Quantitative Analysis of Heparanase Gene Expression in Normal Cervical, Cervical Intraepithelial Neoplastic, and Cervical Carcinoma Tissues

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Abstract: Heparanase is an endoglycosidase that specifically cleaves heparan sulfate side chains of heparan sulfate proteoglycans, the major proteoglycans in the extracellular matrix and cell surfaces. Traditionally, heparanase activity was implicated in cellular invasion associated with angiogenesis, inflammation, and cancer metastasis. More recently, heparanase up-regulation was documented in an increasing number of primary human tumors. In this study, we sought to investigate the expression of heparanase messenger RNA (mRNA) in normal cervical tissue and intraepithelial cervical lesion and its clinicopathologic importance in invasive cervical cancer. Gene expression of heparanase was assessed by quantitative real-time reverse transcriptase polymerase chain reaction in 28 normal cervical, 26 intraepithelial neoplastic, and 48 cervical cancer tissue samples. Heparanase mRNA expression was different between the 3 groups and lower in normal cervical specimens in relationship with intraepithelial cervical lesions and invasive cervical cancer tissue samples ($P = 0.048$). Gradually increasing expression of heparanase was evident as the cells progressed from low-grade to high-grade squamous intraepithelial lesions ($P = 0.002$). In invasive cervical cancer cases, there was a direct correlation between heparanase expression and tumor size ($P = 0.002$). In cases treated with radical hysterectomy and pelvic lymphadenectomy, the heparanase mRNA expression was significantly higher in tumors exhibiting lymph vascular space invasion ($P = 0.044$) and in cases with big tumor size ($P = 0.005$). In our study, we did not find any significant correlation between disease-free and overall survival rates and expression of heparanase ($P = 0.396$ and $P = 0.712$, respectively). The results of this study suggest that the gene expression of heparanase in cervical cancer enhances growth, invasion, and angiogenesis of the tumor and may have therapeutic applications.

Key Words: Heparanase, Cervical cancer, Intraepithelial cervical lesion

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Although cervical cancer incidence and mortality rates have declined for the past 3 decades, the disease remains a serious health threat. Cervical cancer is preventable and curable if detected early. Important strategies to reduce the risk of cervical cancer include massive human papillomavirus (HPV) vaccination of the population for primary prevention and the widest possible regular screening with standard Papanicolaou and HPV-DNA tests as complementary tests for early (noninvasive) detection of the disease. Researchers have identified specific HPV subtypes transmitted through sexual contact, as the main cause of cervical cancer. Tumor cell invasion and subsequent distant spread via blood and lymph

vessels are critical steps in tumor progression including cervical cancer.

Heparan sulfate proteoglycans (HSPGs) are found in extracellular matrices and on cell surfaces, playing critical functions in cell-cell and cell-matrix interactions.[1] In fact, transmembrane HSPGs (syndecans) are emerging as molecules that mediate cell interactions with components of the microenvironment that control cell shape, adhesion, proliferation, and differentiation.[2,3] In addition, cell-associated HS can potentiate the interaction of soluble growth factors with cell surface receptors, and its binding can also protect growth factor cleavage by proteolytic enzymes.[4,5] Furthermore, HSPGs are also prominent components of endothelial cells[6] and the basement membrane.[7]

Heparanase is a mammalian endo-β-D-glucuronidase that is capable of degrading HS chains of proteoglycans, a key component of the extracellular matrix and the basement membrane. The oligosaccharides so generated lead to the release of a variety of bioactive molecules, such as growth factors, chemotactic agents, and angiogenic agents, which are then deposited in the extracellular matrix and basement membrane. These molecules can stimulate cell proliferation, increase cell survival, and promote angiogenesis, morphogenesis, and vascularization.[8] Fragments of HS generated by heparanase can also induce the maturation of dendritic cells and activate macrophages, thereby stimulating the release of factors such as interleukins 1 and 6 and prostaglandin E_2, which modulate immune cell responses.[9,10] Furthermore, protein or messenger RNA (mRNA) expression of heparanase has been identified in various cancer cells, and its overexpression in tumor cells has also been reported to correlate with metastatic potential and poorer prognosis.[11,12]

The purposes of this study were to evaluate the expression of heparanase mRNA in normal cervical tissue and intraepithelial cervical lesion and its clinicopathologic importance in invasive cervical cancer by using a quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) method.

MATERIALS AND METHODS

Patients and Tissue Samples

The patient population consisted of 28 individuals undergoing a surgical intervention for benign gynecological disease with normal cervical tissue, 26 women with intraepithelial cervical lesion histopathologically determined, and 48 women with microinvasive or invasive cervical cancer. Biopsy specimens were obtained before initiation of treatment. All specimens were partitioned into 2 equal portions. One sample was snap frozen and stored at −80°C until required for RNA extraction. The second sample was fixed in 10% formaldehyde solution for histopathological examination. We classified the intraepithelial lesions according to the Bethesda system in 9 low-grade squamous intraepithelial lesion (LG-SIL) and in 17 high-grade squamous intraepithelial lesion (HG-SIL).

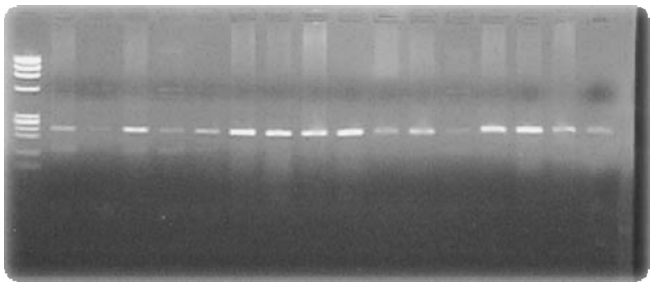

FIGURE 1. Detection of mRNA for PBGD in cervical cancer. It shows the negative expression of housekeeping gene in few tissue samples.

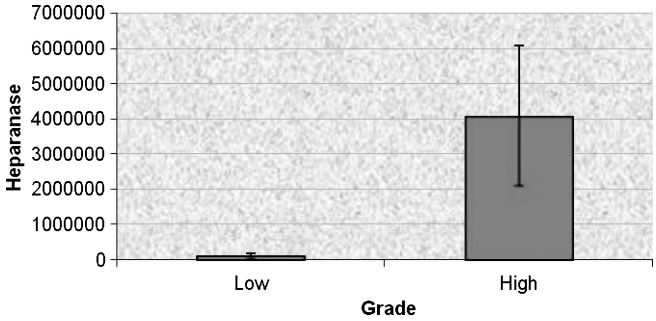

FIGURE 2. Heparanase mRNA expression was significantly higher in HG-SIL than LG-SIL.

Staging of the patients with cervical cancer was based on the International Federation of Gynecology and Obstetrics (FIGO) staging system: 1 was IA1, 20 were IB, 19 II, and 8 III stages. Histological type of tumors was assigned according to the World Health Organization classification: 39 were classified as squamous cell carcinoma, 8 as adenocarcinoma, and 1 as adenosquamous carcinoma. The median age at the time of diagnosis was 54 years (range, 35–80 years). Radical hysterectomy and pelvic lymphadenectomy were performed on 24 patients with stage IB and IIB disease. Total abdominal hysterectomy was performed on 1 patient with stage IA1. Patients with lymph node metastasis, parametrial involvement, and deep stromal invasion were treated with adjuvant external whole pelvic irradiation or adjuvant combination chemotherapy. Twenty-three patients were treated primarily with radiotherapy or concurrent chemoradiotherapy. Disease-free and overall survivals were defined as the interval from the initial therapy to clinically or radiologically proven recurrence and death, respectively. The median duration of follow-up was 23.2 months (range, 2–37 months).

RNA Extraction From Tissue and Quantitative RT-PCR

Total cellular RNA was extracted from the tissue specimens using the TRIZOL reagent (Invitrogen) as described by the manufacturer. Quantitative determination of RNA concentration was performed with NanoDrop 1000 Spectrophotometer (NanoDrop Technologies). Complementary DNA (cDNA) synthesis was performed with the Thermoscript RT-PCR System (Invitrogen) protocol for reverse transcription using 1 μg of total RNA. RNA integrity and cDNA quality were tested for all samples by PCR amplification of the porphobilinogen deaminase (PBGD) housekeeping gene. Using this gene as internal control, we excluded 12, 4, and 2 of the tissue samples of cervical cancer, intraepithelial lesion, and normal cervix, respectively, from the initial total number of samples. The primer sequences for amplification of heparanase gene were 5′-GGG ACC TCA TGG ATT ACT TTC CAA A-3′ (forward) and 5′-GCA ACT TTG GCA TTT CTT ATC ACA A-3′ (reverse), and the probe was 5′ FAM-CAG GAA TTC ACT GGG CTT GCC AGC TTT CTC A-TRAM 3′. The primer sequences for amplification of PBGD gene were 5′-GGT-GGG-TGT-GCT-GCA-CGA-T-3′ (forward) and 5′-ATC-TTC-ATG-CTG-GGC-AGG-GA-3′ (reverse), and the probe was 5′FAM-ATG AAG GAT GGG CAA CTG TAC CTG ACT GG-TRAM 3′. Expected RT-PCR product sizes were 171 base pairs for heparanase gene and 150 base pairs for PBGD. Heparanase cDNA amplification

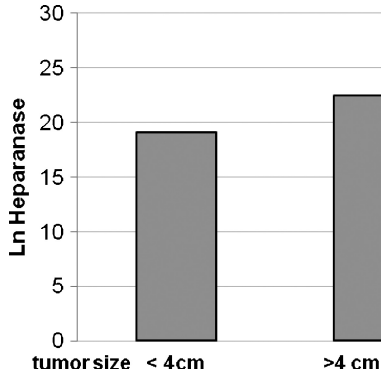

FIGURE 3. Association between heparanase mRNA expression and tumor size in all cervical cancer cases.

was initiated with denaturation for 5 minutes at 94°C followed by 45 cycles of 45-second denaturation at 94°C, annealing at 62°C for 30 seconds, followed by extension at 72°C for 45 seconds. The PCR mixture was maintained at 72°C for 5 minutes for final extension. The RT-PCR products were also visualized on 2% agarose gels stained by ethidium bromide. Ultraviolet-illuminated gels were photographed using Polaroid film with Polaroid Gel Cam, DS-34 machine (Fig. 1).

Quantification of heparanase gene expression was performed with a quantitative real-time RT-PCR in the LightCycler (Roche Diagnostics). We used the absolute value of heparanase mRNA obtained using a serial dilution of DNA internal standard preparation by MCF7 human breast carcinoma cells from the American Type Culture Collection (Manassas, VA),[13] and this result was directly calculated by the LightCycler analysis software.

Statistical Analysis

The statistical analysis of heparanase description was based on the assessment of the geometrical mean with the respective 95% confidence intervals (CIs). The relative mRNA expression levels among clinicopathologic parameters were evaluated by the generalized lineal models. Univariate analysis included χ^2 statistic. The disease-free and overall survival rates with respect to heparanase and prognostic factors were calculated by the Kaplan-Meier method and were evaluated by the log-rank test. The median heparanase values of each group were used for the statistical comparisons, whereas the median value of heparanase between the cancer tissue samples was used for cutoff. $P \leq 0.05$

TABLE 1. Association between heparanase mRNA expression and clinicopathological factors in all cervical cancer cases (n = 48)

Parameters	No. Cases	Heparanase mRNA Expression, Mean (95% CI)	*P*
Parametrial invasion			
No	26	21.983 (20.332–23.635)	NS
Yes	22	20.205 (18.786–21.624)	
Stage			
I	21	21.306 (19.773–22.839)	NS
II	19	21.010 (19.780–22.240)	
III	8	20.966 (18.866–23.067)	
Tumor size, cm			
≤4	26	19.701 (18.406–20.996)	0.002
>4	22	22.487 (21.271–23.703)	
Histological cell type			
SCC	39	20.569 (19.819–21.319)	NS
Non-SCC	9	21.620 (20.036–23.203)	

SCC, squamous cell carcinoma, NS, not significant.

TABLE 2. Association between heparanase mRNA expression and clinicopathological factors in cervical cancer cases undergoing in surgical intervention (n = 25)

Variables	No. Cases	LN Heparanase Expression	*P*
Histological cell type			
SCC	21	23.215	NS
Non-SCC	4	22.580	
Tumor size, cm			
≤4	22	20.334	0.005
>4	3	25.460	
Stromal invasion			
≤1/2	13	23.190	NS
>1/2	12	22.605	
Stage			
I	19	23.415	NS
II	6	22.380	
Lymph node metastasis			
Negative	22	22.724	NS
Positive	3	23.071	
LVS involvement			
Negative	17	21.556	0.044
Positive	8	24.239	

LN, naturale logaritme.

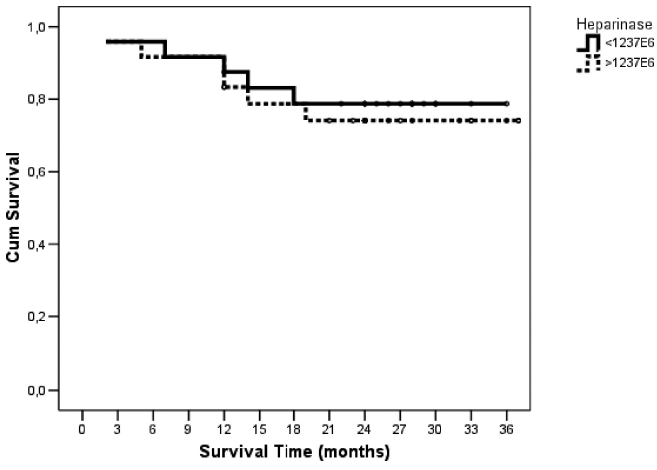

FIGURE 4. Survival curves in the groups with low and high heparanase mRNA expression.

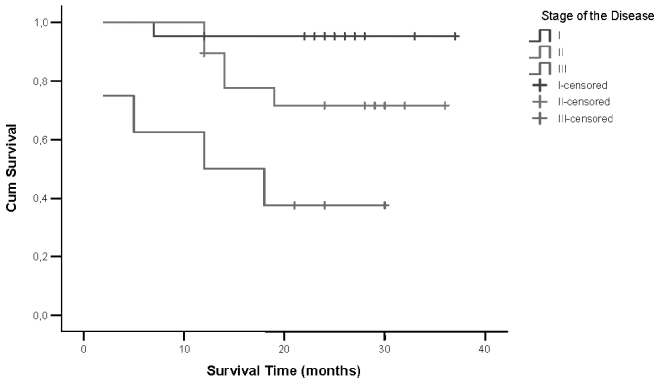

FIGURE 5. Survival curves of the 48 patients with cervical cancer according to the FIGO stage.

was considered statistically significant and $P < 0.1$ as borderline significant.

RESULTS

Using the mean value of mRNA heparanase into the normal controls, we can see that the normal cervical tissue samples had significantly lower mRNA expression (39.3%) in relationship with the other 2 groups (65.4% and 66.7%; $P = 0.048$). Heparanase mRNA expression was significantly higher in HG-SIL comparing to LG-SIL ($P < 0.001$; Fig. 2).

Heparanase mRNA expression was significantly higher in cases with tumor size bigger than 4 cm compared with those 4 cm or smaller ($P = 0.002$; Fig. 3). No meaningful differences in heparanase expression were observed with respect to parametrial invasion, stage and histological cell types (Table 1). In cases treated with surgical intervention, heparanase mRNA expression was significantly higher in tumors exhibiting lymph vascular space invasion ($P = 0.044$) and in tumors with size bigger than 4 cm ($P = 0.005$). No correlation was found between heparanase mRNA expression and other clinicopathological factors (Table 2).

The 48 patients with cervical cancer were divided into 2 subgroups (high heparanase mRNA expression group: >1273E6, n = 24; low heparanase mRNA expression group: <1273E6, n = 24) according to the median heparanase of the 48 tumors. Overall survival was similar in both subgroups (75% for the high heparanase and 79.2% for the low heparanase mRNA expression groups; $P = 0.712$; Fig. 4). Similarly, we did not found any relationship between heparanase expression and disease-free survival. In univariate analysis, the factors that significantly shortened disease-free and overall survival were advanced FIGO stage ($P = 0.065$ and $P < 0.001$, respectively), tumor size (>4 cm; $P < 0.0001$ and $P < 0.001$, respectively), and parametrial invasion ($P = 0.002$ and $P = 0.001$, respectively). On the subsequent Cox multivariate analysis, only the FIGO stage was the independent prognostic factor for the overall survival (95% CI, 1.708–9.734, $P = 0.002$; Fig. 5).

DISCUSSION

In our study, heparanase mRNA expression levels in the normal cervix, squamous intraepithelial lesion, and cervical cancer specimens were investigated. Messenger RNA levels and catalytic activities of heparanase have been demonstrated to be correlated.[14] We found a gradually increasing mRNA expression of heparanase as the histological abnormality progressed from normal cervix tissue through intraepithelial lesion to invasive cervix carcinoma. It has been reported that the expression of heparanase mRNA in normal tissues is restricted to cytotrophoblasts, endothelial cells, keratinocytes, platelets, mast cells, neutrophils, macrophages, and to both T and B lymphocytes.[15–17] We further demonstrate that heparanase expression was significantly higher in HG-SIL than in LG-SIL. As progressing cervix dysplasia from mild to severe form destroys the epithelial organization, atypical vessels appear. This is correlated with the capacity of heparanase to degrade the side chains of HSPGs, one of the main components in cell surfaces, basal membranes, and extracellular matrix.[18] Inki et al[19] showed that the progression of cervical intraepithelial neoplasia grades I to III was associated with reduced syndecan-1, cell surface HSPGs, expression, and localization of syndecan-1 to more superficial cell layers. Furthermore, heparanase may contribute to angiogenesis by releasing-binding angiogenic factors.[20]

We further demonstrate that heparanase mRNA expression is significantly higher in cervical cancers with big size tumor (>4 cm). The present data are also consistent with previous reports that describe a positive correlation between heparanase expression and tumor growth.[21] 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 fibroblast growth factors,[22] vascular endothelial growth factor,[23] hepatocyte growth factor,[24] and platelet-derived endothelial growth factor,[25] which are stored in H–S and possessed H–S–binding sequences. It is quite reasonable to assume that the release of such growth factors may influence tumor growth and angiogenesis as was demonstrated by Biao et al,[21] although this assumption needs further studies to be positively confirmed. Nevertheless, based on the above findings of ours, the heparanase mRNA expression is a clinical marker indicating tumor aggressiveness and therefore could affect the clinical management of a patient (ie, use of combined treatments).

We also showed that heparanase expression is significantly higher in cases where lymph vascular space involvement was evident. Kodama et al[26] reported that heparanase expression is associated with the loss of basement membrane HS-glycosaminoglycan (GAG) expression, which is closely related to lymph node metastasis in invasive cervical cancers and that decreased HS-GAG expression in the basement membrane is associated with tumor progression in endometrial cancer.[27] These results raise the possibility that cleavage of HS-GAG from the basement membrane in these cancers is important for accelerating tumor cell invasion and metastasis. In addition, heparanase expression was correlated with the incidence of lymphatic invasion and venous involvement in invasive esophageal carcinoma[28] and in gastric carcinoma.[29] Intense heparanase immunostaining was observed in breast carcinoma cells that had entered the circulation and in lymph node metastases.[30] The principal cells involved in angiogenesis are endothelial cells which line all blood vessels and constitute virtually the entirety of capillaries. Immunohistochemical staining of several human carcinomas revealed preferential expression of heparanase by endothelial cells of sprouting capillaries in the vicinity of the tumor, but little or no staining of mature, quiescent vessels.[16] Moreover, a correlation between heparanase expression and tumor microvessel density has been reported in multiple myeloma, endometrial, cervical, esophageal, colon, hepatocellular, salivary gland, and bladder cancers.[21,27,31–36]

The gene of human heparanase has been reported that is functionally related to the invasion and metastasis of cancer cells.[37] However, the mechanism leading to the overexpression of heparanase gene in cancer cells remains unclear. Various reports suggest a transcriptional regulation, which has been shown to involve promoter methylation,[38] eukaryotic initiation factor 4E,[39] and the erythroblast transformation specific[40] and Egr1[41] transcription factors.

This may have practical applications in antimetastatic gene therapy of human carcinomas. Generally, little is known about heparanase expression at metastatic sites. Few studies were shown that heparanase expression levels are higher in the primary tumor site than in distant metastases.[42] However, the exact mechanism and functional role of heparanase down-regulation are yet poorly understood.

The overexpression of heparanase is correlated with shorter overall survival in several human tumors such pancreas, esophageal, colon, gastric, endometrial, and cervical invasive cancers.[21,27,29,32,33,42] We investigated the expression of heparanase mRNA in correlation with prognosis in invasive cervical cancer, and we found that the cases a higher mRNA heparanase expression were correlated with shorter posttreatment survival but that did not reach statistical significance. Probable a longer follow-up is required to confirm the above finding.

In conclusion, we showed progressive expression of mRNA heparanase from normal cervix tissue to intraepithelial cervical neoplasia and then to invasive cervical cancer. Furthermore, we found that mRNA heparanase expression was directly correlated with tumor size, lymph vascular space involvement and parametrial invasion, suggesting an association of heparanase mRNA expression with tumor microenviroment alterations that facilitate cervical cancer cell growth, invasion, and metastasis formation. Consequently, by the time when the mechanism of this association will be fully elucidated, the development of drugs acting as inhibitors of heparanase may potentially add a new therapeutic modality in the future treatment of invasive cervical cancer.

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