastic variant is the most common subtype, followed by chondroblastic, fibroblastic, and other subtypes.[13] This difference can be attributed to different geographical location, race, and sample size.

Most of the osteosarcoma in our

study were comprised of highgrade tumors (93.8%), followed by moderate grade tumors and low grade tumors (each 3.1%). This finding was concordant with the previous reportthat showed high grade osteosarcoma asthe majority cases (92-93%), where as low and intermediate grade were only 7-8%.[10]

More advanced disease is represented by positive lymph vascular invasion (LVI) and our study found that majority of cases (71.9%) were with positive LVI. In developed countries, such as Canada[8] lymph vascular invasion was observed in only 27% of the cases. Different onsets at diagnosis period and patient behavior in seeking medical professional help may contribute to the various findings among those studies. In developing countries where there are more barriers for accessing health care, advanced stage is found more often compared to developed countries.[14]

Osteosarcomas with low mitotic rate (<20/10HPF) are the majority type in our study (78.1%). A study in Canada showed a similar resultas 86% of the osteosarcoma showing low mitotic rate.[5] Osteosarcoma with higher mitotic rate significantly has worse prognosis.[6] The majority of osteosarcoma cases in our study show minimal or intermediate TIL (81.3% and 15.6, respectively). Previous study also reported that the majority of osteosarcoma had low or no TIL.[17] Some neoplasms have a mechanism to promote tumor growth through cell contact or paracrine secretion of cytokines, growth factors, and nutrients. Some secreted cytokines may influence tumor-induced immunosuppression. During the tumor development, an inhibitory program for T-cell activation has been activated. Immunosuppressive mechanisms in the tumor microenvironment involve the suppressive action of regulatory T-cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and stromal fibroblasts. These cells suppress T-cell function by increasing expression of surface molecules that bind to the inhibitory receptors, such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), T cell transmembrane, immunoglobulin, mucin (TIM-3), lymphocyte-activation gene 3 (LAG-3), B and T lymphocyte associated (BTLA) protein,[7] resulting in low number of TIL in osteosarcoma, quantitatively and qualitatively.

Positive VEGF expressions were found in 87.5% of the samples. Most of them were moderately positive by the IRS scoring system. Many studies assessing VEGF expression in osteosarcomas revealed different results ranging from 20% to 93%. The thresholds of VEGF positivity were also different across many studies, ranging from 10% to 50% of tumor cells. The scoring systems used were also different. Some used only percentage of positive cells, while others used complex scoring combining intensity and percentage.[15] Until recently, there is no single widely accepted scoring system to assess VEGF expression in osteosarcomas. Vascular endothelial growth factor expression is shown to have significant negative correlation with TIL. Our finding shows that tumors with low TIL tend to have higher VEGF expression. Theoretically, high level of VEGF expression initiates endothelial cell proliferation, which leads to higher density but structurally and functionally abnormal vessels. These vessels have irregular, twisted shape, and are leaky. Although acute VEGF stimulation acts as up-regulator for adhesion molecules and thereby causing leukocyte infiltration, where as chronic VEGF exposure may inhibit interaction of endothelial and leukocytes by lowering expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin. VEGF suppress lymphocyte infiltration

through inhibition of C-X-C motif ligand 10 (CXCL10) and C-X-C motif ligand 11 (CXCL11), which are both chemokines involved in T-cell recruitment.[16] VEGF also decreases proinflammatory cytokines' responsiveness surrounding endothelial cells by repressing nuclear factor (NF)-κB pathways. Inhibition of VEGF, in contrast, as shown in many studies, stimulates the interaction of leukocytes and endothelial cells by increasing adhesion molecules expression. Sunitinib, a VEGF inhibitor, acts as an up-regulator for inflammatory genes *CXCL10* and *CXCL11* that leads to increased T-cell infiltration. Thus, VEGF acts as a barrier for leukocyte infiltration.[16]

The presence of high numbers of cytotoxic T lymphocytes in the tumors correlates with better outcomes. There is also positive correlation of TIL and programmed cell death ligand-1 (PDL-1) gene expression in osteosarcoma.[17] The ability of T-cells to recognize, localize, and kill tumor cells is very promising in cancer immunotherapy research. Vascular endothelial growth factor inhibits maturation and reduces number of dendritic cells. It also inhibits differentiation of progenitor cells into CD4/CD8+ve T cells, suppress proliferation and cytotoxic functions, and upregulates programmed cell death-1 (PD-1), CTLA-4, TIM3 and LAG3. Anti-VEGF, such as bevacizumab or sorafenib, may block immunosuppressive effects of angiogenic protein and enhance immune cell therapeutic effect on neoplasms. Its combination with immunotherapy drugs, such as nivolumab, pembrolizumab, atezolizumab, and avelumab, shows synergic effects in enhancing the activity of cytotoxic T-cells on tumor cells.[18] The finding regarding significant correlation between VEGF expression and TIL provides a valuable opportunity to explore the role of VEGF and lymphocytes in osteosarcoma development and progression which can lead to better understanding of VEGF role in osteosarcoma immunotherapy.

This study showed no significant association between VEGF expression with tumor subtypes, grade, lymph vascular invasion, and mitoticcounts. Similar previous study revealed that there was no association between VEGF expression and tumor grade in osteosarcoma.[19] However, other study showed significant associations between VEGF-C expression with histopathological subtypes and grading in osteosarcoma.[20] Previous in-vitro study in osteosarcoma cell lines showed that VEGF correlates with tumor growth as VEGFR inhibition resulted in the decreases of osteosarcoma proliferation and invasion.[21] Different result in some studies can be attributed to different microenvironments between in-vitro cell lines and human tissue. Various quantification techniques of VEGF expression may also contribute to different results among studies.

CONCLUSION

This study shows significant correlation between VEGF expression and TIL, however, its correlation with tumor subtypes, grading, and mitoticcountin osteosarcoma patientsis not observed. Correlation between TIL and VEGF expression infers that VEGF has immunosuppressive effect in the osteosarcoma microenvironment through inhibition of lymphocyte infiltration in the peritumoral area. Further study is needed to investigate the effect of anti-VEGF on alteration of T-cells cytotoxic activity in osteosarcoma cells.

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