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The prognostic value of heparanase expression and cellular localization in oral cancer

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Abstract

Background—Metastases formation depends on the ability of tumor cells to invade basement membranes in a process involving enzymes capable of degrading extracellular matrix components.

Purpose—To examine the expression of heparanase in oral carcinomas and correlate its staining extent, intensity and cellular localization with patient's outcome.

Results—Quantitative real-time PCR revealed over 4-fold increase in heparanase levels in oral carcinomas compared with adjacent normal tissue. Normal oral epithelium was found negative for heparanase, while all oral carcinomas stained positively for heparanase. Heparanase staining was associated with Ki67 staining, a measure of cell proliferation. Notably, while cytoplasmic localization of heparanase was associated with high grade carcinomas, nuclear localization of the enzyme was found primarily in low grade, well differentiated tumors, and in all oral verrucous carcinomas.

Conclusion—Expression level and cellular localization of heparanase could serve as an important diagnostic marker in oral cancer patients.

Keywords

heparanase; staining; localization; nuclear; verrucous carcinoma

Introduction

Head & neck cancer accounts for about 5% of all cancers ^{1, 2}. Oral cancer accounts for roughly 30% of all head & neck cancers exhibiting high mortality which, despite progress in treatment, have not improved dramatically over the last three decades. Depending on the tumor site, the 5 year survival rate of oral squamous cell carcinoma (SCC) patients ranges from 30-80% ³ (61% for years 1999-2005) ¹. Thus, novel therapeutic modalities are needed

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to improve the prognosis for patients with advanced oral cancer. The etiology of oral cancer is multifactorial, and no single clearly recognizable cause has been characterized.

Heparanase is an endoglycosidase that specifically cleaves heparan sulfate (HS) side chains of HS proteoglycans 4-6, a major constituent of the extracellular matrix (ECM). Traditionally, heparanase activity was correlated with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence of HS cleavage and remodeling of the ECM barrier 7-8. Similarly, heparanase activity was implicated in cell dissemination associated with inflammation and angiogenesis 5. In recent studies, heparanase induction was noted in increasing number of hematological and solid tumors including carcinomas of the head and neck 9-11, conferring an invasive phenotype 12-14 and positioning heparanase as a potential target for anti-cancer drug development 13-15-17. Additionally, there is growing evidence that heparanase can up regulate the expression of genes associated with aggressive tumor behavior. These include VEGF, MMP-9, uPA/uPAR and tissue factor and likely others yet to be discovered 18-20. Elevated levels of heparanase are detected in the urine and plasma of cancer patients 21-23. Moreover, enhanced heparanase expression correlates with metastatic potential, tumor vascularity and reduced postoperative survival of cancer patients 12-14. The role of heparanase in tumorigenesis is further supported by findings that heparanase inhibitors 13-15-24 and gene silencing 25-26 can inhibit tumor growth and metastasis.

The present study was undertaken to investigate the expression, cellular localization and clinical significance of heparanase in oral squamous carcinoma. We report that heparanase is over expressed in the vast majority of oral carcinomas, associating with tumor differentiation level and inversely correlating with patient's survival. Furthermore, we investigated and compared between two types of oral carcinomas, squamous cell carcinoma and verrucous carcinoma which represents a non-metastasizing, well differentiated tumor. While cytoplasmic localization of heparanase was associated with high grade carcinomas, nuclear localization of the enzyme was found primarily in low grade, well differentiated tumors. Altogether, our study could assist in the diagnosis and prognosis of oral cancer patients and promote the incorporation of heparanase inhibitors into therapeutic regimens for oral cancer.

Materials and Methods

Patients

The study included archival paraffin embedded pathological biopsy specimens that were retrospectively collected from 50 oral cancer patients. The study cohort included 8 cases of verrucous carcinoma (Fig. 2C d, e) and 42 cases of SCC (Fig. 2C g, h); 15 lip SCC, and 27 intra-oral SCC. One verrucous carcinoma case was diagnosed as 'hybrid' (i.e., included a combination of verrucous carcinoma and SCC), while all the rest were diagnosed as 'pure' verrucous carcinomas. Patients were diagnosed and treated at the Department of Oral and Maxillofacial Surgery, Rambam Health Care Campus, Haifa, Israel. Inclusion criteria were oral carcinoma patients that were treated surgically according to standard procedures consisting of wide local surgical resection, with or without prophylactic neck dissection. Only patients with a confirmed diagnosis of oral/lip carcinoma, complete clinical and pathological record, and sufficient amount of paraffin embedded material were included in the study. A written informed consent was obtained from each patient before surgery, and the study was approved by the Institutional Ethics Committee, in accordance with the Helsinki guideline. Fresh biopsies were collected after Helsinki approval was issued and informed consent form was signed.

Real-time PCR

Fresh biopsies were collected from tumor lesions and adjacent normal-looking control tissue and total RNA was isolated with TRI Reagent according to the manufacturer's (Sigma, St. Louis, MO) instructions. Half of the normal-looking tissues were sent for pathological analysis to confirm no evidence of malignancy or pre-malignancy. The second half was used as control. Real-time quantitative PCR analysis was carried out essentially as described 27. Briefly, the PCR reaction mix (20 μ l) was composed of 10 μ l of QPCR primer and probe labeled with 6FAM dye (Taqman, Applied Biosystems, Carlsbad, CA), 5 μ l diluted cDNA (1:10), and 0.3 μ M of each primer (final concentration). Large ribosomal protein (RPLPO) primers (Applied Biosystems) were used as a reference gene for total RNA normalization. The sense and antisense primers used for quantitative amplification of heparanase were: forward GGACTTGCACGACTAA, reverse CCGTACGTCAATTGAC.

Immunohistochemistry

Staining of formalin-fixed, paraffin-embedded 5 micron sections was performed essentially as described 10: 28-30. Briefly, slides were deparaffinized with xylene, rehydrated and endogenous peroxidase was quenched using 3% hydrogen peroxide in methanol. Slides were then subjected to antigen retrieval by boiling (20 min) in citrate buffer, pH 6, blocked with 10% normal goat serum and incubated with the primary antibodies [anti-heparanase 733 polyclonal antibody diluted 1:100, or anti-Ki67 monoclonal antibody (MIB-1; Dako)]. Anti-heparanase antibody (733) was raised in rabbits against a 15 amino acid peptide that maps at the N-terminus of the 50 kDa heparanase subunit and preferentially recognizes the active 8+50 kDa heterodimer vs. the 65 kDa latent proenzyme 31. Slides were then extensively washed with PBS containing 0.01% Triton-100 and incubated with a secondary reagent according to the manufacturer's (Envision kit, Dako, Glostrup, Denmark) instructions. Following additional washes, color was developed with the AEC reagent (Sigma), sections were counterstained with hematoxylin and mounted, as described 28: 30. Slides were examined and scored by two expert pathologists blinded to the clinical data. The staining intensity and extent scores were assigned according to the following categories. Intensity: +1 = weak staining, +2 = moderate staining, +3 = high staining intensity. Extent: +1 = staining of less than 15% of the cells, +2 = 15-50% of the cells, +3 > 50% of the cells. Cellular localization of staining (cytoplasmic vs. nuclear) was also recorded. Specimens that were similarly incubated with pre-immune serum, or without the primary antibody, yielded no detectable staining.

Statistical analysis

Association between category groups was tested using the Chi-square or the Fisher exact test. Univariate association with survival and cause-specific survival were evaluated by Kaplan-Meier curves and tested using Log-Rank test. For the multivariate analysis of survival, the Cox's proportional hazard test was used to detect independent parameters that affect patient survival and to estimate relevant odds ratio (OR) with 95% confidence interval. For statistical analyses, cases exhibiting low (+1 and +2) staining intensity and extent were combined and compared with cases diagnosed to bear high levels (+3) of heparanase. Two-tailed p values of 0.05 or less were considered to be statistically significant. The SPSS 16.0 software for windows (SPSS Inc. Chicago, IL) was used for the statistical analyses.

Results

In order to examine the expression levels of heparanase by oral carcinoma, we employed quantitative real-time PCR analysis. Total RNA was isolated from fresh biopsies obtained from the carcinoma lesions and from adjacent normal tissue, and heparanase expression was

evaluated. Heparanase levels were increased 4.7-fold in average in the carcinoma lesion compared with adjacent normal tissue (Fig. 1A, $p=0.03$). A similar increase in heparanase levels was observed in oral carcinomas compared with benign oral lesions (including pre-malignant hyperplasia or hyperkeratosis and friction induced oral fibroma, Fig. 1B, $p=0.04$). Notably, no significant elevation in heparanase levels was observed between normal tissue adjacent to the carcinoma and benign tumors ($p=0.14$, not shown).

In order to further investigate the expression levels and clinical significance of heparanase in oral carcinoma, a retrospective immunohistochemical study was performed including 50 oral cancer patients (34 males and 16 females). Clinical description of patients is presented in Table 1. Median age at diagnosis was 69.2 ± 8 years; the mean follow-up was 49.6 ± 9.2 months for the entire study and 63.45 ± 12.1 months for patients who are still alive. Lower lip and buccal carcinoma were the predominant sites (30% each), followed by carcinomas of the tongue, maxilla and floor of mouth (Table 1). Most patients had early disease, as indicated by the T (primary tumor) and N (nodal metastasis) staging criteria (Table 1).

Expression of heparanase by oral carcinomas

All of the oral cancer biopsies were stained positively for heparanase, and were categorized according to the intensity and extent of staining. Weak heparanase staining (+1) (Fig. 1C, a) was detected in 20% (10/50) of the biopsies, moderate staining (+2) (Fig. 1C, b) was detected in 36% of the biopsies (18/50), and 44% (22/50) of the specimens exhibited a strong staining intensity for heparanase (+3) (Fig. 1C, c). Low heparanase extent (+1, staining of less than 15% of the cells) was detected in 20% (10/50) of the biopsies, moderate extent (+2, 15-50% of the cells) in 38% (19/50) of the biopsies, and high heparanase extent (+3, > 50% of the cells) was observed in 42% (21/50) of the biopsy specimens.

Heparanase staining intensity was found to be associated with the tumor level of differentiation (Table 2). Hence, low heparanase staining was found in 94% of the tumors diagnosed as well-moderate differentiation, whereas strong staining of heparanase was detected in 32% of the cases diagnosed as moderate-poorly differentiated tumors (Table 2; $p=0.01$). A similar correlation was found for the staining extent parameter (Table 2, $p=0.01$). This is emphasized given the correlation established between tumor differentiation level and the survival rate of oral carcinoma patients (Fig. 2A). Hence, patients exhibiting moderate-poorly differentiated carcinomas had poor prognosis compared with patients bearing well-moderate differentiation tumors ($p=0.007$; Fig. 2A).

Notably, high heparanase staining extent (>2) was associated with poor survival ($p=0.005$). We found that 74% of the patients exhibiting low staining extent of heparanase (≤ 2) were still alive at the end of the follow-up period, as opposed to the high heparanase staining group (>2) in which none of the patients survived by the end of the follow-up (Fig. 2B), suggesting that heparanase plays a role in oral cancer progression and patient's outcome.

Cellular localization of heparanase associates with tumor grade and type

Careful examination of the immunostaining revealed a distinct cellular localization pattern of heparanase. In addition to the more commonly observed cytoplasmic staining (Fig. 2C, c), heparanase was noted also to assume nuclear localization (Fig. 2C, f). Heparanase cellular localization was significantly associated with the tumor level of differentiation ($p=0.0007$, Table 2). Accordingly, cytoplasmic localization prevailed in patients with moderate-poorly differentiated carcinomas (63%; Table 2), whereas nuclear localization of heparanase was observed in 85% of the patients with well-moderately differentiated carcinomas (Table 2). Nuclear localization of heparanase was also significantly associated with the tumor type. Notably, in all the biopsies diagnosed with oral verrucous carcinoma (Fig. 2C, d, e)

heparanase staining was detected in the cell nucleus (Fig. 2C, f). In contrast, only 29% of the squamous cell carcinoma (SCC) biopsies exhibited nuclear localization of heparanase ($p=0.001$).

Heparanase staining and cellular localization correlate with Ki67 levels

In order to further reveal the significance of heparanase in oral carcinoma, heparanase staining and cellular localization was examined in relation to Ki67 as a measure for cell proliferation. Ki67 staining extent was found to be associated with tumor size. High extent of Ki67 staining (>2) was more frequent in large tumors (10/18, 56%) compared to low Ki67 staining extent (≤ 2) which was observed in 88% of the small tumors (28/32; Table 3; $p=0.001$), providing a clinical relevance for this parameter. Importantly, a significant association was found between the extent of Ki67 staining and heparanase cellular localization ($p=0.03$; Table 3). Clearly, cytoplasmic localization of heparanase was associated with high Ki67 extent, while nuclear heparanase correlated with low (≤ 2) Ki67 extent (Table 3), in agreement with favorable outcome of patients exhibiting nuclear localization of heparanase 10, 32.

Multivariate analysis was performed for demographic and pathological variables. The most significant factor affecting patients' survival was the tumor level of differentiation ($p=0.004$, Hazard Ratio 5.2). When heparanase extent parameter was included, this variable was found to be the most significant and independent predictor of survival in oral cancer patients ($p=0.02$, Hazard Ratio=5.7). When only well-moderate differentiated carcinomas were included in the statistical analysis, we found that the survival rate of patients diagnosed to bear weak heparanase staining (≤ 2) was 76% while none of the patients that stained strongly (>2) for heparanase survived. Although this difference is clinically important, it was not found to be statistically significant ($p = 0.1$), likely due to the low number of cases in each group. Nevertheless, it suggests that heparanase expression is a critical determinant for survival of oral cancer patients.

Discussion

Apart of specific cell types (i.e., cytotrophoblasts, keratinocytes, platelets, activated cells of the immune system), heparanase is hardly detected in normal epithelium 12. Applying real-time PCR (Fig. 1A, B) and immunohistochemical (Fig. 1C) analyses, the present study strongly supports previous reports documenting a significant increase in heparanase expression in various carcinomas and hematological malignancies 12-14, 24, 33. Notably, high levels of heparanase correlated with Ki67 staining extent (Table 3) and inversely correlated with the survival rates of oral cancer patients (Fig. 2B), depicting the clinical relevance of heparanase in oral cancer and positioning heparanase as a valid drug target. Recent studies performed on cultured cells and biopsy specimens, including head & neck carcinoma 10, indicated that heparanase also assumes nuclear localization. Nuclear localization of heparanase was demonstrated by cell fractionation, immunostaining and immunoblot analyses of cultured cells 34 and in tumor biopsies 32, 35. Notably, nuclear localization of heparanase correlated with sustained cellular differentiation and a favorable outcome in patients with esophageal 35, 36 and head & neck 10 carcinomas, and, as demonstrated here, for oral carcinoma (Table 2). Heparanase levels and cellular localization correlated with both the tumor level of differentiation (Table 2) and tumor type (verrucous vs. SCC). In the non-metastasizing verrucous carcinoma, heparanase exhibited nuclear localization (Fig. 2C f) in all cases, as opposed to SCC which expressed the heparanase protein in the cell cytoplasm in 71% of the biopsy specimens. While a role for nuclear heparanase in the regulation of gene expression and cell differentiation was suggested both *in vitro* and *in vivo* 32, 35-37, the mode of its translocation into the nucleus and the associated effect on gene expression, have not been elucidated. Transfection of MCF-7

breast carcinoma cells with a plasmid vector that targets heparanase exclusively into the nucleus induced the appearance of lipid droplets typical of differentiated mammary epithelial cells 37. This and other results further substantiate a role of heparanase in cellular differentiation, as also suggested by the presently observed preferential nuclear localization of heparanase in well differentiated vs. poorly differentiated SCC (Table 2). Heparanase was noted to translocate from the cytoplasm to the nucleus during esophageal cell differentiation, correlating with induction of keratinocyte differentiation markers such as p27 and involucrin. p27 and involucrin induction was significantly reduced upon inhibition of heparanase enzymatic activity 36, suggesting that HS degradation by heparanase directly affects gene expression, in agreement with previous studies demonstrating nuclear localization of HS 38, 39. Notably, appreciable degradation of heparan sulfate takes place at nearly physiological pH (i.e., pH 6.8-7.0) 40, well above the pH optimum of heparanase. Thus, the enzyme is capable of degrading HS in the nucleus, as in fact suggested by our studies with nuclear preparations (34; and data not shown).

HS has been associated with several functional roles within the nucleus, including suppression of gene transcription via inhibition of topoisomerase I and HAT activity 41, 42. HATs regulate gene expression by catalyzing acetylation of the N-terminus region of histones, thereby modifying chromatin structure in a manner that facilitates transcriptional activation 43. Decreased nuclear syndecan-1 following up-regulation of heparanase expression was recently reported 44, possibly leading to increased histone acetylation with an associated induction of gene transcription. It should be noted that both heparanase and syndecan-1 lack a characteristic nuclear localization signal 45 and thus may require a binding partner to shuttle into the nucleus. It has been recently speculated that heparanase acts as a master regulator of the aggressive tumor phenotype 19, 27. This is apparently accomplished via heparanase effects on multiple cell functions including gene expression. It is conceivable that heparanase regulation of gene expression may be related to its ability to inhibit accumulation of HSPGs within the nucleus 44. Thus, strategies to enhance nuclear HS levels may prove effective in blocking at least some of the heparanase-mediated effects that promote tumor growth and metastasis 44. Cell lines originating from verrucous carcinoma may serve as a good model system to study nuclear translocation of heparanase and its consequences in term of gene transcription and cellular differentiation.

Taken together, heparanase expression is induced in oral cancer as revealed by real-time PCR and immunohistochemical analyses. Immunohistochemical analysis identifies heparanase levels critical for oral cancer patients' survival, likely due to enhanced Ki67 labeling as indicative of cell proliferation. Furthermore, by multivariate analysis, heparanase staining extent was found to be the most significant and independent predictor of survival in oral cancer. Heparanase levels and cellular localization may aid in the diagnosis of oral cancer patients, while heparanase inhibitors are hoped to advance future therapeutic modalities for cancer patients.

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Figure 1.

Heparanase expression by oral cancer. A-B. Real-time PCR. **A.** Total RNA was isolated from fresh biopsies obtained from oral carcinoma lesions (■) and adjacent normal-looking control tissue (□). Heparanase expression was determined by real-time PCR analysis as described under 'Material and Methods' and presented as fold-increase ($\pm$ SE) in the carcinomas compared with control adjacent tissue set arbitrarily to a value of 1. **B.** Total RNA was isolated from benign (□) and malignant SCC (■) and heparanase expression was determined as above. **C.** Immunohistochemical analysis. Formalin-fixed, paraffin-embedded 5 μ m sections of well-moderate SCC were subjected to immunostaining as described under 'Materials and Methods'. Shown, are representative photomicrographs of heparanase staining diagnosed as low (+1, **a**), moderate (+2, **b**) and strong (+3, **c**) staining intensity. **d-i.** Staining localization. Nuclear staining, mostly seen in oral verrucous carcinoma, is shown in (**f**), while cytoplasmic localization prevails in SCC (**i**). Hematoxylin/eosin staining of verrucous carcinoma is shown in (**d**, **e**). These lesions are characterized by hyperkeratosis with a verruciform surface, wide and elongated rete ridges, and minimal cytological atypia. Hematoxylin/eosin staining of SCC is shown in (**g**, **h**). No detectable staining was noticed in control sections incubated with preimmune serum instead of the primary antibodies. Original magnification: A-C, F, I $\times$ 200; D, G $\times$ 50; E, H $\times$ 100.

Figure 2.

Patients' survival. **A.** Overall survival curves of patients with oral cancer stratified by the tumor level of differentiation. Kaplan-Meier survival analysis revealed poor survival of patients with moderate-poorly differentiated tumors compared to those with well-moderate differentiated tumors. As expected, patients diagnosed with moderate-poorly differentiated tumors had a significantly poor prognosis ($p=0.0072$, Log-Rank) as compared to well-moderate differentiated tumors. Verrucous carcinomas were graded as well differentiated carcinomas. **B.** Overall survival curves of patients with oral cancer stratified by heparanase staining extent. Kaplan-Meier survival analysis revealed poor survival of patients expressing high levels of heparanase (>2). All patients exhibiting high heparanase staining ($+3$) died at the end of follow-up (median survival time 59.9 months, $p = 0.0056$, Log-Rank), while 74% of the patients exhibiting low heparanase staining (≤ 2), were still alive at the end of the follow-up period (median survival time not reached).

Table 1

Clinical description of patients

Parameter	Patients [n (%)]
Site of tumor	
Lip (lower)	15 (30)
Flore of mouse	5 (10)
Buccal mucosa	15 (30)
maxilla	6 (12)
tongue	7 (14)
other	2 (4)
T stage	
T0-2	41 (82)
T3	9 (18)
T4	0
N stage	
N0	41 (82)
N1	4 (8)
N2	5 (10)
N3-4	0
Tumor differentiation	
Well	25 (50)
Well-moderate	3 (6)
moderate	15 (30)
Moderate-poor	5 (10)
poor	2 (4)

Table 2

Correlation between heparanase levels, cellular localization and tumor differentiation.

	Differentiation levels (%)		Total	P (Chi square)
	Well-moderate	Moderate-poor		
Heparanase intensity				
≤2	32 (94)	2 (6)	34	0.01
>2	11 (68)	5 (32)	16	
Total	43	7	50	
Heparanase extent				
≤2	28 (96)	1 (4)	29	0.01
>2	15 (71)	6 (29)	21	
Total	43	7	50	
Heparanase localization				
Cytoplasm	11 (37)	19 (63)	30	0.0007
Nucleus	17 (85)	3 (15)	20	
Total	28	22	50	

Table 3

Ki67 staining extent correlates with tumor size and heparanase cellular localization.

	Heparanase localization (%)		p	Tumor size (%)		p
	Cyto.	Nuclear		<2.5 cm	>2.5 cm	
Ki67 extent			0.03			0.001
<2	20 (51)	19 (49)		28 (88)	4 (12)	
>2	10 (90)	1 (10)		8 (44)	10 (56)	
Total	30	20		36	14	