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ORIGINAL ARTICLE

Caveolin-1 Expression Together with VEGF can be a Predictor for Lung Metastasis and Poor Prognosis in Osteosarcoma

Fatma El-Zahraa Ammar Mohamed[1] · El Zahraa Ibrahim Khalil[1] · Nisreen D. M. Toni[1]

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Abstract

Caveolin-1, the major protein component of caveolae, plays vital functions in tumorigenesis and metastasis. Previous evidence demonstrated the positive role of Caveolin-1 in the regulation of endothelial cell differentiation and the involvement of Caveolin-1 in vascular endothelial growth factor (VEGF) mediated angiogenesis. The correlation of Caveolin-1 expression and angiogenesis is not yet elucidated in osteosarcoma. This study aimed to investigate the expression levels of Caveolin-1 and VEGF in osteosarcoma and their associations with clinicopathological data. This study included 66 formalin-fixed and paraffin embedded osteosarcoma tissue samples. The expression levels of Caveolin-1 and VEGF were assessed by immunohistochemistry. Then associations with clinicopathological variables and the correlation between both markers were evaluated statistically. We also investigated the expression of Caveolin-1 and VEGF values in gene microarrays of osteosarcoma patients and cell lines by using GEO data sets on https://www.ncbi.nlm.nih.gov. Caveolin-1 and VEGF were expressed in 19.6% and 77.3%, respectively. Caveolin-1 expression was associated positively with osteoblastic histological subtype ($P < 0.0001$). VEGF expression showed positive association with patient age, histological grade and clinical stage ($P = 0.031$, $P = 0.024$ and $P < 0.001$; respectively). An inverse correlation between Caveolin-1 and VEGF expressions in osteosarcoma was found ($r = 0.2$ $P = 0.04$). In silico analysis of Caveolin-1 and VEGF expression supported our results. Our results suggest that Caveolin-1 may act as a tumor suppressor in osteosarcoma. Down-regulation of Caveolin-1 can be used as an indicator for poor prognosis in osteosarcoma patients. Meanwhile, overexpression of VEGF is a predictor of pulmonary metastasis and poor prognosis.

Keywords Caveolin-1 · Vascular endothelial growth factor · Osteosarcoma · Immunohistochemistry

Introduction

Osteosarcoma (OS) is the most common primary sarcoma of the bone in children and adolescents, constituting 3-4% of all malignancies and about 30% of malignant bone tumors in adolescents [1]. OS arises from primitive mesenchymal bone-forming cells which are characterized by production of osteoid. OS can also produce varying amounts of cartilage and/or fibrous tissue. The conventional OS, a high grade primary central OS, is the most common type, accounting for 75 to 85% of all OS [2, 3]. According to the World Health Organization, conventional OS is subdivided in terms of the predominant matrix into osteoblastic, chondroblastic, and fibroblastic subtypes [4]. Despite their heterogeneous histomorphology, most cases of high-grade OS are treated in the same way using neoadjuvant chemotherapy [5]. In spite of the development of current treatment modalities which have significantly improved OS outcome, there is still a high mortality rate in the OS patients. This is due to pulmonary metastasis that occurs in approximately 40–50% of the patients [6]. Moreover, up to 20% of the cases are presented with metastasis at initial diagnosis [7]. Therefore, further work towards finding the factors controlling metastasis is urgently needed.

It is well known that angiogenesis is one of the hallmarks of tumor as it induces proliferation and migration of endothelial cells to form new capillaries which are crucial for the growth, invasion and metastasis of cancer cells [8]. Angiogenesis is regulated by the balance of positive and negative factors. One of these factors is VEGF, which is a homodimeric protein identified as a specific mitogen for endothelial cells that

✉ El Zahraa Ibrahim Khalil
zahraa333eg@gmail.com

[1] Pathology Department, Faculty of Medicine, Minia University, Minia, Egypt

showed over-expression in a variety of tumors and other inflammatory diseases [9–11].

Caveolin proteins (caveolin-1, caveolin-2, and caveolin-3) have been proven to participate in human disease processes such as diabetes, cancer, and a variety of degenerative muscular dystrophies [12]. They also act as scaffolding proteins, which are capable of recruiting numerous signaling molecules to caveolae and regulating their activity [13].

Caveolin-1(Cav-1) is the major protein component of caveolae [14]. It is abundant in terminally differentiated mesenchymal cells, as adipocytes, endothelial cells, fibroblasts [15], and osteoblasts [16, 17]. Cav-1 has been identified as either a tumor suppressor or a tumor promoter; hence, its role in malignancy is very complex and varies considerably. In sarcomas, lung carcinoma, and ovarian carcinoma, Cav-1 had tumor suppressor activity and was down-regulated during the development of these tumors [18–20]. Conversely, up-regulation of Cav-1 has been demonstrated in esophageal squamous cell carcinoma and prostate cancer, and such up-regulation has been correlated with metastases and poor prognosis [21, 22]. In OS, loss of Cav-1 has been reported to induce metastasis in experimental conditions and correlates with unfavourable clinical course [23].

Previous studies have demonstrated that Cav-1 plays an important positive role in the regulation of endothelial cell differentiation and its function is a prerequisite for angiogenesis [24–27], but the exact role of Cav-1 as a stimulator or inhibitor of angiogenesis is controversial [26, 28–30]. Many studies have also reported the involvement of Cav-1 in VEGF mediated angiogenesis [31–33]. However, the correlation of Cav-1 expression and angiogenesis in tumor cells is still not elucidated.

For this reason, using immunohistochemistry and gene microarray analysis, this study aimed to investigate the expression levels of Cav-1 and VEGF in OS and their association with clinicopathological data. The correlations among the immunohistochemical markers were also studied.

Materials and Methods

Tissue Samples

This study comprised of 66 randomly selected OS cases that were obtained from Pathology Department, Minia University Hospital and Minia Oncology Center, Egypt during the period from March 2000 to December 2008. Hematoxylin and Eosin (H&E) stained slides for all cases were reviewed to confirm the diagnosis. Staging was done according to Enneking system for staging malignant musculoskeletal tumors [4]. Clinical data for patients were collected from the medical records after approval of the corresponding hospital districts and the study protocol was approved by Local Ethics Research Committee of Faculty of Medicine, Minia University.

In Silico Study (Data Set Analysis)

To validate our results, we investigated the expression of Cav-1 and VEGF genes values in OS patients or cell lines in the previous gene microarrays studies through the use of GEO datasets on https://www.ncbi.nlm.nih.gov. The values of these two genes were extracted from each data set by selecting two groups e.g. tumor and non-tumor, metastatic or not, then the value of each gene in the microarray was calculated. The following datasets were investigated:

- GSE12865 OS tissue ($n = 12$) and osteoblasts ($n = 2$)
- GSE14827 chondroblastic OS type ($n = 3$) and osteoblastic OS type ($n = 21$)
- GSE85537 primary OS (n = 3) and metastatic lung OS (n = 3)
- GSE14359 primary OS ($n = 10$) and metastatic lung OS ($n = 8$)
- GSE49003 metastatic cell ($n = 6$) and non-metastatic cell line (n = 6)
- GSE16008 this data set contains multiple tumors, we select only the OS samples ($n = 9$)

Immunohistochemistry

Four micrometer tissue sections on positive charged slides were deparaffinized and rehydrated through xylene and graded ethanol solutions and then treated for 30 min with 3% hydrogen peroxide to block the endogenous peroxidase activity. For antigen retrieval, sections were treated with 0.1 mol/L citrate, pH 6.0, in a 700-W microwave oven for 20 min. Primary antibodies used were a polyclonal rabbit Cav-1antibody, (1:100; Santa Cruz Biotechnology, USA) and monoclonal mouse VEGF antibody Ab-7 (clone VG1, ready to use, Thermo Fisher Scientific/ Lab vision corporation, USA. Both antibodies were incubated overnight at (4 °C). The reaction was detected with the avidin-biotin detection kit using diaminobenzidine (DAB) as chromogen. Finally, sections were counterstained with Mayer's hematoxylin. Endothelial cells of capillaries within the stained sections were evaluated as an internal positive control for both Cav-1 in VEGF antibodies. Negative control tissue sections were processed by omitting the primary antibody and the slides were incubated with phosphate buffered saline (PBS).

Immunohistochemical Scoring

The slides were independently reviewed by two pathologists. Regarding Cav-1, a semiquantitative estimation of Cav-1 expression was made using score obtained by adding the values of the intensity and percentage of immunoreactive cells. The

intensity was graded as 0 negative; 1 weak; 2 moderate and 3 intense staining. The percentage of positive cells was graded from 0 to 4 (0 < 5% positive cells; 1: 5–25%; 2: 26 –50%; 3: 51–75%; 4: 76 –100%). High Cav-1 expression was defined if the score was 6 or 7. Cases with scores between 0 and 5 were considered low Cav-1 expression [34]. For VEGF immunostaining, scoring was done according to the percentage of positive tumor cells; low VEGF expression (≤30% of tumor cells) and high VEGF expression (>30% of tumor cells) [9].

Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 17 software. Firstly, a descriptive analysis of clinicopathological features was performed. Chi-square and Fisher's exact tests were used to compare categorical variables. Correlation between markers was evaluated using Spearman's correlation coefficient. Results were considered statistically significant when P value <0.05.

Results

Clinicopathological Parameters

The mean patients' age was (14 ± 2.8 SD) years; with a median age of 13 years (range: 8-18 years). Forty-two patients were males (63.7%) and 24 were females (36.3%). All cases were diagnosed as of the conventional high-grade type with predominance of osteoblastic subtype (54.5%), followed by chondroblastic and fibroblastic subtypes (36.5 and 9%, respectively). Forty-eight (72.7%) cases were stage II and 18 (27.2%) were stage III. Twelve cases (22.8%) were obtained from lung metastatic OS, while 54 cases (77.2%) were primary OS samples.

Immunohistochemical Results of Cav-1

Cav-1 staining was seen in the cytoplasm of OS cells. Cav-1 expression was found to be absent or significantly reduced in OS cells comparable to endothelial cells of capillaries within the stained sections (P < 0.001). Low Cav-1 expression was observed in 53 (80.4%) cases, while high Cav-1 expression was seen in 13 (19.6%) cases (Fig. 1).

There was a significant positive association between Cav-1 immunostaining and histological tumor subtype (P < 0.0001), where all chondroblastic and fibroblastic subtypes were negative for Cav-1 staining. However, no significant association was observed between Cav-1 expression and patients' age nor sex (P = 0.995 and P = 0.074, respectively). As for tumor stages and tumor site whether primary OS or metastatic OS, absent or low Cav-1 expression was more seen in stage III compared to stage II (83.3% versus 79.2%) and in metastatic OS compared to primary OS samples (83.3% versus 79.6%), although the difference was not significant (P = 0.705 and P = 0.770, respectively). The association between Cav-1 expression and different clinicopathological features is presented in Table 1.

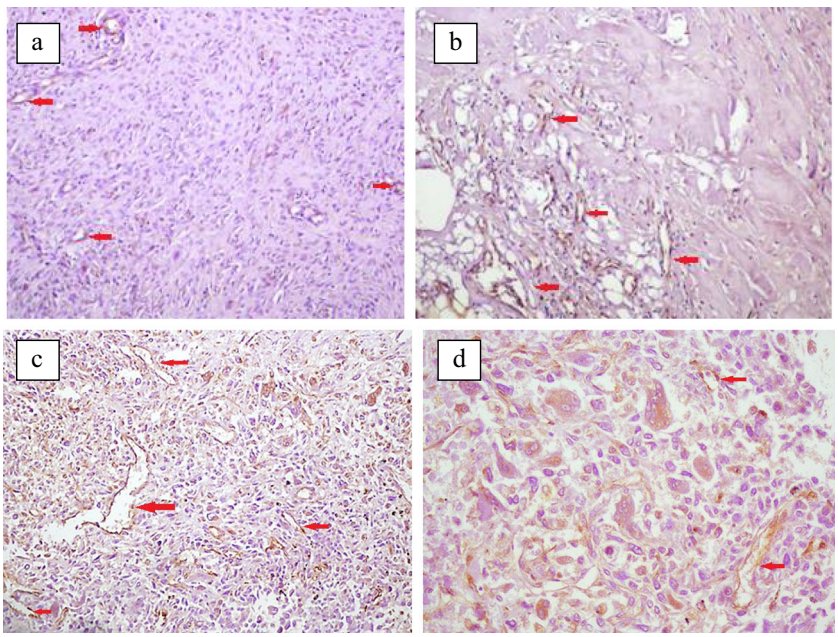

Fig. 1 Caveolin-1 expression in osteosarcoma. Immunohistochemical staining of Cav-1 showed absence of Cav-1 in OS cells (a) and (b). High Cav-1 expression in the cytoplasm of tumor cells (c). Higher magnification 200x showed expression of Cav-1 in tumor cells (d). Endothelial cells were stained as an internal control (arrow)

Table 1 Relationship between Caveolin1 expression and different clinicopathological parameters in OS

Clinicopathological variables	Total $N = 66$	High Cav-1 $N = 13$	Low Cav-1 $N = 53$	P value
Age		14 ± 3.15	14 ± 1.96	0.995
Sex				
Male	42	11 (26.2%)	31 (73.8%)	0.074
Female	24	2 (8.3%)	22 (91.7%)	
Histologic subtype				
Chondroblastic	24	0	24	$P < 0.0001$
Osteoblastic	36	13	23	
Fibroblastic	6	0	6	
Stage				
Stage II	48	10 (20.8%)	38 (79.2%)	0.705
Stage III	18	3 (16.7%)	15 (83.3%)	
Site				
Extremities	54	11 (20.4%)	43 (79.6%)	0.770
Metastatic lung OS	12	2 (16.7%)	10 (83.3%)	

Immunohistochemical Results of VEGF

VEGF positive expression was seen uniform cytoplasmic and/or membranous staining. High VEGF expression was found in 51(77.3%) of the OS cases (Fig. 2a and c).

Table 2 showed the association between VEGF expression and different clinicopathological variables. VEGF expression was significantly associated with age, being high with increased age ($P = 0.031$). There was a significant positive association between high VEGF expression and stage ($P < 0.001$). In line with this, lung metastatic samples showed significantly higher VEGF expression than primary OS samples ($P = 0.024$). No significant association was detected between VEGF expression and sex nor histological subtype ($P = 0.121$ and $P = 0.143$, respectively).

Correlation Between Cav-1 Expression and VEGF Expression

Spearman's correlation coefficient revealed a statistically significant inverse correlation between Cav-1 expression and

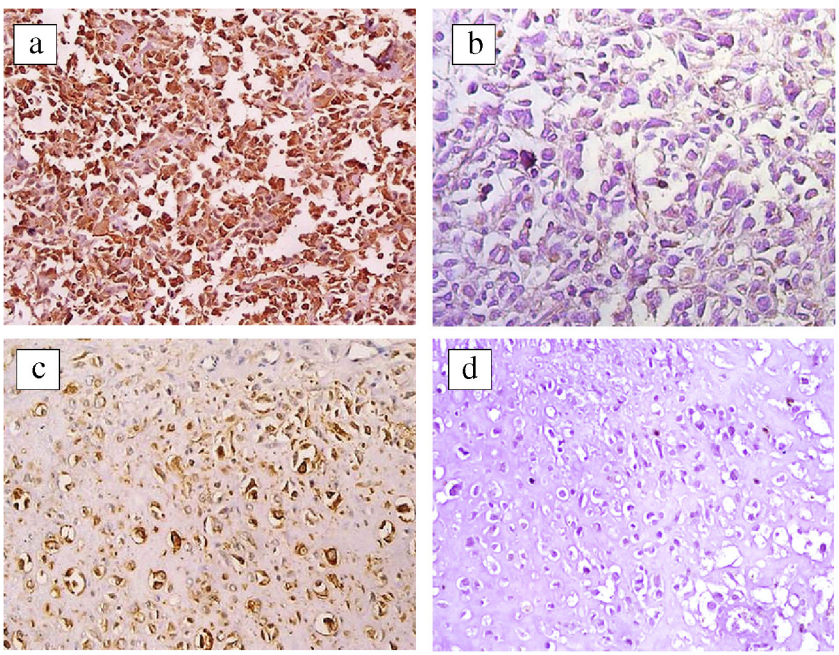

Fig. 2 VEGF expression in osteosarcoma and the correlation between VEGF and Cav-1 expressions in OS. Immunohistochemical staining of VEGF showed high VEGF expression in osteoblastic OS (a), and the absence of Cav-1 in the same case (b). High VEGF expression in chondroblastic subtype of OS (c) and absence of Cav-1 in the same case (d)

Table 2 Relationship between VEGF expression and different clinicopathological parameters in OS

Clinicopathological variables	Total $N=66$	Positive VEGF $N=51$	Negative VEGF $N=51$	P value
Age		14.4 ± 2.19	12.60 ± 4.27	0.031
Sex				
Male	42	30 (71.4%)	12 (28.6%)	0.121
Female	24	21 (87.5%)	3 (12.5%)	
Histologic subtype				
Chondroblastic	24	18 (75%)	6 (25%)	0.143
Osteoblastic	36	30 (83.3%)	6 (16.7%)	
Fibroblastic	6	3 (50%)	3 (50%)	
Stage				
Stage II	48	33 (68.75%)	15(31.25%)	$P<0.0001$
Stage III	18	18 (100%)	0	
Site				
Extremities	54	39 (72.2%)	15 (27.8%)	0.028
Metastatic lung OS	12	12 (100%)	0	

VEGF expression ($r=-0.2$, $P=0.033$), where Cav-1 was absent in cases which showed high VEGF expression (Fig. 2).

In Silico Analysis of Caveolin-1 and VEGF Expression in Osteosarcoma Tissue and Cell Lines

In the data set GSE12865, there was a significant reduction of Cav-1 in OS (12 cases) compared to normal osteoblast (2 cases) ($P<0.0001$) (Fig. 3a). No significant differences were found between OS subtypes, whether osteoblastic ($n=21$) or chondroblastic ($n=3$) in the data set GSE14827 ($P=0.7$) (Fig. 3b). In the data set GSE85537, the expression of Cav-1 was examined in conventional OS and lung metastatic OS. There was significant loss of Cav-1 expression in the lung metastatic OS compared to primary OS ($P=0.02$) (Fig. 3c). On the other hand, the data sets GSE14359 and GSE49003 showed different results. In the data set GSE14359, the metastatic lung OS showed significant increase in Cav-1 compared to the primary OS (Fig. 3d), while in the data set GSE49003, there were no expression differences of Cav-1 in four different cell lines of OS, two of them were metastatic (KHOS, KRIB) and the other two were non-metastatic (HOS, U2OS) ($P=0.9$) (Fig. 3e).

VEGF expression was also studied in the data sets GSE14359 and GSE12865, there was a significant increase in VEGF in OS compared to normal osteoblast in the data set GSE14359 ($P=0.0004$) (Fig. 3f), but not in the data set GSE12865 ($P=0.07$) (Fig. 3g).

There was inverse correlation between Cav-1 and VEGF ($r=-0.2$ and -0.1) in the GSE16008, GSE12865 data sets respectively; however, these values did not reach the significant levels Fig. 3h and i.

Discussion

Metastasis is a major determining factor of OS patients' outcome. Thus, there is a great demand towards finding new targets involved in OS metastasis. As we mentioned previously, the role of Cav-1 in cancer development has been the subject of close scrutiny, whether it is a tumor promoter or a tumor suppressor [12, 22, 35, 36]. Cav-1 involvement in tumor growth has also been discussed in relation to several cancers [18–22]. As for OS, only one study has investigated Cav-1 in OS [23]. Therefore, lack of data about Cav-1 significance in the tumorigenesis of OS has been the main impetus for the present study. Moreover, the exact role of Cav-1 as a stimulator or inhibitor of angiogenesis is controversial [27–30]. This is the first study to investigate immunohistochemical expression of Cav-1 in OS cases, its association with clinicopathological data and the correlation between Cav-1 and VEGF expressions in OS.

Consistent with previous literature (reviewed in Wiechen et al. 2001) [18], Cav-1 expression was obviously down-regulated in this series. The majority of OS cases studied in the present study showed low or absence of Cav-1 expression especially metastasized tumors and in advanced stages. **Wiechen et al.** [18] studied Cav-1 in a variety of normal mesenchymal tissues and mesenchymal tumors and was found to be expressed in benign mesenchymal tumors at high levels comparable to normal mesenchymal tissues, while Cav-1 expression was strongly reduced in sarcomas and was considered a tumor suppressor gene in human sarcomas. Likewise, **Cantiani et al.** [23] found that Cav-1 was down-regulated in OS samples by means of quantitative PCR. These results suggest that Cav-1 can function as a tumor suppressor rather than an oncogene in OS.

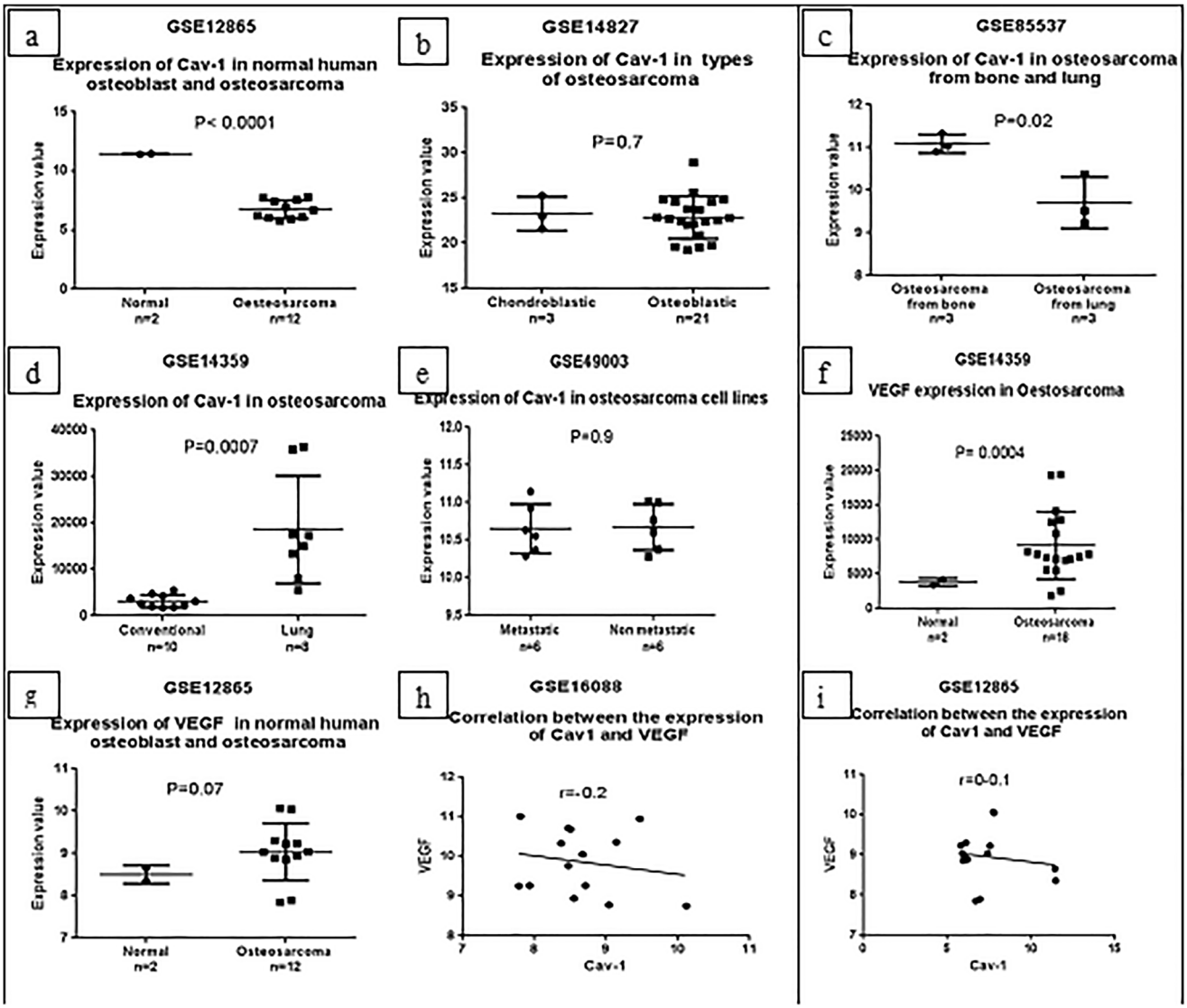

Fig. 3 In silico analysis of Cav-1 and VEGF expression in osteosarcoma tissue and cell lines. **a** Graph showed Cav-1expression significantly reduced in OS tissue compared to normal osteoblast in GSE12865. **b** Graph showed no significant differences in Cav-1expression between chondroblastic and osteoblastic types of OS in GSE14827. **c** Graph showed significant reduction of Cav-1 expression in lung metastatic samples compared to primary OS tissue in GSE85537. **d** Graph showed significant increase of Cav-1expression in lung metastatic samples compared to primary OS tissue in GSE14359. **e** Graph showed no significant differences of Cav-1expression in metastatic to non-metastatic type in GSE49003. **f** Graph showed VEGF expression significantly increased in OS tissue compared to normal osteoblast in GSE14395. **g** Graph showed VEGF expression value increased in OS tissue compared to normal, but it didn't reach significant level in GSE12865. **h** and **i** Graphs showed inverse correlation between Cav-1 and VEGF expression values in GSE16088 and 12865data sets

Moreover, in several in vitro studies, Cav-1 was down-regulated in tumor cells obtained from cervix, ovary, breast and lung, where the oncogenic transformation of cells was associated with a reduction in Cav-1 expression [35–37]. On the contrary, elevated Cav-1 expression has been negatively correlated with patient survival in esophageal carcinoma, bladder carcinoma, prostate carcinoma and Ewing's sarcoma, [38–41], suggesting an oncogenic role for Cav-1.

The contradictory findings in Cav-1 might be explained by the varied effects of Cav-1 mediated by different molecules which interact with Cav-1 different regions [12, 42]. **Cohen et al.** [12] suggested that down-regulation of Cav-1 could be explained by hyper-methylation of Cav-1 gene promoter which abolished its expression. Additionally, a dual role for the scaffolding domain of Cav-1 molecule was demonstrated, which acts as an anchor for different proteins within caveolae either inhibiting or enhancing protein's signaling activity [12].

In the present study, we observed that Cav-1 is significantly expressed in osteoblastic OS subtype. No significant associations were found between Cav-1 expression and patients' age, sex, tumor stage, and whether primary tumor or metastatic OS. The lack of significant association between Cav-1 expression, tumor

stage and tumor site may be attributed to the disproportionate distribution of the cases included in this study. **Cantiani et al.** [23] showed down-regulation of Cav-1 potentiated both Src family kinase and Met signaling, which provide OS cells with the capacity of invading neighboring tissues. Consequently, abrogation of Cav-1 induces metastasis in experimental conditions. Further, they found that high levels of Cav-1 measured by quantitative PCR were associated with a favorable overall survival in OS patients. These findings coincide with our results that low or absence of Cav-1 is associated with more aggressive tumors and may suggest that Cav-1 expression could be a biomarker for predicting OS behavior and clinical outcome.

Several studies have assessed the expression of VEGF in OS; the majority reported VEGF over-expression in OS, which coincides with our results [9, 43–45]. Conversely, few studies reported low expression of VEGF in OS [46, 47]. This discrepancy between different studies may be attributed to using different scoring systems. Our results showed that VEGF over-expression was significantly associated with adverse prognostic factors of OS including advanced stage and metastatic OS patients. These findings are in agreement with several reports demonstrating that higher VEGF levels indicate poor prognosis and less survival [9, 43–56], supporting the hypothesis that VEGF is crucial for tumor growth and progression. Moreover, the over-expression of VEGF might be a predictor of metastasis in OS.

In the current work, positive VEGF expression was significantly associated with increased age, in contrast to **Kaya et al.** [9] who found no association between VEGF expression and age, but their study included different age range (9 to 82 years) from ours. In agreement with previous studies [9, 43, 55], our results showed no significant association between VEGF expression and histological subtype of OS. Furthermore, there was no significant association between VEGF expression and gender of patients, agreeing with **Becker et al.** [47] and **Baptista et al.** [48].

Few studies have been performed to investigate the relation between Cav-1expressed in tumor cells and tumor-associated angiogenesis. The results of these studies were contradictory. **Tang et al.** [57] reported a positive association between Cav-1 and VEGF expression in hepatocellular carcinoma, whereas, in mucoepidermoid carcinoma of salivary glands, no correlation was found between Cav-1 and VEGF expression [32]. Conversely, reduced expression of Cav-1 was correlated with increased VEGF in the current study.

Many hypotheses were assumed for the relation between Cav-1 and VEGF in endothelial cells. It was hypothesized that many angiogenic regulators are normally held in inactive units and located in the caveolae of capillary endothelial cells. When angiogenesis is stimulated, these inactive units detach, allowing angiogenic regulators to become active. It was postulated that the presence of Cav-1 can inhibit proangiogenic factors [58]. In line with this, **Wu et al.** [59] reported that the over-expression of Cav-1 reduces VEGF-ERK2/1 activation and proliferation response in fetoplacental artery endothelial cells. Furthermore, Cav-1 was considered a negative regulator of VEGFR2 activity in VEGF-induced signaling in endothelial cells. On the other hand, **Liu et al.** [60] reported that angiogenesis activators, such as VEGF, down-regulate Cav-1 in human endothelial cells, and the down-regulation of Cav-1 may be pivotal for endothelial cell proliferation.

In view of the relation between Cav-1 and VEGF in endothelial cells, in the current study, we observed significant inverse correlation between Cav-1and VEGF in tumor cells, a relation similar to that found in endothelial cells. However, since angiogenesis includes wide array of angiogenic regulators, their relation with Cav-1 in OS still needs further investigation.

To further confirm our results, Cav-1 and VEGF genes expression values were studied in gene microarray studies of OS already published on PUBMED. In line with our results, Cav-1 was significantly down-regulated in OS tumor cells compared to normal osteoblast in the data set GSE12865, Cav-1 was also significantly reduced in metastatic OS samples in the data set GSE85537. Moreover, VEGF was significantly over-expressed in OS tumor cells compared to normal osteoblast in the data set GSE14359. Interestingly, the inverse correlation between Cav-1 and VEGF in OS samples in the data sets GSE16088 and GSE12865 remained, although it was not significant. Contrary to our results, no significant difference was seen between osteoblastic and chondroblastic types in the data set GSE14827. This may be due to low number of cases in the chondroblastic group and large variation of Cav-1 expression values in the osteoblastic group.

In conclusion, the current study showed a remarkable low Cav-1expression in OS, especially in late stages and metastatic OS samples. Additionally, VEGF was positively associated with metastasis and poor prognosis of OS patients. Furthermore, there was a significant inverse correlation between Cav-1 and VEGF. In view of these data, both markers appeared to be predictors of clinical outcome and may provide valuable clues for new therapeutic plans.

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Compliance with ethical standards

Conflict of Interests All the authors have no potential conflicts (financial, professional, or personal) relevant to the manuscript to disclose.

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