

Received: 2016.03.31
Accepted: 2016.07.01
Published: 2017.02.06

Correlations Between Hector Battifora Mesothelial-1 (HBME-1) Expression and Clinical Pathological Characteristics and Prognosis of Osteosarcoma Patients

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Source of support: Departmental sources

Background: The aim of this study was to investigate the correlation between Hector Battifora mesothelial-1 (HBME-1) expression and the clinical pathological characteristics and prognosis of osteosarcoma (OS).

Material/Methods: HBME-1 expression was assessed using immunohistochemistry in OS tissues (n=152), osteochondroma tissues (n=91), and normal bone tissues (n=74). We carried out a follow-up lasting 8-60 months to investigate HBME-1 expression and its correlations with the clinical pathological characteristics and prognosis of OS.

Results: HBME-1 was highly expressed in OS tissues compared with osteochondroma tissues and normal bone tissues, and was highly expressed in osteochondroma tissues compared with normal bone tissues (all $P < 0.05$). HBME-1 expression was correlated with clinical stages, postoperative recurrence, metastasis, and 5-year survival (all $P < 0.05$). The area under the receiver operating characteristics curve of HBME-1 expression was 0.864, with sensitivity of 80.92%, specificity of 91.89%, and accuracy of 84.51%. The survival rate was lower in the HBME-1 positive expression group than the HBME-1 negative expression group ($P < 0.05$). Clinical stages, metastasis, and HBME-1 expression were independent risk factors for the survival of patients with OS (all $P < 0.05$).

Conclusions: HBME-1 expression was correlated with the occurrence and development of OS. HBME-1 positive expression was a risk factor for the prognosis of OS.

MeSH Keywords: **Osteochondroma • Osteosarcoma • Recurrence**

Full-text PDF: <http://www.medscimonit.com/abstract/index/idArt/898820>

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Background

Osteosarcoma (OS), sharing the histological finding of osteoid production in association with malignant mesenchymal cells, is the most common bone malignancy, with aggressive invasion and metastasis [1–3]. Based on the predominant morphology of the neoplastic cells and quality of the intercellular matrix, OS can be classified into the following subtypes: osteoblastic, chondroblastic, and fibroblastic [4]. OS is considered an “orphan” disease, with an overall incidence of 0.2–3/100,000 per year [5]. OS often primarily occurs in the proximal tibia, distal femur, and proximal humerus, with over 50% originating around the knee [1]. Typical symptoms of OS include history of pain, localized swelling, and limitations in joint movement, and typical results from X-rays, but definite diagnosis requires histological examination of tumor material, generally by open biopsy [5].

Current preferable treatments for OS include multi-agent chemotherapy and surgical resection of all involved sites [6]. Survival rates of OS, however, continue to be unsatisfactory for metastatic and relapsed patients [7]. The 5-year survival rate is at least 70% in patients with localized disease, while long-term survival for patients with metastatic or recurrent disease is less than 20% [6]. Identifying prognostic factors for OS is thus of great significance for OS research and stratification and consolidation of the therapy and potentially prioritizing clinical trials of new therapeutic agents.

Hector Battifora mesothelial-1 (HBME-1), a membrane antigen that exists in the microvilli of the mesothelioma cells and other epithelial cells, has been used for the diagnosis of tumors originating from mesothelial cells [8–10]. HBME-1 has been reported to be a promising biomarker in thyroid pathology and also a universal marker of malignancy due to its high expression in several aggressive tumors [11,12]. Also, HBME-1, as a marker of mesothelial cells, has been suggested to participate in an epithelial-to-mesenchymal transition linked to metastasis in tumors of mesenchymal origin [13]. Thus, it is hypothesized that HBME-1 may have a role in tumors of mesenchymal origin (such as OS) through epithelial-to-mesenchymal transition. Epithelial cells have been reported to be able to down-regulate epithelial characteristics and acquire mesenchymal characteristics, which is commonly known as epithelial–mesenchymal transition (EMT), illustrating an inherent plasticity of the epithelial phenotype [14]. Accumulating studies demonstrated associations between EMT and OS. Ru et al. showed that SPRY4 Intronic Transcript 1 promoted EMT via association with Snail1 in OS [15]. Liu et al. reported that microRNA-128 inhibited EMT of human OS cells through directly targeting integrin $\alpha 2$ [16]. Considering the connection between HBME-1 and EMT, and also between EMT and OS, we put forward a hypothesis that HBME-1 might be associated with OS. In this study,

therefore, we aimed to determine associations between the immunohistochemical marker HBME-1 and the clinical pathological characteristics and prognosis of OS.

Material and Methods

Study subjects

The OS samples for the study were from 152 patients with primary OS admitted at Children’s Hospital of Zhengzhou City from Jan. 2007 to Dec. 2009, all of whom were confirmed by clinical, pathological, and imaging diagnoses. Inclusion criteria were: (1) the cases were confirmed with OS on the basis of clinical data, imaging and pathological examinations; (2) the cases were primary cases, receiving no therapies (e.g., radiotherapy, chemotherapy) targeting tumor cells before biopsy or operation; (3) the cases had the diseased part at the bone of limbs. Exclusion criteria were: (1) the cases had history of other malignant or benign bone tumor or tumor like diseases which might lead to secondary OS (e.g., osteochondroma, chondrosarcoma, and fibrous dysplasia); (2) the cases had not received the complete chemotherapy according to the treatment plan; (3) the cases were not accessible; (4) the cases had history of metabolic bone disease and major organ failure (e.g., liver, kidney, and heart). Of the 152 cases, 98 were male and 54 female (the M-F ratio was 1.81: 1), the age ranged from 9 to 50 years, with the median age of 19 years old. Pathological types were determined according to WHO OS classification [17]; 132 cases were with conventional OS, including osteoblast type (n=105), fibroblast type (n=20), and chondroblast type (n=7), and 20 cases had other types. Clinical stages were determined based on Enneking surgical staging [18]; 18 cases were at stage I, 38 at IIA, 60 at IIB, and 36 at stage III. As for diseased sites, 64 cases were at proximal tibia, 70 at distal femur, and 18 cases at other sites. Metastasis occurred in 99 cases, of which pulmonary metastasis occurred in 69 cases. Osteochondroma specimens were collected as controls for our study, which were from 91 patients with osteochondroma at the Department of Pathology of Children’s Hospital of Zhengzhou City from Jan. 2007 to Dec. 2009. In addition, fresh normal bone tissues from 74 subjects with no tumors were used as normal controls. This study was discussed and approved by the Ethics Committee of Children’s Hospital of Zhengzhou City and all the study subjects signed the informed consent.

Immunohistochemistry

The tissue samples went through gradient dehydration with alcohol (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China), clearing by xylene (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China), embedding with paraffin (Fuzhou Maixin Biotechnology Development Co., Ltd.,

Fuzhou, China), and slicing (with slide glass pre-coated with polylysine) (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China). The paraffined slices were then soaked in xylene for dewaxing, followed by gradient hydration with alcohol, phosphate-buffer saline (PBS) washing, antigen retrieval in a pressure cooker (Hong Hong Electrical Appliance Co., Ltd., Sichuan, China). Each slice was added with 1 drop of peroxidase (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for solution blocking, followed by incubation at room temperature for 10 min and PBS washing. Added with normal nonimmune animal serum, the slices were incubated for another 10 min, and then incubated overnight with first antibody [mouse anti-human HBME-1 monoclonal antibody, with PBS as control] (Santa Cruz Biotechnology, CA, USA), followed by PBS washing, incubation for 10 min with biotin (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) labeled second antibody, washing with PBS. After the addition of streptomycin anti-biotin enzyme (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) solution, the slices were incubated for 10 min, washed with PBS, added with diaminobenzidine (DAB) liquid (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China), and washed again with PBS, followed by double-staining with hematoxylin (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China), blue staining, gradient hydration with alcohol, clearing with xylene, and sealing with neutral gum (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China).

Staining score

HBME-1 was mainly located in cytoplasm and partly in cell membrane, and was considered to be positively expressed when brown yellow granules appeared in cell membrane and cytoplasm, with nuclear membrane not stained. With a high-power V-130B10C microscope (Shenzhen Boshida Optical Instrument Co., Ltd., Shenzhen, China), 5 high-power fields were selected for each slice at the 4 corners and the center to calculate the proportion of positive cells of 100 cancer cells; a mean was obtained from the 5 calculation. The percentage of positive cells was categorized on a scale of 0=less than 10% positives; 1=10~40% positives, 2=40~70% positives, and 3 is equal to or over 70% positives. The intensity was scored on 0=no coloring, 1=pale yellow, 2=brown and yellow, 3=brown. The final score for the staining was determined by multiplying the above 2 scores together: 0~3=negative (-), 4~6=positive (+), 7~9=positive (++), 10~12=positive (+++) [19].

Follow-up

Follow-up was carried out in all the patients until June 2015 (8~60 months, average 49.80 months), mainly via telephone, outpatient, or medical records review. At the end of the follow-up, 47 cases had died. Altogether, 16 cases were lost in the follow-up.

Statistical methods

Statistical analysis of all data was performed using SPSS 21.0 statistical software (SPSS Inc, Chicago, IL, USA). Measurement data were presented with mean \pm standard deviation ($\bar{x}\pm s$); comparison between 2 groups was testified using *t*-test and comparison among groups by analysis of variance. Enumeration data were presented as percentage or rate, and *chi*-test was applied for comparison between groups. Kaplan-Meier curve was applied for survival analysis. Comparison between groups was validated by log-rank test, and Cox regression model was used for multivariate analysis. A $P<0.05$ was considered statistically significant.

Results

Results of immunohistochemistry

HBME-1 was positively expressed mainly in cytoplasm and had distinct staining in cell membrane (significantly higher than background staining), presenting as yellow or brown particles (Figure 1). The positive rate was shown in Table 1. Of the 152 cases with OS, 123 cases had positive expression and the positive rate was 80.92%, of which 32 cases had strong positive (+++) expression, 54 had moderate positive (++) expression, 37 had weak positive (+) expression, and 29 cases had negative (-) expression. The positive expression rate of HBME-1 in osteochondroma tissues was 31.87%, of which 29 cases had positive expression and 62 had negative expression. The positive expression rate of HBME-1 in normal bone tissues was 8.11%; only 6 cases had weak positive expression and the others had negative expression. Expression of HBME-1 in OS tissues was significantly higher than that in osteochondroma and normal bone tissues, and HBME-1 expression in osteochondroma tissues was higher than that in normal bone tissues (all $P<0.05$).

Correlations between HBME-1 expression and the clinical pathological characteristics

Table 2 showed the relationships between HBME-1 expression and the clinical pathological characteristics of OS. The positive expression rate of HBME-1 was significantly higher in the patients with postoperative recurrence of OS than in non-recurrent patients (87.12% vs. 40.00%, $P<0.001$). The positive expression rate of HBME-1 was significantly higher in the patients with metastasis than those without metastasis (97.98% vs. 49.06%, $P<0.001$). The positive expression rate of HBME-1 was also significantly higher in the IIB + III patients than in the I + IIA ones (92.71% vs. 60.71%, $P<0.001$); as well as in the patients with survival less than 5 years than those with survival over 5 years (95.83% vs. 74.04%, $P<0.001$). The positive expression rate of HBME-1 was significantly higher in the

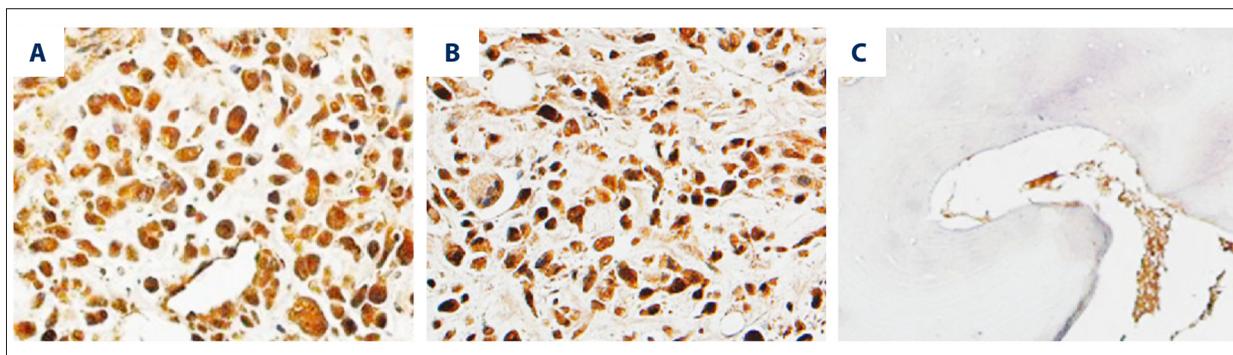


Figure 1. Expression of HBME-1 (×100). (A) positive expression of HBME-1 in osteosarcoma tissues; (B) Positive expression of HBME-1 in osteochondroma tissues; (C) Negative expression of HBME-1 in normal bone tissues; HBME-1, Hecto Battifora mesothelial-1.

Table 1. Positive rate of HBME-1 expression in each group.

| Group | n | - | + | ++ | +++ | Positive rate (%) | F/χ ² | P |
|-----------------------|-----|----|----|----|-----|-------------------|------------------|---------|
| Normal bone tissue | 74 | 68 | 6 | 0 | 0 | 8.11 | | |
| Osteochondroma tissue | 91 | 62 | 9 | 15 | 5 | 31.87 | 125.7 | < 0.001 |
| Osteosarcoma tissue | 152 | 29 | 37 | 54 | 32 | 80.92 | | |

All $P < 0.05$, compared between each two groups; HBME-1 – Hecto Battifora mesothelial-1.

patients with moderately and lowly differentiated OS than in the patients with highly differentiated OS (97.73% vs. 57.81%, $P < 0.001$). The positive expression rate of HBME-1 was not related to the indices, such as age, sex, pathological type, or lesion sites (all $P > 0.05$).

Diagnostic value of HBME-1 for OS

A receiver operating characteristics (ROC) curve was drawn on the basis of HBME-1 expression of the normal bone tissues as control and OS tissues (Figure 2). The area under the curve was 0.864; 95% confidence interval (95% CI) was 0.812–0.916 ($P < 0.001$); the sensitivity, specificity, and accuracy were 80.92% (123/152), 91.89% (68/74), and 84.51% (191/226), respectively; the positive and negative predictive value were 95.35% (123/129) and 70.10% (68/97), respectively.

Correlations between HBME-1 expression and the prognosis of OS

According to the HBME-1 immunohistochemical expression in OS, the patients were divided into a negative expression group and a positive expression group. The Kaplan-Meier survival curve of HBME-1 expression was shown in Figure 3. The survival rate of the patients was significantly lower in the positive expression group than the negative expression group ($P < 0.05$). A Cox proportional hazard model was built based on HBME-1 protein expression and the clinical pathological

factors of the patients with OS (Table 3), demonstrating that clinical stage, metastasis, and HBME-1 expression were the independent risk factors for the survival rate of patients with OS (all $P < 0.05$). Among all patients with OS, there were 30 patients with moderate and high differentiation but no metastasis. Among the patients with high differentiation but no metastasis, the survival rate of patients with positive HBME-1 expression was significantly lower than that of patients with negative HBME-1 expression of ($P = 0.027$). Figure 3B presented patients' survival curve.

Discussion

In our study, we determined HBME-1 expressions in OS, osteochondroma, and normal bone tissues based on immunohistochemistry and found that HBME-1 expression was higher in OS tissues than in osteochondroma tissues and normal bone tissues, and higher in osteochondroma tissues than normal bone tissues. Then we suggested HBME-1 expression as a potential marker for OS. We obtained a high sensitivity (80.92%) in the ROC curve, indicating the diagnostic value of HBME-1 expression for OS; consistently, Liu et al. reported a higher sensitivity (85.3%) when assessing the diagnostic performance of HBME-1 as a single protein marker [10].

HBME-1 expression was correlated with the clinical pathological characteristics of OS, including clinical staging, metastasis,

Table 2. Correlations between HBME-1 expression and clinical pathological characteristics of osteosarcoma.

| Clinical pathological characteristics | Cases (n) | Negative expression (case) | Positive expression (case) | Negative rate (%) | F/ χ^2 | P |
|---------------------------------------|-----------|----------------------------|----------------------------|-------------------|-------------|---------|
| Age | | | | | | |
| <18 years old | 70 | 12 | 58 | 82.86 | 0.13 | 0.723 |
| ≥18 years old | 82 | 17 | 65 | 79.27 | | |
| Gender | | | | | | |
| Male | 98 | 21 | 77 | 78.57 | 0.61 | 0.437 |
| Female | 54 | 8 | 46 | 85.19 | | |
| Pathological types | | | | | | |
| Osteoblast | 105 | 17 | 88 | 83.81 | 1.94 | 0.163 |
| Chondroblast | 7 | 1 | 6 | 85.71 | | |
| Fibroblast | 20 | 6 | 14 | 70.00 | | |
| Others | 20 | 5 | 15 | 75.00 | | |
| Lesion sites | | | | | | |
| Proximal tibia | 64 | 14 | 50 | 78.13 | 2.50 | 0.287 |
| Distal femur | 70 | 14 | 56 | 80.00 | | |
| Other sites | 18 | 1 | 17 | 94.44 | | |
| Clinical stages | | | | | | |
| I + IIA | 56 | 22 | 34 | 60.71 | 23.450 | <0.001 |
| IIB + III | 96 | 7 | 89 | 92.71 | | |
| Metastasis | | | | | | |
| Yes | 99 | 2 | 97 | 97.98 | 53.520 | <0.001 |
| No | 53 | 27 | 26 | 49.06 | | |
| Recurrence | | | | | | |
| Yes | 132 | 17 | 115 | 87.12 | 24.98 | <0.001 |
| No | 20 | 12 | 8 | 40.00 | | |
| Differentiation degree | | | | | | |
| High | 64 | 27 | 37 | 57.81 | 38.24 | <0.001 |
| Moderate & low | 88 | 2 | 86 | 97.73 | | |
| Survival | | | | | | |
| ≤5 years old | 48 | 1 | 46 | 95.83 | 12.660 | < 0.001 |
| >5 years old | 104 | 28 | 77 | 74.04 | | |

HBME-1 – Hector Battifora mesothelial-1.

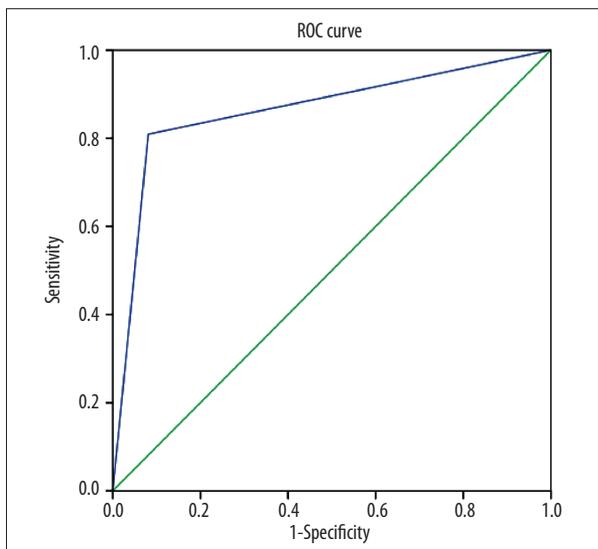


Figure 2. ROC curve of HBME-1 expression for diagnosing osteosarcoma. ROC – receiver operating characteristic; area under the curve: 0.890; 95% CI: 0.761~1.000.

and 5-year survival. Our findings also identified that HBME-1 played a critical role in the prognosis of OS by the evidences that HBME-1 positive expression was higher in the recurrent group than in the non-recurrent group, and that the survival rate of the patients in the HBME-1 positive expression group was significantly lower than that in the HBME-1 negative expression group. When diagnosed with OS, 10~20% of patients showed metastasis, most commonly (90%) in the lung, also in

bone (8~10%), and rarely in lymph nodes [1]. Therefore, systemic staging should focus on the lung and skeleton, where the majority of metastases arise [5]. About 30~40% of patients with localized OS developed a local or distant recurrence [20]. OS recurrences were associated with poor prognosis [20,21]. The 5-year overall survival for patients with recurrent OS was reported to be 23~29% [22]. Previous studies have demonstrated the association between inadequate surgical resection, local recurrence and morphologic progression, which was associated with a poor prognosis [4,23].

The mechanism behind the association between HBME-1 expression and OS might be inferred from previous studies. Tang et al. reported that over-expression of metadherin mediated metastasis of OS by regulating EMT [24]. Lv et al. demonstrated that down-regulation of tumor suppressing STF cDNA 3 promoted EMT and tumor metastasis of OS via the Wnt/GSK-3 β / β -catenin/Snail signaling pathway [25]. Hou et al. reported that Cyr61 promoted EMT and tumor metastasis of OS via Raf-1/MEK/ERK/Elk-1/TWIST-1 signaling pathway [26]. Studies also showed that microRNA-503 repressed EMT and inhibited metastasis of OS by targeting c-myc, and that microRNA-204 inhibited proliferation, migration, invasion and EMT in OS cells via targeting Sirtuin 1 [27,28]. During EMT, epithelial cells undergo the loss of their junctions and apical-basal polarity, reorganization of their cytoskeleton and a change in the signaling programs that defines cell shape and reprogram gene expression, which improves the motility of individual cells and enables the development of an invasive phenotype [29,30]. EMT has

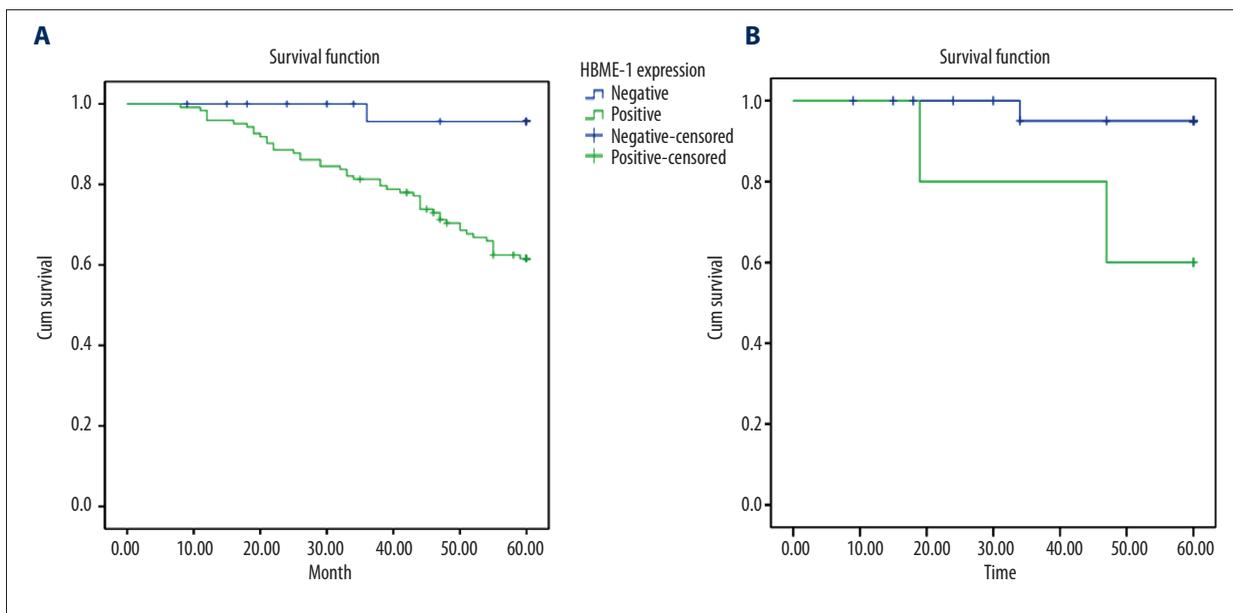


Figure 3. Kaplan-Meier curves of HBME-1 expression and living condition of the patients with osteosarcoma. (A) Comparison of living condition between patients with positive HBME-1 expression and negative HBME-1 expression; (B) Comparison of living condition between positive and negative HBME-1 expression among osteosarcoma patients with high differentiation but no metastasis.

Table 3. COX regression analysis of the factors for patients with osteosarcoma.

| Factors | B | SE | Wald | P | RR | 95% CI | |
|------------------------|--------|-------|-------|-------|--------|-------------|-------------|
| | | | | | | Lower limit | Upper limit |
| Pathological types | -0.132 | 0.192 | 0.469 | 0.493 | 0.877 | 0.601 | 1.278 |
| Lesion sites | -0.25 | 0.224 | 1.239 | 0.266 | 0.779 | 0.502 | 1.209 |
| Clinical stages | 1.025 | 0.433 | 5.605 | 0.018 | 2.787 | 1.193 | 6.509 |
| Metastasis | 0.78 | 0.333 | 5.496 | 0.019 | 2.182 | 1.136 | 4.191 |
| HBME-1 expression | 2.695 | 1.054 | 6.543 | 0.011 | 14.813 | 1.878 | 116.85 |
| Differentiation degree | -0.127 | 0.845 | 0.022 | 0.711 | 0.881 | 0.168 | 4.612 |

B – beta; SE – standard error; RR – relative risk; CI – confidence interval; HBME-1 – Hector Battifora mesothelial-1.

been recognized integral to development, and the processes underlain can be reactivated in cancer progression [14]. Taking the studies mentioned above together, it is clear that OS, especially metastasis, is affected greatly by EMT, although specific entities were different. As it is known that HBME-1 is located in the microvilli of the mesothelioma cells and other epithelial cells, we may assume that HBME-1 might participate in the EMT for its location and thus associate with OS. Furthermore, some other diagnostic markers of OS have been found. Yu et al. revealed that the P15 gene mutation was significantly correlated with osteosarcoma formation and metastasis towards the pulmonary tissue, suggesting its potency as a novel biological marker for early diagnosis of osteosarcoma [31]. Hu et al. reported that Src and p-Src can be used as an auxiliary indicator to determine a malignant phenotype of bone tumors, and the combined detection of Src and p-Src may indicate the prognosis of osteosarcoma [32]. Liu et al. demonstrated that osteosarcoma metastasis-related gene PMP22 participates in the proliferation, invasion, migration, and colony formation of osteosarcoma cells possibly via the MAPK signal transduction pathway, providing evidences for further investigation of metastatic mechanism of osteosarcoma [33]. Liu et al. suggested that survivin can function as a new diagnostic biomarker for osteosarcoma and be used as a reference index to determine pathology classification of osteosarcoma, providing new targets for gene therapy of osteosarcoma [34].

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Conclusions

We detected the HBME-1 expressions in OS, osteochondroma, and normal bone tissues based on immunohistochemical technique, finding the correlation between high expression of HBME-1 and the clinical pathological characteristics and prognosis of OS. We identified that HBME-1 expression was implicated with the disease occurrence and progression and thus might be a diagnostic marker of OS. However, with high rate of positive staining and small sample size due to limitations in time and funds, it is indeed hard to see how statistically significant results were obtained in multivariable analysis if the known prognostic factors were accounted for first. Therefore, the clinical application of HBME-1 expression in the diagnosis of OS needs further investigation and confirmation on the basis of studies with large sample sizes.

Acknowledgments

We would like to give our sincere appreciation to the reviewers for their helpful comments on this article.

Competing interests

The authors have declared that no competing interests exist.

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