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Original Article

Vitronectin significantly influences prognosis in osteosarcoma

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Abstract: Vitronectin (Vn), a multifunctional adhesive protein, is found in association with tumor progression, angiogenesis and metastasis in a variety of (human) tumors. But no studies concerning its correlation to osteosarcoma prognosis were found. Hence, we aimed to investigate the prognostic value of Vitronectin (Vn) in osteosarcoma. Here, we studied the expression of VN in the tumor tissues from 67 patients with osteosarcoma and 20 patients with osteochondroma using immunohistochemistry and estimated the effects of VN expression in osteosarcoma on progression-free survival (PFS) and overall survival (OS) using the Kaplan-Meier curve and COX proportional hazards regression model. Increased expression of VN in osteosarcoma tissue compared to no VN expression in osteochondroma tissue was shown in immunohistochemical assay. No associations were observed between VN expression and osteosarcoma patients' gender (P = 0.675), age (P = 0.813), tumor size (P = 0.436), histologic subtype (P = 0.0.543) or tumor location (P = 0.456). Univariate survival analysis demonstrated significant correlations of high VN expression with shorter PFS (P = 0.002) and OS (P = 0.001); multivariate survival analysis revealed high VN expression as a significant independent prognostic indicator for shorter PFS (HR 2.788, P = 0.003) and OS (HR2.817, P = 0.003). In conclusion, the high expression of VN in tumor cells independently indicated poor clinical prognosis in patients with osteosarcoma, other than large tumor size and non-neoadjuvant chemoradiotherapy, suggesting that VN may serve as a potential therapeutic target in osteosarcoma.

Keywords: Vitronectin, immunohistochemistry, osteosarcoma, prognosis

Introduction

Osteosarcoma is the most frequently seen primary malignant bone tumor and reaches its peak morbidity rate predominantly in childhood and adolescence. The prognosis of osteosarcoma in younger patients has improved markedly, mainly due to the introduction of adjuvant and neo-adjuvant chemotherapy [1]. However, there still lacks favorable clinical outcome for patients with overt metastasis or recurrent disease. Unfortunately, increase in survival rate has not been witnessed in the last decade. As a result, it is indispensable to precisely recognize the key molecular mechanisms of tumor invasion and metastasis as well as resistance to chemoradiotherapy to improve prognosis for patients with malignant osteosarcoma.

It is well known that the tumor microenviroment plays an important role during the progression from primary tumor growth to metastasis [2]. Extracellular matrix (ECM) is one important component of the microenviroment containing a variety of matricellular proteins, which have structural and biological roles [3]. Extracellular matrix (ECM) glycoproteins participate in angiogenesis in many physiological and pathological processes, including ischemic vascular diseases, inflammation, tumor growth and metastasis [4, 5]. Thus, its synthesis and degradation are associated with tumor invasion and metastasis.

Vitronectin (VN) is an important ECM glycoprotein that presents in plasma and is localized into the ECM of various tissues. Most plasma VN is an inactive monomer [4]. ECM VN, which is present as an active multimeric form that binds to various ligands such as integrins, plasminogen activator inhibitor-1 and urokinase

receptors [6-8], is an adhesive protein that mediates the interactions between cells and their microenvironment. Integrin is recognized to be the primary receptor for VN. Integrin αvβ3-VN interaction promotes hypoxia resistance and tumor cell invasion through a mechanism that involves integrin-linked kinase (ILK) [9]. VN-binding integrins (both on tumor and endothelial cells), together with uPAR system, are essential for tumor progression and neovascularization [10-12]. The role of VN in carcinoma is well-documented. VN plays a role in promoting disease progression of several cancers including human breast cancer, melanoma, hepatocellular carcinoma and colorectal adenocarcinoma etc. [13-16].

In osteosarcoma lines, migration (haptotaxis) and invasive potentiation (haptoinvasion) of osteosarcoma (MG-63) cells can be mediated by vitronectin, and this process could be inhibited by high molecular weight kininogen (HK) [17]. Vitronectin improved the adhesive potency of osteosarcoma (MG-63) cells via directly associating with integrin αvβ3 and αvβ5 [18]. Integrin αvβ3 expression accelerated the metastatic potential and migratory and chemotactic ability of human osteosarcoma cells through adhesion to vitronectin [19]. The above studies indicated VN plays a crucial role in the invasion and metastasis of osteosarcoma cell lines. Whereas, it is worth noting that the relationship between VN and osteosarcoma prognosis has not been tapped into. Besides, considering the features of osteosarcoma early metastasis and the mechanism where VN promotes the invasion and metastasis of tumor cells, investigating the correlation between VN expression and osteosarcoma survival rate is a novel and significant attempt. In the present study, VN expression in osteosarcoma tissues was examined and the influence of its expression on PFS and OS in patients with osteosarcoma was evaluated, which would assist in judging whether the inhibition of VN could work as therapeutic target for osteosarcoma.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of Fujian Medical University. Written informed consents were taken from all the participants and ethical guidelines under Declaration of Helsinki were followed.

Clinical data

Specimens were obtained from archived, paraffin-embedded tissue sections from 67 patients with osteosarcoma in the First Affiliated Hospital of Fujian Medical University between January 1st 2006 and December 31st 2012 as experimental group. 20 osteochondroma tissue samples were acquired from homochronous surgical specimens and utilized as control group. Histopathological diagnosis of all specimens was confirmed by two trained pathologists. In the experimental group, thirty-eight patients (56.7%) were male and twenty-nine patients (43.3%) were female. The age at diagnosis ranged from 11 to 75 years (median age 20 years). Forty-one patients received neoadjuvant chemotherapy, and the remainder underwent operation without any preoperative therapy. Upon completion of the surgery, all patients received normative post-operative chemotherapy using methotrexate, cisplatin and doxorubicin. Following chemotherapy, the patients were undergoing lung checkup through X-ray or CT scans every 3 months during the first 3 years after chemotherapy, every 4 months during the fourth and fifth years and every 6 months thereafter. The X-ray or CT scans were applied for the evaluation of the development of local recurrence and distant metastases.

All patients were followed up until December 2014 either by phone call or outpatient visit. The period of time between the date of surgical excision and first tumor progression (or death, or the latest follow-up) was determined as Progression-free survival (PFS). Overall survival (OS) was calculated according to the time between surgery and death (or the latest follow-up). All dead cases died of osteosarcoma, other than other diseases.

Protein expression of vitronectin in osteosarcoma

Tissues were obtained from patients during surgical excision. Paraffin-embedded specimens were cut into 4 μm thick slices, which were baked for 1-2 h in an incubation of 60-65°C to prevent slice detachment. Next, xylol and alcohol were used for dewaxation and hydration. During antigen retrieval process, these slices were placed in citrate buffer (pH = 6.0) in an autoclave sterilizer for approximately 2 min. To block the endogenous enzymes, they

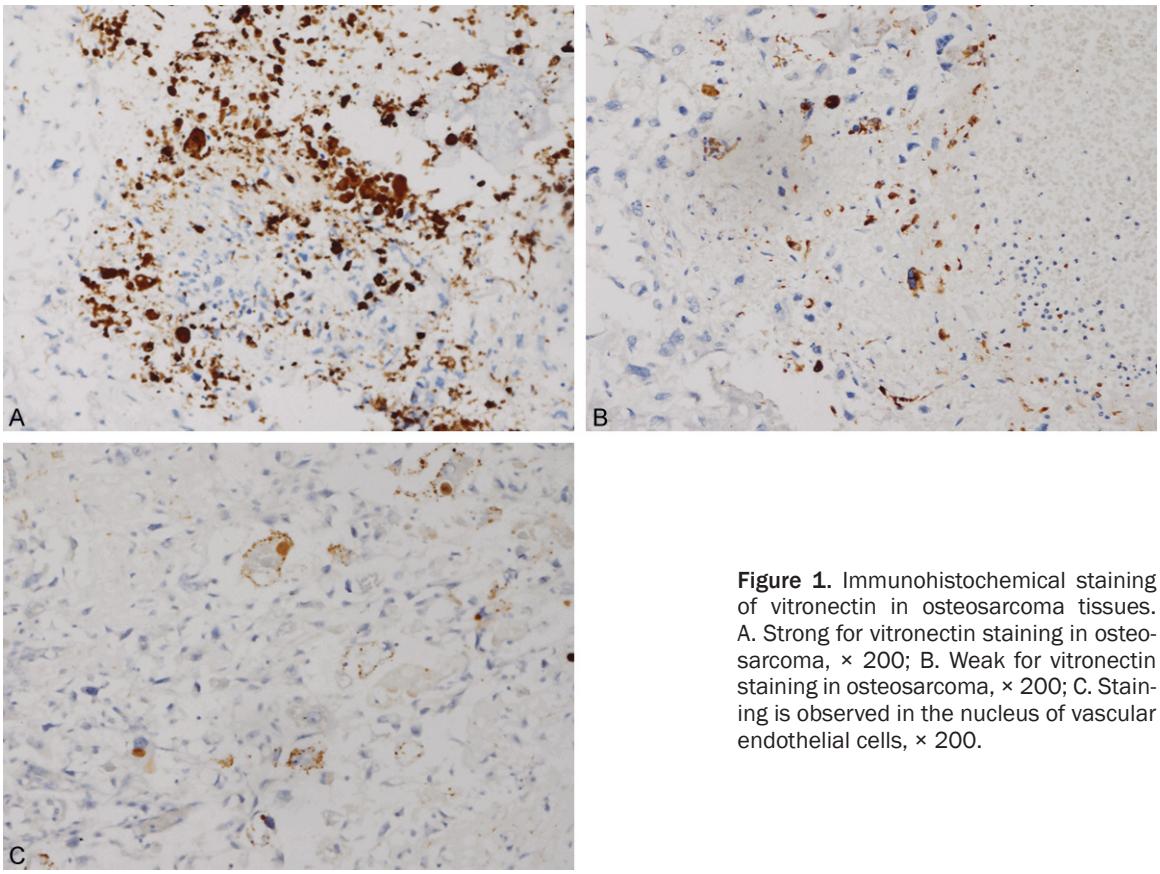

Figure 1. Immunohistochemical staining of vitronectin in osteosarcoma tissues. A. Strong for vitronectin staining in osteosarcoma, × 200; B. Weak for vitronectin staining in osteosarcoma, × 200; C. Staining is observed in the nucleus of vascular endothelial cells, × 200.

were rinsed in PBS and immersed in 3% H_2O_2 for 12 min once cooling to room temperature. Then, the specimens were incubated for 2 h at room temperature with an antibody against vitronectin (EP781Y, rabbit monoclonal antibody, Abcam, UK) at a dilution of 1:200. Next, the sections were exposed to polyperoxidase-anti-rabbit/mouse IgG (Maixin Biotechnology Co, Fuzhou, China) for 30 min following the manufacturer's instruction. With the help of diaminobenzidine detection kit (Maixin Biotechnology Co) under light microscope, immunoreaction was identified. Hematoxylin was employed to counter stain the slices and gelatin to cover them. Phosphate buffer solution (0.01 M, pH 7.2), rather than the primary antibody, was used as negative control, and known positive human lung tissue as positive control.

Assessment of vitronectin expression in osteosarcoma patient specimens

Two pathologists participated in the assessment of tumor tissue specimens independently. Brown granules occurring in the nucleus or cytoplasm of tumor cells were determined as positive vitronectin expression under light microscope. Cells with no positive staining were rated as '0', '±' if only a few scattered positive cells were present, '+' if a cluster(s) of positively stained cells (≤ 30% within a visual field) were present and '++' if there were cluster(s) of positively stained cells (> 30% within a visual field) were present. For the statistical analysis in the osteosarcoma cells lines, no positive expression or the positive expression rates lower than 10% were determined as weak expression, stained neoplastic cells of higher than 10% positive expression were considered as strong expression.

Statistical analysis

SPSS 22.0 statistical software was utilized for statistical analyses. The correlations between vitronectin expression and clinical variables (gender, age, tumor size, histologic subtybe and tumor location) were analyzed using Chi-square test. How vitronectin expression related with PFS and OS was indicated by log-rank tests and

Table 1. Relationships between Vitronectin expression and clinicopathological characteristics

Clinicopathologic data	Case number	Vitronectin		*P* Value (Chi-square test)
		Weak	Strong	
Gender				
Male	38	19	19	0.675
Female	29	13	16	
Age (years)				
< 18	24	11	13	0.813
≥ 18	43	21	22	
Tumor size				
< 5	16	9	7	0.436
≥ 5	51	23	28	
Histologic subtype				
Conventional	62	30	32	0.543
Special	5	2	3	
Tumor location				
Tibia or Femur	43	22	21	0.456
Other	24	10	14	

presented as Kaplan-Meier curves. In multivariate analysis, the COX proportional hazards model and stepwise regression analysis were adopted to estimate the influences of vitronectin expression on PFS and OS. Besides, hazard ratios (HR), together with the corresponding 95% confidence intervals (CI), were calculated. *P* value\0.05 (two-sided) was proved to be statistically significant.

Results

Topology of vitronectin expression in osteosarcoma tissues

Immunohistochemical analysis demonstrated that vitronectin expression, primarily localized in osteosarcoma cell nuclear or cytoplasm, was found in 50 out of 67 (74.6%) cases. In the osteosarcoma cells, VN immunoreactivity was scored as '-' in 25.4% (n = 17), '+/-' in 22.4% (n = 15), '+' in 32.8% (n = 22) and '++' in 19.4% (n = 13) of the samples. Among the 67 osteosarcoma tissue samples, we discovered strong expression (**Figure 1A**) in 35 samples (52.2%) and weak vitronectin expression (**Figure 1B**) in 32 samples (47.8%). Apart from carcinoma cells, we also detected immunohistochemical staining in vascular endothelial cells (**Figure 1C**). However, there was no significant vitronectin staining observed in osteochondroma tissue samples.

Associations between vitronectin expression and clinicopathological characteristics

Of 67 osteosarcoma samples, 38 patients (56.7%) were male and 29 patients (43.3%) were female. Median age at diagnosis was 20 years old (range 11-75). Median tumor size at diagnosis was 7 cm (range 2-17). Histologic subtype classification indicated there were 62 cases of conventional osteosarcoma and 5 cases of special osteosarcoma. With regard to tumor location, 43 cases were located in the tibia or femur and 24 in other locations. In order to estimate the effect of clinical characteristics on vitronectin expression, we analyzed the relationships between vitronectin expression and clinical factors, including gender, age, tumor size, histologic subtype, and tumor location. However, our findings suggested that there were no statistically noticeable correlations between vitronectin expression and patient gender (P = 0.675), age (P = 0.813), tumor size (P = 0.436), histologic subtype (P = 0.543) or tumor location (P = 0.456) (**Table 1**).

Relationships of vitronectin expression with clinical outcome in osteosarcoma

To evaluate the influence of percentage of vitronectin-positive expression on patient prognosis, corresponding PFS and OS were calculated. Out of 67 patients, the median PFS and OS were 25 months [95% confidence interval (CI) 20-33] and 31 months (95% CI 27-39), respectively. Univariate survival analysis (**Table 2**) showed stronger vitronectin expression was correlated with shorter PFS (P = 0.002, log-rank test, **Figure 2A**) and OS (P = 0.001, log-rank test, **Figure 2B**). In addition, similar results were achieved for tumor size (**Figure 2C**, **2D**) and neo-adjuvant chemotherapy (**Figure 2E**, **2F**). However, we observed no significant difference between gender, age, histologic subtype, tumor location and PFS or OS. Multivariate survival analysis (**Table 3**) revealed strong vitronectin expression could serve as a significant prognostic predictor of shorter PFS (HR 2.788, P = 0.003), independent of tumor size and neo-adjuvant chemotherapy; similar results were identified for OS (HR 2.827, P = 0.003). In the same line, big tumor size and non-neoadjuvant

Table 2. Univariate analysis of variables associated with PFS and OS by log-rank test

Variables	Case number	PFS (months)			OS (months)		
		Median	95% CI	*P* value	Median	95% CI	*P* value
Vitronectin							
Low	32	44	31-57	0.002	52	46-58	0.001
High	35	19	8-30		27	14-40	
Gender							
Male	38	22	13-31	0.316	28	16-40	0.283
Female	29	43	19-67		48	31-65	
Age (years)							
< 18	24	43	21-72	0.606	51	24-85	0.555
≥ 18	43	25	16-34		36	22-50	
Tumor size							
< 5	16	59	29-88	0.043	52	36-68	0.044
≥ 5	51	22	16-28		30	21-39	
Histologic subtype							
Conventional	62	28	9-47	0.806	39	27-51	0.739
Special	5	43	14-73		51	13-89	
Tumor location							
Tibia or Femur	43	24	14-33	0.264	36	15-57	0.343
Other	24	56	27-85		45	25-65	
Neoadjuvant							
Yes	41	43	12-74	0.022	56	35-77	0.027
No	26	21	17-25		36	26-34	

chemoradiotherapy were also proved to be independent indicators of PFS and OS.

Discussion

Osteosarcoma is the most common primary malignant bone tumor [20], and has a high capacity for distant metastasis, predominantly seen in lung tissues. With the application of comprehensive treatment including preoperative chemotherapy, operation and postoperative chemotherapy, 5-year survival rate of patients has remarkably enhanced to 50%-60% [21, 22] while metastasis or recurrence of the disease still remains as high as 30 to 40% and the disease is fatal to the majority of the patients [23]. Thus, to explore the tumor metastasis-associated biomarkers of osteosarcoma is of great significance and urgent need.

Vitronectin plays a role in guiding cell adhesion, migration, cell cycle progression and cell differentiation. It is noteworthy that vitronectin is involved in facilitating accelerated disease progression of many cancers, such as human breast cancer [13], melanoma [14], hepatocellular carcinoma [15] and colorectal adenocarcinoma etc. [16]. In this paper, we demonstrated for the first time that high percentage of vitronectin expression in osteosarcoma is a predictor of poor survival, importantly, it is an independent outcome factor in patients with osteosarcoma, suggesting that vitronectin is associated with osteosarcoma progression and metastasis.

There may be several mechanisms for vitronectin to enhance the progression and metastasis of osteosarcoma. It had been demonstrated that vitronectin may induce cancer stem cell differentiation by downregulation of stem cell genes through αvβ3 integrin-mediated mechanisms [24] and seems to influence cell adhesion and motility as well as formation of cancer cell aggregates [25]. On the cellular phase, VN-binding integrins (both on tumor and endothelial cells) and uPAR system together play an essential role for tumor progression and neovascularization, and these cell surface molecules may offer feasible therapeutic alternatives for imaging and cancer treatment [10-12]. VN binding played a key role in tumor growth through accelerating tumor activity of uPAR in vivo, and host deficiency in VN greatly damaged tumor formation [26]. Recent studies show that vitronectin significantly promoted Vascular Endothelial Growth Factor-Mediated Angiogenesis [27]. And VEGF overexpression indicated a poor prognosis for patients with osteosarcoma had been verified by a meta-analysis [28]. Tumor angiogenesis is a prerequisite for solid tumor growth and contributes to the initial step of metastatic spreading, facilitating the dissemination of tumor cells into the blood circulation [29]. Meanwhile, as a main

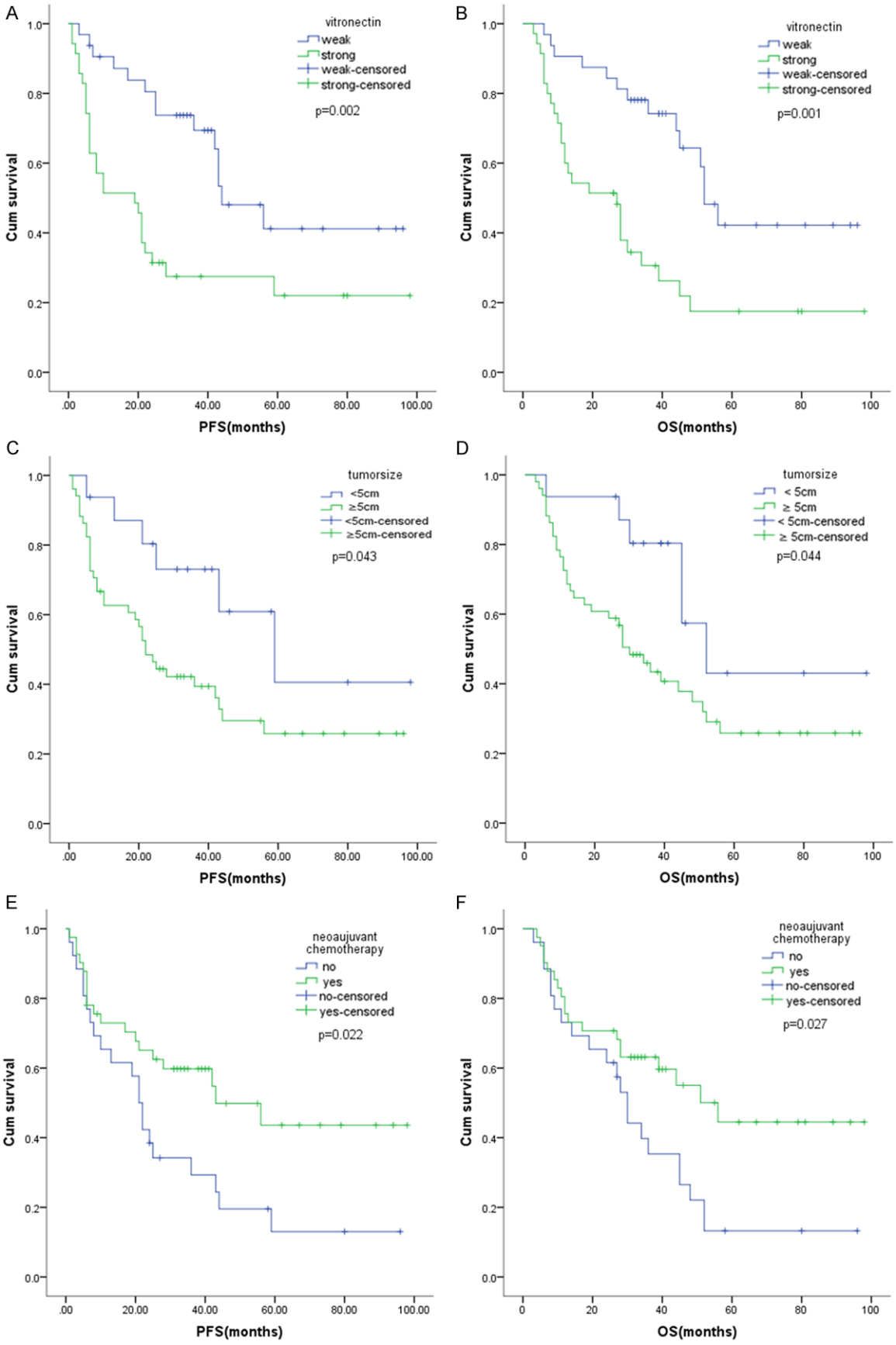
A
vitronectin
weak
strong
weak-censored
strong-censored
p=0.002
Cum survival
PFS(months)
B
vitronectin
weak
strong
weak-censored
strong-censored
p=0.001
Cum survival
OS(months)
C
tumorsize
<5cm
≥5cm
<5cm-censored
≥5cm-censored
p=0.043
Cum survival
PFS(months)
D
tumorsize
< 5cm
≥ 5cm
< 5cm-censored
≥ 5cm-censored
p=0.044
Cum survival
OS(months)
E
neoaujuvant chemotherapy
no
yes
no-censored
yes-censored
p=0.022
Cum survival
PFS(months)
F
neoaujuvant chemotherapy
no
yes
no-censored
yes-censored
p=0.027
Cum survival
OS(months)

Figure 2. Kaplan-Meier curves showing a correlation of percentage of vitronectin positive tumor cells with PFS (A) and OS (B), tumor size with PFS (C) and OS (D) and neo-adjuvant chemoradiotherapy with PFS (E) and OS (F) in the entire cohort. The osteosarcoma patients with vitronectin-strong expression demonstrated significantly poorer OS and DFS rates than those with vitronectin-weak expression, so do osteosarcoma patients with tumor size > 5 cm or without neoadjuvant chemotherapy.

Table 3. Multivariate analysis of variables associated with OS and PFS

Variables	OS			PFS		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Vitronectin	2.827	1.438-5.554	0.003	2.788	1.420-5.474	0.003
Neoaujuvant	0.478	0.251-0.911	0.025	0.446	0.234-0.851	0.014
Tumor size	3.037	1.244-7.412	0.015	3.357	1.355-8.317	0.009

Receptor, αvβ3 integrin expression improved the metastatic potential and migratory and chemotactic ability of human osteosarcoma cells. And echistatin, a RGD-containing peptide antagonist of αvβ3, decreased osteosarcoma cell adhesion to vitronectin [30]. Thus, vitronectin blockade is an attractive candidate for therapy in humans with osteosarcoma to cut down metastasis from primary to lung tissue and potentially improve patient outcomes.

Vitronectin expression was also seen in vascular endothelial cells in our study, suggesting that the cross talk between vascular endothelial cells and sarcoma cells might be mediated by vitronectin signal molecular, which could facilitate tumor angiogenesis and even may be associated with invasion and metastasis. Furthermore, we found there were no associations between vitronectin expression and gender, age, tumor size, tumor location, implying that patient age, gender or performance status might not influence vitronectin expression level.

Whereas, some limitations are still found in our study. Firstly, as a retrospective study and being specifically designed, it is highly possible that some biases may occur when collecting patient information. Secondly, sample size of 67 cases is relatively small, which would require further large-scale studies to be done in order to offer more convincing evidence for the future clinical employment of VN inhibition.

In conclusion, the findings of our study revealed that higher vitronectin expression was noted in osteosarcoma tissues compared with osteochondroma tissues and its overexpression in patients with osteosarcoma had no correlation with clinicopathological characteristics including gender, age, tumor size, histologic subtype and tumor location. And we confirmed that the high percentage of vitronectin positive tumor cells in osteosarcoma was considered as a vital independent predictor for poorer prognosis, indicating that the overexpression of VN is a promising prognostic factor for osteosarcoma progression and metastasis. Hence, our study suggested that the inhibition of vitronectin may be a potential therapeutic target in osteosarcoma.

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Disclosure of conflict of interest

None.

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