

Clinicopathological significance of heparanase and basic fibroblast growth factor expression in human esophageal cancer

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Abstract

AIM: Human heparanase is an endo-D-glucuronidase that degrades heparan sulfate/heparin and has been implicated in a variety of biological processes. The objective was to investigate the expression of heparanase (Hps) and basic fibroblast growth factor (bFGF) and their relationship to neoangiogenesis and metastasis of human esophageal carcinoma.

METHODS: Seventy-nine patients who had undergone esophageal resection for esophageal carcinoma without preoperative treatment were included in the present study. Immunohistochemistry was used to study the expression of Hps, bFGF and microvessel density (MVD) in 79 cases of esophageal carcinoma. bFGF and Hps were quantitatively detected with immunohistochemistry in 79 cases of human esophageal carcinoma and 19 cases of adjacent normal human esophageal carcinoma. Cd34 was used to explore the MVD as a marker of endothelial cells.

RESULTS: Hps and bFGF expression in tumor tissue, being remarkably higher than that in normal esophageal tissue, were significantly correlated with clinicopathological features (depth of invasion, lymph-node metastasis and TNM stage) and MVD.

CONCLUSION: The results of this study suggest that the coexpression of Hps and bFGF plays a key role in angiogenesis, invasion and metastasis of esophageal carcinoma. Hps and bFGF may serve as a predictor of progression in esophageal carcinoma. The expression of heparanase in esophageal carcinoma enhances growth, invasion, and angiogenesis of the tumor, and bFGF seems to be a potent antigenic factor for esophageal carcinoma.

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INTRODUCTION

For a malignant tumor cell to metastasize, it must break away from its neighbors, force its way through the surrounding stroma, and penetrate basement membranes to enter the circulation. When it arrives at its destination, these steps must be repeated in reverse order. A critical event in the process of cancer invasion and metastasis is therefore degradation of various constituents of the extra cellular matrix (ECM) including collagen, lamina, fibronectin, and heparin sulfate proteoglycans. The cell is able to accomplish this task through the concerted action of enzymes such as metalloproteinase, serine proteases, and endoglycosidases. Two essential processes required for metastasis are neoangiogenesis and tumor cell invasion of the basement membrane and ECM^[1]. H-S is an essential component of the extra cellular matrices of most tissues and is also a prominent component of blood vessels, which is essential for insolubility of the extra cellular components, cell adhesion, and locomotion^[2-10]. Accordingly, cleavage of H-S by heparanase enzyme may play a decisive role in extravasations and invasion of tumor cells. So far, heparanase activity has been detected in various tumors and was found to correlate with their metastasis potentials^[2,11-15]. Meanwhile, heparanase may also contribute to angiogenesis by releasing the H-S-bound growth factors such as basic fibroblast growth factor (bFGF)^[6]. However, because the characterization and cloning of the enzyme has remained elusive until the recent reports of Hullet *et al*^[2], and Vlodaysky *et al*^[6], studies aiming at detection and evaluation of heparanase production and its *in vivo* biological role in patients with different malignancies have been hindered. Moreover, many study groups have evaluated the role of different growth factors aiming at elucidation of the biological predictor of angiogenesis in tumor.

bFGF is a potent antigenic growth factor that requires heparin or H-S for its biological activity mediated through tyrosine kinase signaling^[16-20]. The activity of bFGF is stringently controlled because it can be inactive in normal tissues and becomes activated upon tissue injury, inflammation, and tumor invasion^[21]. Heparanase enzyme possesses the ability to activate bFGF through structural modulation of the cell surface H-S proteoglycan^[22]. Accordingly, heparanase and bFGF could play complementary biological roles

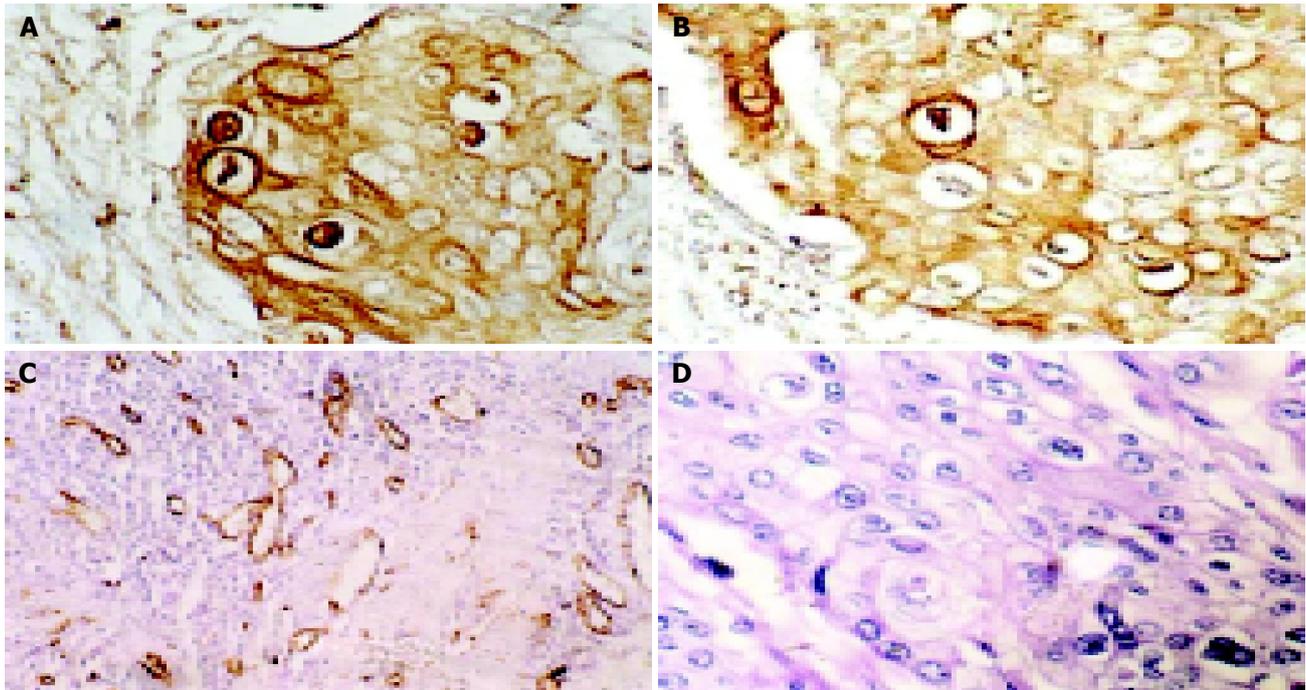


Figure 1 Immunohistochemistry SP staining and HE staining A: Heparanase-positive (nucleolus, cytoplasm, amphithecium) SP×400 B: bFGF-heparanase-positive (cytoplasm, amphithecium) SP×400 C: MVD as evaluated by immunohistochemistry with CD34 (SP×200) D: HE staining of EC SP×400.

in tumor angiogenesis and invasion. To our knowledge, expression of heparanase and its biological role in connection with bFGF expression in human esophageal carcinoma have not been evaluated so far. In the present study, we tried to find out whether bFGF and heparanase were both directly correlated to angiogenesis in human esophageal carcinoma, whether heparanase expression was associated with the degree of tumor invasiveness or not, and whether the coexpression of heparanase and bFGF enhanced tumor angiogenesis compared with expression of either factor alone.

MATERIALS AND METHODS

Patients

Seventy-nine patients undergoing esophageal carcinoma resection between 1996 and 2002 were included in the present study. None of the patients had received preoperative chemo- or embolic therapy.

The patients' ages ranged from 40 to 73 years. Tumors were staged at the time of surgery by the standard criteria for TNM staging using the Unified International esophageal carcinoma staging classification and the following morphological details were recorded: depth of invasion (pT category), lymph node involvement (pN category).

Tissue specimens

Esophageal carcinoma tissues from all of the patients were taken from the areas of tumor immediately after surgical resection. Nineteen surrounding esophageal carcinoma tissues were included. Surrounding esophageal carcinoma tissue specimens were obtained from tissues at a clear distance from the tumor edge (>5 cm), there was no evidence of nearby tumor invasion.

Antibodies and other chemicals

The following reagents were purchased from Maxim Biotech (Macim Biotech Inc., South San Francisco, CA, USA); heparan sulfate proteoglycan (RT-794), fibroblast growth factor, basic (b-FGF) (RAB-0305), CD34 (MAB-0034).

Immunohistochemistry

Immunohistochemistry was performed as described before with minor modifications^[19]. Briefly, 5- μ m sections were deparaffinized and rehydrated. Tissue was then denatured for 3 min in a microwave oven in citrate buffer (0.01 mol/L, pH 6.0). Blocking steps included successive incubations in 0.2% glycine, 3% H₂O₂ in methanol, and 5% goat serum. The first two steps were followed by two washes in phosphate-buffered saline (PBS). Sections were incubated with a monoclonal anti-human heparanase antibody diluted 1:15 in PBS, followed by incubation with horseradish peroxidase-conjugated goat-anti-mouse IgG+IgM antibodies. The preparation and specificity of this mAb have been previously described and demonstrated^[19]. Color was developed using either Sigma Fast 3,3'-diaminobenzidine tablet sets for 10 min followed by counterstain with Mayer's hematoxylin.

Statistics

The correlation between the expression of heparanase or bFGF and micro vessel density (MVD) and clinicopathological features was analyzed. For statistical significance, the χ^2 test was used. Postoperative survival periods were computed by the method of Kaplan-Meier and compared by using the log-rank test. $P < 0.05$ was taken as the level of significance for all tests. All statistical analyses were performed using the SPSS 10.0 statistical package (USA).

Table 1 Expression of Hps, bFGF, MVD in esophageal carcinoma and normal esophageal tissue

Type	n	Heparanase-positive (%)	bFGF-positivity (%)	MVD
Esophageal carcinoma	79	52 (65.8)	57 (72.2)	30.59±13.38
Normal esophageal tissue	19	0	3 (15.8)	13.82±4.16

Table 3 Relationship between MVD and heparanase and bFGF expression

Parameter	Hps and bFGF	bFGF and MVD	HPS and MVD
Correlation coefficient (r)	0.553	0.687	0.760
P	0.000 ^b	0.000 ^b	0.000 ^b
Remark	Significant linear correlation	Significant linear correlation	Significant linear correlation

Note: 0.01 (two-tailed); it shows that coexpression of both heparanase and bFGF was significantly correlated with MVD (^b $P < 0.001$).

RESULTS

Expression and clinical significance of heparanase

In Table 1 Fifty-two (65.8%) esophageal carcinoma were heparanase-positive (Figure 1A and B) and 27 (34.2%) tumors had no detectable level of heparanase. Expression of heparanase was significantly higher in tumors than in normal esophageal tissue ($P = 0.01$). Heparanase expression was also associated with depth of invasion, clinical stages and lymph-node metastasis. All were significantly higher in heparanase-positive tumors compared with heparanase-negative tumors ($P < 0.05$). However, there was no significant correlation between heparanase expression and tumor cell differentiation, age and gender ($P > 0.05$).

Expression of bFGF and clinicopathological features of esophageal carcinoma

The bFGF-positive rate was significantly higher in esophageal carcinoma compared with that in the surrounding normal tissues ($P < 0.01$), so was heparanase expression. There was a significant correlation between bFGF expression and depth of invasion, clinical stages and lymph-node metastasis ($P < 0.05$ Table 2).

Expression of bFGF and heparanase in tumor was

significantly positively correlated with MVD expression (Figure 1C and D) ($P < 0.001$; Table 3). A simple regression model was used to evaluate the correlation between MVD and bFGF and heparanase. There was a direct linear relationship between the positive expression of bFGF and heparanase and the MVD in each individual tumor ($P < 0.001$). This indicates that bFGF and heparanase are directly correlated with angiogenesis in esophageal carcinoma.

Table 3 shows that coexpression of both heparanase and bFGF was significantly correlated with MVD ($P < 0.001$).

Based on the level of heparanase-positivity, we divided heparanase-positivity into three groups (high; moderate; low). Postoperative survival periods were computed by the method of Kaplan-Meier and compared by using the log-rank test. (Table 4) Level of heparanase-positivity negatively correlated with the postoperative survival periods (Figure 2, Table 5, $P < 0.001$).

Analysis of prognosis was computed by the method of Cox proportional hazard model. It suggested that positive expression of heparanase and bFGF, lymph node and distant metastasis and MVD were hazardous factors of postoperative survival periods.

Table 2 Expression of Hps, bFGF, MVD in esophageal carcinoma

Parameter	Hps				bFGF			MVD		
	n	+	Positive ratio(%)	P	+	Positive ratio(%)	P	Mean	SD	P
Gender										
Male	60	40	66.7	0.787 ^a	43	71.7	0.864 ^a	35.32	7.55	
Female	19	12	63.2		14	73.7		37.23	4.17	0.317 ^a
Age (yr)										
<60	50	33	66	1 ^a	35	70	0.614 ^a	35.59	3.87	0.876 ^a
≥60	29	19	65.5		22	75.9		36.07	6.11	
Tumor cell differentiation										
High	27	17	63.0	0.921 ^a	20	74.1		33.13	7.93	
Moderate	33	22	66.7		23	69.7	0.918 ^a	31.92	5.29	0.878 ^a
Low	19	13	68.4		14	73.7		32.52	6.17	
Depth of invasion										
T ₁ +T ₁ +T ₂	28	10	35.7	0 ^c	12	42.9	0 ^c	23.78	4.16	0 ^c
T ₃ +T ₄	51	42	82.4		45	88.2		42.01	5.40	
Lymph-node metastasis										
N ₀	32	14	43.8	0.001 ^c	14	43.8		31.87	7.17	
N ₁	47	38	80.9		33	70.2	0.019 ^b	38.44	5.74	0.051 ^a
Clinical stages										
0+I+II	38	20	60.5	0.017 ^b	23	60.5		35.95	5.19	
II+IV	41	32	70.7		34	82.9	0.026	43.19	7.15	0.375 ^a

Note: ^a $P > 0.05$ no significant difference; ^c $P < 0.05$ statistical difference; ^b $P < 0.001$ significant difference.

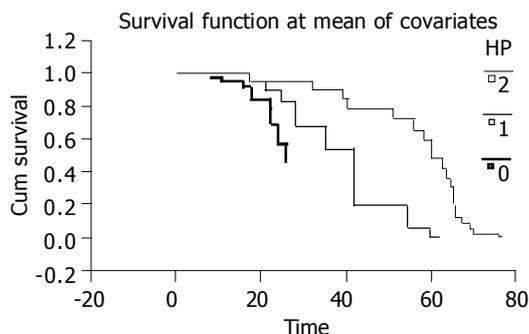


Figure 2 Heparanase-negative; 1 = heparanase-positive; 2 = strongly heparanase-positive.

DISCUSSION

H-S proteoglycan is present in the basement membrane of every vascularized organ and in the tumor stroma of several human cancers^[22]. A major function of the proteoglycans is attributed to the properties of H-S^[23], which is essential for insolubility of the extracellular components, cell adhesion, and locomotion^[6-10]. H-S also works as a storage depot for active growth factors, of which bFGF is the most extensively studied^[24]. Thus, acquisition of heparanase and splitting of H-S by a given tumor would offer such a tumor two essential features of malignancy: volatilization of the other extracellular matrix constituents, facilitating tumor invasion through blood vessels and tissues; and releasing and activation of H-S-binding growth factors and, hence, enhancing the tumor angiogenesis.

In our results, there was a direct correlation between heparanase expression and angiogenesis in esophageal carcinoma. Tumors with positive heparanase expression had a significantly higher MVD compared with heparanase-negative tumors. Accordingly, we can conclude that heparanase expression has an axial role not only in the tumor growth and invasion but also in the angiogenesis of esophageal carcinoma.

In the present study, the expression of bFGF was significantly higher in esophageal carcinoma compared with that in the surrounding and normal esophageal tissue, indicating that its up-regulation was involved in the tumor biology. Moreover, bFGF was directly correlated with MVD of esophageal carcinoma. bFGF induces neovascularization through various mechanisms including a potent mitogenic effect on the vascular and capillary endothelial cells^[19,22], stimulation of endothelial migration and capillary formation, and production of plasminogen activators, proteases that are involved in the invasive property of endothelial cells during angiogenesis^[25].

Table 4 Log-rank analysis of postoperative survival periods

	Statistics	df	P
Log rank	19.64	2	0.0001 ^b
Breslow	15.66	2	0.0004 ^b
Traone-ware	17.62	2	0.0001 ^b

^bP<0.001 significant difference.

Table 5 Variables in the equation

Variables	P	Odds ratio	95% CI for Exp(B)
T	0.366	1.181 ^a	0.824-1.692
N	0.035	2.430 ^a	1.065-5.543
BFGF	0.021	2.032 ^a	1.111-3.716
MVD	0.047	1.038 ^a	1.000-1.077
M	0.002	11.221 ^a	-2.427-51.880
Hps	0.000	3.390 ^a	2.046-5.615
Clinical stage	0.207	1.256 ^a	0.819-1.924

Remark: ^aP>1 hazardous factor.

Moreover, heparanase expression was directly correlated with tumor size. Generally, the tumor size reflects tumor growth that is the outcome of many integrated factors, including the availability of enough nutritional support through abundant blood supply (angiogenesis) and of proliferation stimuli from active growth factors. Heparanase may influence the bioavailability of different growth factors including FGFs^[26-29], vascular endothelial growth factor^[30], hepatocyte growth factor^[31], and PDGF^[32], which are stored in H-S and possess H-S-binding sequences. It is quite reasonable to assume that the release of such growth factors may influence tumor growth and angiogenesis.

In the current study, there was an obvious synergetic effect of heparanase and bFGF expression in tumor. Coexpression of both factors was associated with higher MVD compared with expression of either factors alone. Such synergetic action may be attributed to the direct ability of heparanase to solubilize the components of ECM, which enhances endothelial cell migration during neovascularization. On the other hand, heparanase increases the biological activity of bFGF as well as of other H-S-bound growth factors like VEGF, PDGF, and HGF^[33-37], which are expected to further enhance the angiogenic effect.

In conclusion, expression levels of heparanase correlate negatively with patient survival, suggesting a role of basement membrane and ECM-degrading enzymes in tumor microenvironment alterations that facilitate esophageal carcinoma cell growth, invasion, and metastasis formation. Therefore, the development of drugs acting as inhibitors or blocking agents of hps action may add a new therapeutic modality in the future treatment of pancreatic cancer.

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