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ORIGINAL PAPER

Heparanase expression correlates with poor survival in oral mucosal melanoma

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Abstract Oral mucosal melanoma (OMM) is a lethal cancer with a poor prognosis. Despite the great interest in heparanase (HPSE) as a potential anticancer therapy target, the prognostic role of HPSE in oral mucosal melanoma has not been elucidated. In this study, we investigated HPSE expression in OMM tissues and examined its association with clinical outcome. A total of 81 patients with OMM were enrolled in this study. We examined the expression of HPSE in OMM, and its staining extent, intensity and cellular localization were analyzed for clinical significance. HPSE staining was positive in 81 % of tumors (66 of 81 patients) and was negative in the remaining 19 % (15 patients). The median survival time and the 5-year survival rate were 12 months and 7.0 % in the high-heparanase group, 35 months and 36.4 % in the low-heparanase group and 62 months and 53.3 % in the none-heparanase group ($P = 0.001$). In univariate survival analysis of oral mucosal melanoma, AJCC Stage, heparanase level, heparanase location and tumor size were the clinical parameters related to overall survival. In Cox analysis, overall survival time was significantly dependent on AJCC stage and heparanase level, but not tumor size and heparanase location. Heparanase is frequently expressed in oral mucosal melanoma, and its expression levels inversely correlate with the survival rates of OMM patients, clearly indicating that heparanase is a reliable prognostic factor for this malignancy and an attractive target for anticancer drug development.

Keywords Heparanase · Oral mucosal melanoma · Survival · Staining · Localization

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Despite being first reported over 150 years ago, oral mucosal melanoma (OMM) remains a mystery which associated with a poor outcome. It represents 0.2–8 % of all melanomas in Europe and the United States [1] and 0.26 % of all oral cavity cancers [2] and is clearly distinct from its cutaneous counterpart in biology, clinical course and prognosis [3]. Oral mucosal melanomas are more aggressive than cutaneous melanomas and are more inclined to metastasize or recur after resection and treatment. Their 5-year survival rate is 15–38 % [4–6]. To date, we have a limited understanding of the etiopathogenesis of OMM. The rarity of these cancers, the heterogeneity of clinical and histopathologic appearances and the paucity of molecular and genetic studies have been the main reasons. Surgery continues to be the mainstay of treatment for these

patients, but once it has progressed to the metastatic stage, it is extremely difficult to treat and does not respond to current therapies. Therefore, functional studies of the biological nature involved in its aggressive growth and metastasis are important to develop new treatment modalities for this disease.

Tumor metastasis depends on the ability of cancer cells to invade tissue barriers composed of basement membrane and extracellular matrix [7]. Heparan sulfate (HS) proteoglycans are essential components of the cell-surface and extracellular matrix which provide structural integrity. Heparanase (HPSE) is the only mammalian endoglycosidase known that cleaves HS, thus contributing to matrix degradation and cell invasion [8]. Heparanase has been reported to be overexpressed in many types of human malignancies, including tongue cancer, ovarian carcinoma, carcinomas of the lung, alveolar and embryonal rhabdomyosarcoma, bladder cancer and renal cell carcinoma [9–15]. Previous studies have shown that increased expression of heparanase is correlated with reduced total survival rates, increased lymph node and distant metastasis. However, Because of the rarity of this disease entity, the published data on the epidemiology, tumor behavior, treatment and prognostic information on primary OMMs are sparse and are mainly based on single case reports or small series. The expression and functions of heparanase in OMM have not been well studied. Therefore, in this study, we investigated 81 OMMs, using heparanase immunohistochemistry. The aim of our retrospective study was to evaluate the correlation between heparanase expression and clinicopathological features of OMM and its correlation with patients' survival. Our data showed that heparanase is frequently overexpressed in OMM samples, and its overexpression indicated a poor prognosis. Therefore, targeting heparanase may represent a new approach for the treatment of OMM.

Patients and methods

Patients and tumor tissue samples

We performed a retrospective study of primary oral mucosal melanoma diagnosed in the Ninth People's Hospital of Shanghai and Wuxi People's Hospital of Nanjing Medical University between January 1998 and March of 2007. Data were retrieved and analyzed; 81 patients with OMM were found, and archival paraffin-embedded pathologic material was available for immunohistochemical analysis; histological confirmation of the lesion was required for all the cases. Clinical data of demographic information, site of origin, staging, tumor size and outcomes were analyzed. The Clinical stage according to the seventh AJCC staging system for head and neck mucosal melanomas [16]. This study was approved by the medical ethics committee of Shanghai Jiaotong University.

Immunohistochemistry

We investigated the heparanase protein expression using polyclonal rabbit anti-human heparanase-1 (H-80, Santa Cruz Biotechnology, Santa Cruz, CA), which specifically interacts with one peptide located in the 50 kDa active human heparanase enzyme. As a result, the antibody can be used to signify the 50 kDa heparanase-active subunit [17]. The paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated firstly, and the endogenous peroxidase activity was blocked by 3 % hydrogen peroxide (30 min). Then, antigen retrieval was performed using a steamer containing 10 mmol citrate buffer (pH 6.0) at 100 °C for 30 min. After that, sections were immunostained using the DAKO EnVision System Kit according to the manufacture's instruction (Dako Diagnostics, Zug, Switzerland). To be more specific, the sections were first blocked with 10 % normal goat serum for 60 min, and then incubated with the primary antibody H-80 antibody (1:50) at 4 °C over night, while normal mouse serum was used instead of heparanase-1 antibody in the negative control group. Slides were washed with PBS containing 0.01 % Triton X-100 and then incubated with the secondary reagent (Envision kit) according to the manufacturer's (Dako) instructions. After additional washes, the binding of the antibodies was detected with the 3-aminoethylcarbazole (AEC) substrate chromogen (DakoCytomation), and then, the sections were counterstained with hematoxylin and mounted [9, 10, 12].

Evaluation of heparanase expression

Two investigators who were unaware of the clinical data independently evaluated heparanase staining under a light microscope. Discordant cases were reviewed. This evaluation was decisive for the final score. The samples were scored according to the intensity of staining recommendations by Nagler and Rafael [9]. We modified the scoring system slightly as follows: 0: none, 1: weak; and 2: strong. Specimens that were similarly stained with preimmune serum, or applying the above procedure but lacking the primary antibody, yielded no detectable staining. In all tumors diagnosed as heparanase-positive, >50 % of the cells reacted with the anti-heparanase antibody, either nuclear or cytoplasmic, were also assessed.

Statistical analysis

Statistical analysis was performed with the use of SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL). The

Fisher exact test and chi-square tests were used to compare categorical data. Survival was estimated according to the Kaplan–Meier method, and the statistical significance of differences in survival was assessed by the log-rank test. Overall survival was calculated as the time from the date of diagnosis to the date of death or to the date of last follow-up if death did not occur. To evaluate independent prognostic factors associated with survival, multivariate Cox proportional-hazards regression analysis was used. All statistical tests were 2-sided, and a P value <0.05 was considered significant.

Results

Heparanase immunohistochemistry

Eighty-one primary oral mucosal melanomas were analyzed. The clinical description of patients and heparanase expression were shown in Table 1. Among the 81 patients, 15 (19 %) stained negatively for heparanase (Fig. 1a); weak staining was found in 38 % (31 of 81) (Fig 1b), whereas 43 % (35 of 81) were stained strongly (Fig. 1c). Adjacent, normal-looking tissue was not stained by the anti-heparanase antibody, thus serving as internal controls. Patients with an advanced AJCC stage had significantly higher heparanase expression than those with a low AJCC level ($P = 0.001$). A total of 47 patients presented with distant metastasis. Patients with distant metastasis had significantly higher heparanase expression than those without ($P = 0.006$). Heparanase immunostaining was not significantly different between males and females ($P = 0.603$), subsite and tumor size ($P = 0.966$, 0.658).

Table 1 Clinical description of patients and heparanase expression

Clinical features	No. of patients	Heparanase level (%)			P value
		0	1	2	
Gender					
Male	50	10 (20.0)	17 (34.0)	23 (46.0)	0.603
Female	31	5 (16.1)	14 (45.2)	12 (38.7)	
Subsite					
Palate	47	9 (19.1)	19 (40.4)	19 (40.4)	0.966*
Gum	27	5 (18.5)	9 (33.3)	13 (48.1)	
Others	7	1 (14.3)	3 (42.9)	3 (42.9)	
AJCC Stage					
III	42	12 (28.6)	15 (35.7)	15 (35.7)	0.001*
Iva	23	1 (4.3)	15 (65.2)	7 (30.4)	
Ivb	13	2 (15.4)	1 (7.7)	10 (76.9)	
Ivc	3	0	0	3 (100)	
Tumor size					
≥2 cm	52	10 (19.2)	18 (34.6)	24 (46.2)	0.658
<2 cm	29	5 (17.2)	13 (44.8)	11 (37.9)	
Metastasis					
Yes	47	5 (10.6)	15 (31.9)	27 (57.4)	0.006
No	34	10 (29.4)	16 (47.1)	8 (23.5)	

* Fisher's Exact Test

Univariate analysis of heparanase expression for survival rate of OMM patients

On univariate survival analysis, the conventional prognostic markers, AJCC stage, tumor size reached significance for overall survival (Table 2). The strong-heparanase group had significantly shorter survival than the low-heparanase group and none-heparanase group (Fig. 2, $P = 0.001$). The median survival time and the 5-year survival rate were 12 months and 7.0 % in the high-heparanase group, 35 months and 36.4 % in the low-heparanase group and 62 months and 53.3 % in the none-heparanase group. Moreover, we compared the distinct cellular localization pattern of the heparanase-positive group. Thus, in 66 stained positively for heparanase, 42 (64 %) heparanase staining appeared cytoplasmic (Fig. 1c); whereas in the remaining 24 of 66 specimens (36 %), heparanase also was localized in the cell nucleus (Fig. 1d). It is noteworthy that nuclear localization of heparanase was associated with a favorable outcome in patients with OMM. Clearly, patients who had specimens that stained negatively for heparanase or that exhibiting nuclear localization of the enzyme had a significantly longer overall survival than patients who had specimens with only cytoplasmic staining ($P = 0.047$) (Table 2; Fig. 3).

Multivariate analysis of heparanase expression for survival rate of OMM patients

Multivariate survival analysis was performed on all parameters that were found to be significant on univariate analysis. The results of comparing the conventional prognostic factors to heparanase level and location were shown in Table 3. Overall survival time was significantly dependent on AJCC stage and heparanase level but not tumor size and heparanase location. Additionally, heparanase level was shown to be an independent predictor of poor prognosis even when compared to tumor size, which was a significant marker in the univariate analysis.

Discussion

Oral mucosal melanoma is one of the most aggressive malignant tumors, and its prognosis is worse than those of

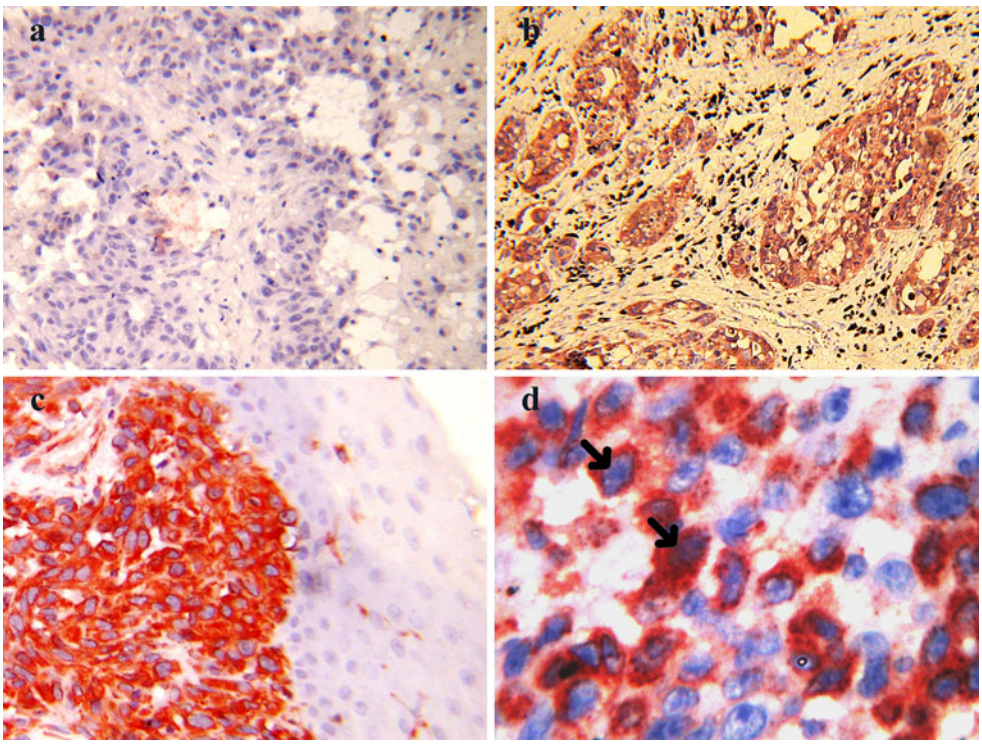

Fig. 1 Immunohistochemical stain of representative case. *Description* **a** Negative heparanase expression in OMM specimens. **b** Weak heparanase expression in OMM specimens. **c** Strong heparanase expression in OMM specimens (cytoplasmic). **d** Nuclear localization of heparanase expression in OMM specimens

other head and neck malignancies. The clinical outlook for patients with OMM is still very poor, due to the high rate of local and distant metastasis. It was reported that the AJCC stage and tumor size are the most powerful predictive factors for evaluating tumor bioactivity and predicting treatment outcomes [18–20]. However, the clinical course of patients with the same disease stage and the same tumor size has different outcomes from the same therapy. Clearly, considerable efforts should be made to discover new biological markers that can accurately predict the disease metastasis and lead to better targeted and more effective treatment. However, because of the rarity of this entity, it is difficult to gather an adequate number of patients; most articles on the subject represent case reports with review of previously published cases which have focused on epidemiological data. Due to China's large population and a higher incidence [2, 21], we collected 81 OMM patients to investigate the relationship between the heparanase expression and the patients' survival.

Heparanase cleaves heparin sulfate (HS), a major constituent of the extracellular matrix (ECM) and basement membranes, is considered an important step for breaking down the ECM barrier and penetrating the blood vessel basement membrane required for tumor cell metastasis. In this study, we found that heparanase were expressed in OMM specimens.

Heparanase overexpression was associated with a higher frequency of distant metastasis and poor overall survival for patients with OMM. Moreover, patients who had specimens that stained exhibiting nuclear localization of the enzyme had a significantly longer overall survival than patients who had specimens with only cytoplasmic staining. Consistent with our results, heparanase up-regulation has been shown in increasing numbers of human carcinomas and hematologic malignancies. In many cases, over expression of heparanase was associated with increased tumor metastasis, vascular density and a lower survival rate [22–26]. Similarly, Rivera et al. [27] has reported that heparanase was not expressed in the oral melanotic macule, but atypical melanocytes and melanoma cells expressed heparanase. In his research, an intense expression was noted in the early invasive phase, which marks the crucial transition from in situ to the invasive phase suggesting that heparanase plays an important role in the progression of OMM. More recently, Leiser et al. [26] utilized quantitative real-time polymerase chain reaction (PCR) to examine the expression of heparanase in oral carcinomas and revealed that expression level and cellular localization of heparanase could serve as an important diagnostic marker in patients with oral cancer. On the basis of these findings, we performed multivariate analysis and showed that heparanase overexpression was a prognostic factor for overall

Table 2 Univariate 5-Year Overall Survival of OMM Patients

Parameter	No. of patients (%)	Median survival time (months)	5-year OS (%)	*P*
Age				
<60	52 (64.2)	25	32.7	0.318
≥60	29 (35.8)	17	18.8	
Color				
Pigmented	72 (88.9)	21	26.8	0.58
Amelanotic	9 (11.1)	35	33.3	
AJCC Stage				
III	42 (51.9)	39	39.9	0.000
Iva	23 (28.4)	25	21.7	
Ivb	13 (16.0)	7	0	
Ivc	3 (3.7)	5	0	
Tumor size				
≥2 cm	52 (64.2)	13	20.5	0.027
<2 cm	29 (35.8)	32	40	
Gender				
Male	50 (61.7)	22	29.6	0.846
Female	31 (38.3)	25	24.7	
Subsite				
Palate	47 (58)	26	30.4	0.422
Gum	27 (33.3)	21	25.9	
Others	7 (8.7)	12	14.3	
Heparanase level				
0	15 (18.5)	62	53.3	0.001
1	31 (38.3)	35	36.4	
2	35 (43.2)	12	7	
Heparanase localization				
No	15 (18.5)	62	53.3	0.047
Nucleus	24 (29.6)	25	33.7	
Cytoplasm	42 (51.9)	21	12.3	

survival independent of AJCC stage and tumor size in patients with OMM.

Another impressive result of our study was the correlation between the cellular localization of heparanase and patient survival. Clearly, nuclear localization of heparanase predicted a favorable outcome (Fig. 3); however, on multivariate analysis, overall survival time was significantly dependent on AJCC stage and heparanase level, but not heparanase localization (Table 3). This result appears somewhat inconsequential in the outcomes reported by Doweck et al. [28]; they revealed that cytoplasmic versus nuclear heparanase localization stands as an independent parameter for patient with head and neck cancer. The mechanisms underlying this association are not clear. We assume that heparanase translocated from the cytoplasm to the nucleus and cleaved HSPGs in the nucleus, which in

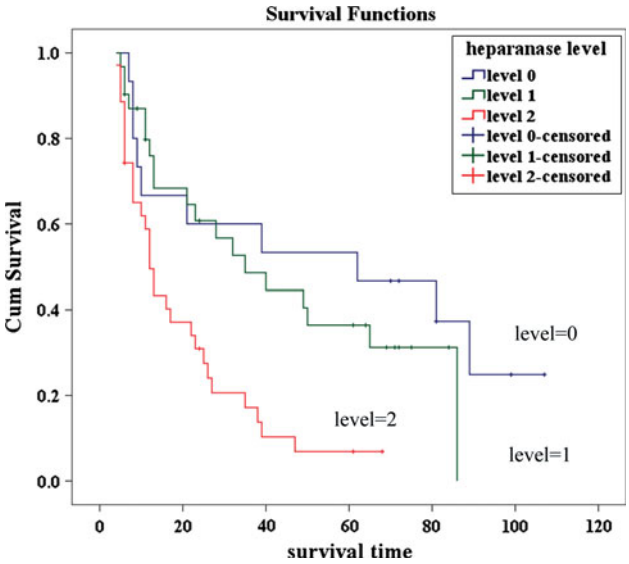

Fig. 2 Overall Survival of Oral Mucosal Melanoma Patients by different heparanase level. Description: The Kaplan–Meier curve for overall survival according to the different heparanase level is shown

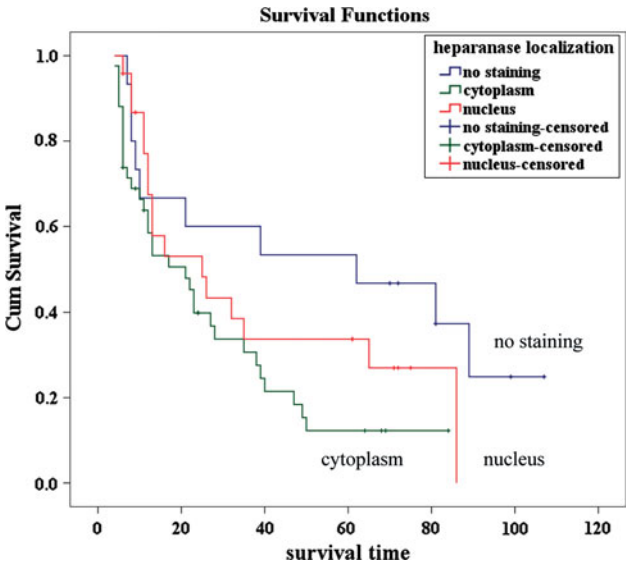

Fig. 3 Overall Survival of Oral Mucosal Melanoma Patients by the heparanase localization. *Description* The Kaplan–Meier curve for overall survival according to the heparanase localization is presented

turn modulated cell growth through regulation of cell cycle as reported previously by Ohkawa et al. [29].

In conclusion, our study demonstrated that heparanase is overexpressed in patients with OMM. High level of heparanase expression is associated with a higher frequency of distant metastasis, poor prognosis and is an independent prognostic marker for OMM. Therefore, targeting heparanase may represent a novel target for the treatment of oral mucosal melanoma.

Table 3 Multivariate survival analysis (Cox regression model) of conventional prognostic factors and heparanase

	Hazard ratio	95 % confidence interval	*P* value
AJCC Stage	2.59	1.767—3.794	0.000
Tumor size	NA	NA	0.063
Heparanase level	1.66	1.099—2.516	0.016
Heparanase localization	NA	NA	0.062

Method = Forward Stepwise (Likelihood Ratio)

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Conflict of interest We declared that we have no conflict of interest.

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