Loss of basement membrane heparan sulfate expression is associated with tumor progression in endometrial cancer

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Summary

Perlecan is a major heparan sulfate proteoglycan (HPSG) of the basement membrane (BM) and binds to various cytokines and growth factors via its heparan sulfate glycosaminoglycan (HS-GAG) chains. The aim of this study was to investigate BM HS-GAG expression in endometrial cancers. We investigated the expression of BM HS-GAG by immunohistochemistry in 109 endometrial cancers and analyzed correlations with various clinicopathological features. The HS-GAG expression index was significantly lower in cases of advanced stage, high-grade, deep myometrial invasion, positive peritoneal cytology, lymph vascular space invasion and lymph node metastasis. There was no association between HS-GAG expression status and patient outcome. Decreased HS-GAG expression of BM is associated with tumor progression, but is not be a useful prognostic factor in patients presenting with endometrial cancer.

Key words: Heparan sulfate; Basement membrane; Endometrial cancer.

Introduction

Proteoglycans are ubiquitous components of extracellular matrix and cell surfaces. Heparan sulfate proteoglycans (HSPGs) consist of a core protein to which heparan sulfate glycosaminoglycan (HS-GAG) chains are covalently attached. These molecules are classified into several families according to the amino acid sequence of the core protein, such as syndecans and perlecan [1, 2]. Perlecan is a major HSPG of the basement membrane (BM). The core protein of perlecan is divided into five domains based on sequence homology to other known proteins. The N-terminal domain I of mammalian perlecan is substituted with three HS-GAG chains that can bind a number of matrix molecules, cytokines, and growth factors. Perlecan plays important structural roles in BM through its HS-GAG chains and core protein [3].

An endo-beta-D-glucuronidase, heparanase, is capable of specifically cleaving glycosidic bonds of HS-GAG via a hydrolase mechanism, and is thus distinct from another bacterial HS-GAG degrading enzyme, heparitinase, which depolymerizes HS-GAG by eliminative cleavage [4]. Since the first cloning of *heparanase* cDNA, overexpression of heparanase has been observed in many human tumors, suggesting an involvement of heparanase in tumor cell invasion, metastasis and angiogenesis [5]. Furthermore, treatment with a heparanase inhibitor or antisense *hepranase* gene has been shown to reduce the incidence of metastasis in experimental animals [6, 7]. Therefore, heparanase expression may also result in HS-GAG degradation in the BM and may contribute to tumor invasion and metastases in endometrial cancer.

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We investigated the expression of BM HS-GAG in 109 endometrial cancers. We then analyzed correlations with various clinicopathological features, including patient outcome.

Materials and Methods

Patients and tissue samples

The patient population consisted of 109 individuals presenting with endometrial cancer. All patients underwent hysterectomy, bilateral salpingo-oophorectomy, pelvic and/or paraaortic lymphadenectomy and partial omentectomy at the Department of Obstetrics and Gynecology of Okayama University Graduate School of Medicine and Dentistry. Tumor specimens were obtained at the time of surgery and immediately fixed in 10% neural-buffered formalin and embedded in paraffin. Informed consent was obtained from each patient before sample collection. Histological cell types were assigned according to the WHO classifications: 104 were classified as endometrioid adenocarcinomas, four as adenosquamous and one as serous carcinoma. Histological grades according to the FIGO (International Federation of Gynecology and Obstetrics) staging classification were as follows: 37 were grade 1, 59 were grade 2 and 13 were grade 3. Surgical staging was reviewed based on the FIGO staging system: 58 were allocated in Stage I, ten in Stage II, 35 in Stage III and six in Stage IV. The median age at the time of surgery was 58 years (range 28-85 years). Patients with grade 3 tumor, non-endometrioid histologic subtype, deep myometrial invasion or extrauterine disease were treated with adjuvant combination chemotherapy consisting of etoposide, epirubicin and cisplatin or paclitaxel, pirarubicin and carboplatin. Both disease-free and overall survival rates were defined as the interval between the initial operation to either clinically or radiologically proven recurrence and death, respectively. The end-date of the follow-up study for analysis was September 30, 2004 and the median duration of the follow-up was 45 months (range 2-85 months).

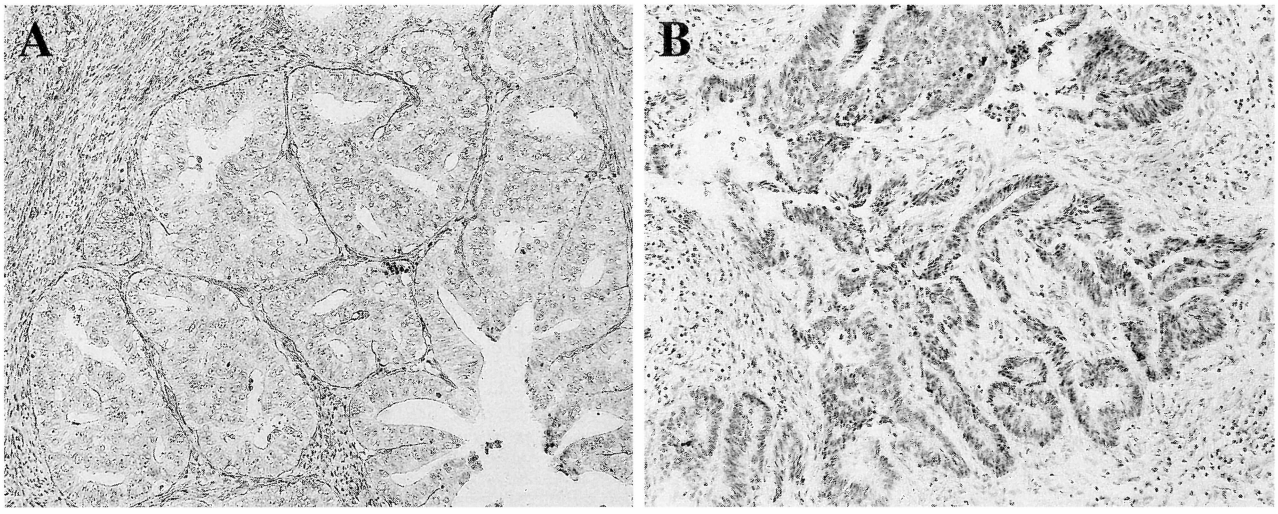

Figure 1. — Immunohistochemical staining of HS-GAG in endometrial cancers. The expression indices of HS-GAG are 3 (A) and 0 (B), respectively.

Immunohistochemistry

Sections 4 μm thick from several representative areas of the tumor specimens were put onto glass slides and immunostained according to the labeled streptavidin biotin procedure of the DAKO LSAB kit (DAKO, CA, USA). Briefly, after the slides were dewaxed in xylene and rehydrated with an alcohol series, antigen retrieval was performed in a microwave oven in 10 mM citric acid buffer (pH 6.0) for 3 x 10 min. The sections were incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity followed by incubation with normal horse serum for five minutes at room temperature. Immunostaining was then performed by incubation with a 1:100 dilution of mouse monoclonal anti-human HS-GAG (clone: F58-10E4; Seikagaku Corporation, Tokyo, Japan) for two hours at room temperature. The sections were next incubated for 20 minutes with biotinylated goat anti-mouse immunoglobulin followed by peroxidase-conjugated streptavidin for 20 minutes and ten minutes with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) containing hydrogen peroxide. Finally, the slides were counterstained with Mayer's hematoxylin and mounted in aqueous mounting medium. At each step the slides were washed carefully in phosphate buffered saline (pH 7.4). As negative controls, the sections were incubated with normal mouse serum (DAKO, Copenhagen, Denmark) at a concentration of 10 μg/ml. As positive controls, normal cervical squamous epithelia were available for analysis.

Staining evaluation

Four-grade semi-quantitative scoring (expression index) was used to evaluate the staining patterns according to continuity of BM staining: 3; continuous BM staining in more than 50% of the cancer nests, 2; continuous BM staining in less than 50% of the cancer nests; 1; BM staining with discontinuity and 0; absent BM staining. Microscopic analyses were evaluated independently by two of the authros with no prior knowledge of the clinical data. Final decisions in controversial cases were made using a conference microscope.

Statistical analyses

Univariate analysis included the Mann-Whitney *U* test. Survival rates were calculated by the Kaplan-Meier method and differences were examined by the log-rank test. These analyses were performed utilizing Stat-View 5.0 software (Abacus Concepts, Berkeley, USA). Probability values less than 0.05 were considered statistically significant.

Results

Figure 1 illustrates representative immunostaining of BM HS-GAG in endometrial cancers. As shown in Table 1, HS-GAG expression index was significantly lower in cases of advanced stage, high-grade, deep myometrial invasion, positive peritoneal cytology, lymph vascular space invasion and lymph node metastasis (Table 1). No meaningful differences, however, in the expression index of HS-GAG with respect to age, cervical invasion and ovarian metastasis was observed. Figure 2 shows that there was no association between HS-GAG expression status and patient outcome.

Discussion

Invasion and metastasis are characteristics of malignant solid tumors. Many mechanisms are involved in these processes and loss of BM integrity is critical. The basement membrane is composed of two networks, one formed by laminin and the other formed type IV collagen. These two networks are connected by entactin. Perlecan makes such supramolecular architecture more stable by interacting with liminin, type IV collagen, and entactin [3]. Proteases, such as the matrix metalloproteinases, and heparanase are believed to cooperate in the degradation of the BM [10].

In a previous report, we showed that heparanase mRNA expression is associated with the loss of BM HS-GAG expression in invasive cervical cancers [11]. Xu *et al.* have also reported that a lack of HS-GAG in the BM of thyroid papillary carcinomas inversely correlated with heparanase expression [12]. Furthermore, it is noteworthy

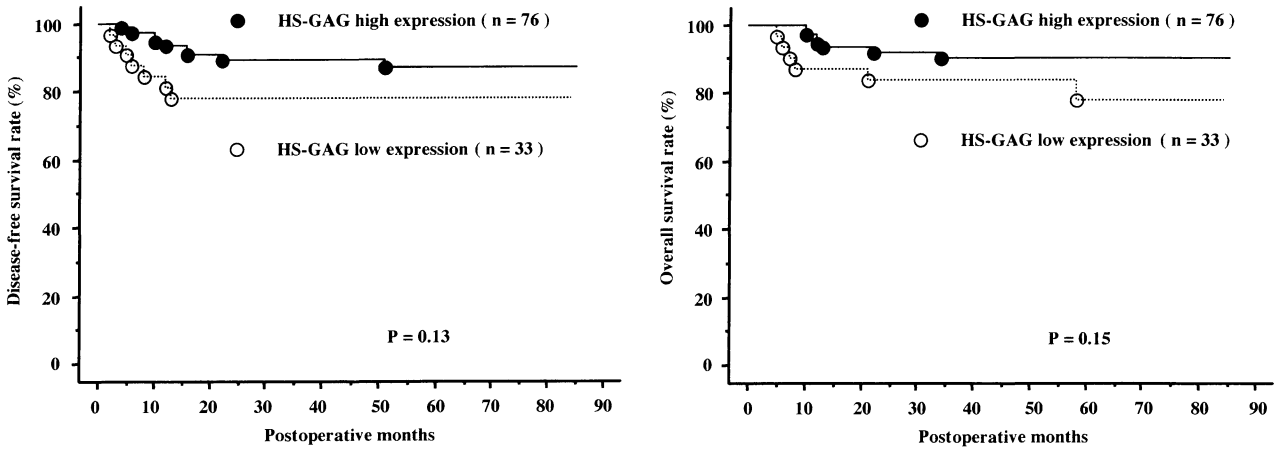

Figure 2. — (A) Disease-free and (B) overall survival curves of the 109 patients displaying endometrial cancer according to their HS-GAG expression status.

Table 1. — *Association between HS-GAG expression index and clinicopathological factors in endometrial cancers.*

Variables	No. of cases	HS-GAG (mean ± SE)	p value[a]
Age (years)			NS
< 60	60	2.08 ± 0.12	
≧ 60	49	2.04 ± 0.13	
FIGO stage			0.0004
I+II	68	2.31 ± 0.11	
III+IV	41	1.68 ± 0.15	
FIGO grade			< 0.0001
1	37	2.57 ± 0.12	
2+3	72	1.81 ± 0.11	
Depth of myometrial invasion			0.0002
≦ 1/2	67	2.31 ± 0.11	
> 1/2	42	1.67 ± 0.13	
Cervical involvement			NS
negative	86	2.08 ± 0.10	
positive	23	2.00 ± 0.22	
Lymph node metastasis			0.0009
negative	95	1.29 ± 0.22	
positive	14	2.18 ± 0.10	
LVS involvement			0.005
negative	76	1.70 ± 0.16	
positive	33	2.22 ± 0.11	
Ovarian metastasis			NS
negative	97	1.75 ± 0.31	
positive	12	2.10 ± 0.09	
Peritoneal cytology			0.01
negative	84	1.64 ± 0.20	
positive	25	2.19 ± 0.10	

LVS: lymp-vascular space; NS: not significant; [a]Mann-Whitney *U*-test.

that Reiland *et al.* demonstrated that heparanase specifically degrades HS chains of purified perlecan HS isolated from WiDr cells [13]. In this study, HS-GAG expression was found to be significantly lower in advanced cases. Although we did not investigate heparanase expression in the present study, Watanabe *et al.* recently reported that heparanase expression is significantly correlated with stage, the presence of lymph-vascular space involvement, lymph node metastasis and histological tumor grade in endometrial cancers. These results raise the possibility that cleavage of HS-GAG from the perlecan core protein, which is mediated by heparanase, is very important for accelerated tumor cell invasion and metastasis. Many biological active molecules, including growth factors, cytokines and angiogenic factors, bind to HS-GAG in the BM [3]. Heparanase has been shown to release these molecules and might affect the progression of cancer cells through these molecules. However, perlecan core protein expression was not examined in the present study, and further studies are needed to elucidate the function of perlecan in tumor invasion and metastasis.

Correlations between HS-GAG expression levels and patient prognosis were examined in the present study. HS-GAG expression was found not to be a significant prognostic factor either for progression-free or overall survival rates of endometrial cancer. Thus, HS-GAG expression of the BM should not be considered as a useful prognostic factor in patients presenting with endometrial cancer.

Conclusion

In conclusion, our findings provide evidence that the loss of BM HS-GAG expression is closely related to tumor progression in endometrial cancers.

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