

Loss of Syndecan-1 and Increased Expression of Heparanase in Invasive Esophageal Carcinomas

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Heparan sulfate proteoglycans play important biological roles in cell-cell and cell-matrix adhesion, and are closely associated with growth factor actions. Loss of syndecan-1, a cell surface-bound heparan sulfate proteoglycan, has been reported for advanced head and neck carcinomas, and expression of endoglycosidic heparanase, which cleaves heparan sulfate glycosaminoglycans (HS-GAGs), is associated with invasion and metastatic potential of malignant tumors. Paraffin sections of 103 primary esophageal squamous cell carcinomas were immunohistochemically examined for the expression of syndecan-1 core protein, HS-GAGs and heparanase protein, and the results were compared with various clinicopathological parameters, such as invasion depth. For 16 cases, fresh tumor samples were quantitatively analyzed for heparanase and syndecan-1 mRNA expression by real-time RT-PCR in addition to the immunohistochemical studies. Syndecan-1 core protein and HS-GAGs expression was significantly decreased in pT2 and pT3 cases compared with their pTis and pT1 counterparts. Decreased expression of core protein and HS-GAGs was correlated with the incidence of lymphatic invasion, and venous involvement. Furthermore, decreased expression of HS-GAGs was correlated positively with the incidence of nodal metastasis and distant organ metastasis, and negatively with the grade of tumor cell differentiation. The percentage of cytoplasmic heparanase protein-positive cases increased significantly in pT2 and pT3 cases compared to that in pTis and pT1 cases, and this was associated with lymphatic invasion, and venous and lymph nodal involvement. The level of heparanase mRNA was inversely correlated with the degree of HS-GAGs expression rather than core protein. In conclusion, loss of syndecan-1 and heparanase overexpression in esophageal squamous cell carcinomas are closely associated with malignant potential. Regarding the mechanism of loss of HS-GAGs, heparanase upregulation appears to play an important role.

Key words: Esophageal squamous cell carcinoma (ESCC) — Heparan sulfate proteoglycan (HSPG) — Heparanase — Syndecan-1

Esophageal squamous cell carcinomas (ESCCs) are relatively common in East Asia, such as in Japan and China, and are generally associated with a poor prognosis.¹⁾ Therefore, elucidation of the mechanisms of invasion and metastasis is a high priority, and establishment of useful biomarkers predicting prognosis is very important. Previously, we reported that increased proliferative activity of malignant cells and accumulation of p53 proteins was linked with a tendency of mucosal esophageal carcinoma invasion in early stages,²⁾ and increased expression of matrix metalloproteinases-7, 9 and membrane type 1-

matrix metalloproteinase was closely associated with submucosal invasion of cancer cells and venous involvement.³⁾

Proteoglycans are complex molecules consisting of core proteins and various glycosaminoglycans chains. Heparan sulfate proteoglycans (HSPGs), in which heparan sulfate glycosaminoglycan (HS-GAG) chains are covalently attached, are classified into several families according to the amino acid sequence of the core protein, such as perlecan, basement membrane-bound, and syndecans, cell surface-bound HSPGs.^{4,5)} The syndecan family comprises at least four members, namely 1 through 4,⁶⁾ syndecan-1 being the best studied in human tissues.

It is expressed in normal stratified squamous epithelium, especially in the suprabasal cell layer, and it has

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been reported that this is diminished during carcinogenesis.⁷⁻¹⁰ In human tumors, decreased expression of syndecan-1 has been reported in head and neck, uterine cervix, skin and hepatocellular carcinomas.¹¹⁻¹⁷ However, the situation regarding ESCCs is unclear.

Endoglycosidic heparanase, which cleaves HS-GAGs at the linkage between GlcUA and GlcNAc, degrades HS-GAGs into fragments with characteristic large molecular sizes, identified in murine metastatic melanoma and lymphoma cells.¹⁸⁻²⁰ The cDNA sequence of *heparanase* gene was recently reported, and its mRNA found to be preferentially expressed in various human tumor tissues.²¹⁻²⁵

It is unclear whether loss of syndecan-1 in malignant tumors is due to decreased synthesis or accelerated degradation by enzymes, such as protease and heparanase. Therefore, in the present study, we examined the expression of syndecan-1 core protein, HS-GAGs and heparanase in surgically resected ESCCs tissues, and analyzed correlations with various clinicopathological parameters, including invasion and metastasis. Furthermore, using frozen carcinoma tissues, heparanase and syndecan-1 mRNA expression was examined by quantitative real-time RT-PCR ("TaqMan" EZ RT-PCR) to cast light on regulation at the gene level.

MATERIALS AND METHODS

Materials One hundred and three cases, which were histologically diagnosed as squamous cell carcinoma of the esophagus between 1994 and 1999, were randomly selected from the patients' file of Tokyo Medical and Dental University (TMDU) Hospital, and were investigated in the present study. Patients who had received irradiation and/or chemotherapy before surgery were excluded. Esophagectomy and lymph node dissection were performed in all cases, and specimens were fixed in buffered formalin solution, and routinely processed for paraffin embedding. One representative block for each case, in which the carcinoma showed the deepest invasion, was employed for the histological analyses. For sixteen cases fresh tissue samples were immediately mounted in OCT compound (Sakura Finetechnical, Tokyo), frozen in liquid nitrogen, and stored at -80°C until use for immunohistochemistry and quantitative RT-PCR. Sections were cut serially at $4\ \mu\text{m}$ from the paraffin blocks, and at $6\ \mu\text{m}$ from the frozen material.

Following the World Health Organization²⁶ and TNM classification,²⁷ cases were classified according to the depth of carcinoma invasion, as follows: 22 mucosal carcinoma cases (m, pTis and pT1a), including carcinomas *in situ* and carcinoma whose invasion is limited to the lamina propria; 25 submucosal carcinoma cases (sm, pT1b); and 56 more advanced cases (pT2 and pT3). Data including age, sex, nodal metastasis, distant metastasis, histological

grading, lymphatic invasion and venous involvement were collected for each patient from the patients' files at the Department of Pathology, TMDU Hospital. This study group comprised 88 (85%) male and 15 (15%) female patients. The ages at the time of diagnosis ranged from 43 to 84 (mean age: 64). Staging was done according to the TNM classification.²⁷ Other clinicopathological parameters are summarized in Table I.

Preparation of anti-heparanase polyclonal antibody Heparanase cDNA cloned from the cDNA library of SV40-transformed WI38 fibroblasts was amplified by PCR using a forward primer containing a restriction enzyme *EcoRI* site in front of the ATG methionine site and a reverse primer containing a restriction enzyme *XhoI* site behind the stop codon.²⁴ The amplified PCR product of the heparanase cDNA was subcloned into a pFastBac donor plasmid, and the recombinant plasmid was transformed into DH10Bac competent cells, that contained the bacmid in the presence of helper plasmid. Colonies containing the recombinant bacmid were identified by disruption of the *lacZ* gene. Recombinant bacmid was prepared from selected *E. coli* clones and used to transfect Sf-9 insect cells. Sf-9 insect cells were infected with high titer virus for 48 h and the conditioned medium was recovered. The supernatant was subsequently subjected to heparin-Sepharose affinity chromatography and concanavalin A-Sepharose affinity chromatography to afford purified recombinant heparanase in a 65 kDa proform. The purified heparanase was used to immunize New Zealand white rabbits with an incomplete adjuvant. The whole blood was harvested when the titer of anti-heparanase activity reached its highest, and the serum was prepared. Anti-heparanase polyclonal antibody was further purified by affinity chromatography using a column of Sepharose coupled with the recombinant heparanase. The polyclonal antibody reacts with human heparanase in both 65 kDa proform and 50 kDa mature forms. The reactivity was confirmed with natural human heparanase of 50 kDa purified from SV40-transformed WI38 fibroblasts using western blot.

Immunohistochemistry Mouse monoclonal antibody (clone: B-B4) against human syndecan-1 core protein (CD138) was purchased from Serotec (Oxford, UK), and mouse monoclonal antibodies (clone: F58-10E4, HepSS-1) against human heparan sulfate from Seikagaku Corporation (Tokyo). F58-10E4 antibody was obtained after immunization with HS-GAGs isolated from human fetal lung fibroblasts,²⁸ and HepSS-1 with murine methylcholanthrene-induced fibrosarcoma.²⁹ These antibodies recognize epitopes in HS-GAGs, and do not react with other types of GAGs.

Paraffin sections were mounted on aminopropyltrimethoxysilane-coated slides, dried at 37°C for up to 24 h, deparaffinized in xylene and rehydrated through graded

Table I. Relationships between Syndecan-1 (Core Protein and HS-GAGs), Heparanase Expression and Clinicopathological Parameters in Esophageal SCCs

Factor	Syndecan-1 core protein positive cases % (n)	HS-GAGs positive cases % (n)	Heparanase positive cases % (n)
Depth of carcinoma invasion			
pTis, pT1a (m, n=22)	82 (18)	73 (16)	18 (4)
pT1b (sm, n=25)	52 (13) }*	52 (13) }n.s.	44 (11) }n.s.
pT2, 3 (mp and a, n=56)	38 (21) }**	29 (16) }***	66 (37) }***
m: mucosa, sm: submucosa, mp: muscularis propia, a: adventitia			
Lymph nodal metastasis			
pN0 (n=49)	59 (29)	63 (31) }***	35 (17) }**
pN1 (n=54)	43 (23)	26 (14) }	80 (35) }
Distant organ metastasis			
pM0 (n=72)	54 (39)	54 (39) }***	49 (35)
pM1 (n=31)	42 (13)	19 (6) }	55 (17)
Histological grading			
Well-moderately diff. (n=73)	51 (37)	51 (37) }*	53 (39)
Poorly diff. (n=30)	50 (15)	27 (8) }	43 (13)
Pathological stage			
pStage 0-2 (n=51)	55 (28)	61 (31) }***	31 (16) }***
pStage 3, 4 (n=52)	46 (24)	27 (14) }	69 (36) }
Lymphatic permeation			
ly (-) (n=36)	67 (24) }*	69 (25) }***	33 (12) }**
ly (+) (n=67)	42 (28) }	30 (20) }	60 (40) }
Venous involvement			
v (-) (n=29)	76 (22) }**	69 (20) }**	24 (7) }***
v (+) (n=74)	41 (30) }	34 (25) }	61 (45) }

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s.: not significant.

ethanol solutions. Frozen sections on aminopropyltrimethoxysilane-coated slides were fixed in acetone for 5 min and washed in distilled water. To quench endogenous peroxidase, slides were immersed in a solution of 0.3% H₂O₂ (volume/volume) in methanol for 10 min, transferred to phosphate-buffered saline (PBS) solution, pH 7.2, and incubated for 20 min in a 1.5% solution of normal rabbit serum (DAKO, Glostrup, Denmark) or normal goat serum (DAKO) for the detection of heparanase. After PBS washing, they were incubated for 2 h at room temperature with a 1:100 dilution of mouse monoclonal anti-human syndecan-1 core protein (B-B4), or a 1:100 dilution of mouse monoclonal anti-human HS-GAGs (F58-10E4, HepSS-1), or a 30 µg/ml of rabbit polyclonal antibody against human heparanase.

After incubation with primary antibody, slides were incubated with a 1:200 dilution of biotinylated rabbit anti-mouse IgG (DAKO) or biotinylated goat anti-rabbit IgG

(DAKO) for the detection of heparanase for 30 min at room temperature. They were incubated with a 1:200 dilution of peroxidase-conjugated streptavidin (DAKO) for 30 min at room temperature, and stained with 0.02% 3,3'-diaminobenzamide (Sigma, St. Louis, MO) at pH 7.6 in 0.05 M Tris buffer plus 0.015% H₂O₂ for 5-7 min. Slides were washed three times in 0.25% Tween 20 in PBS for 5 min between each step, and all incubations were carried out in a humidified chamber.

Sections treated with 1.5% normal rabbit serum (DAKO) and 1.5% normal goat serum (DAKO) for the detection of syndecan-1 and heparanase, respectively, in 0.25% Tween 20 in PBS without primary antibody served as negative controls. As a positive control for syndecan-1 (core protein and cell surface HS-GAGs), normal esophageal epithelium was used. As a positive control for heparanase protein, an esophageal cancer specimen, in which a high level of heparanase mRNA expression was

confirmed by "TaqMan" EZ RT-PCR, was used. To evaluate immunoreactivity for syndecan-1, the entire tumor area was assessed microscopically, and the samples in which the percentage of cell-surface syndecan-1-positive tumor cells was more than 50% were considered as positive. HS-GAGs were positively stained at both cell and basement membranes. However, only cell-surface expression of HS-GAGs was evaluated in the present studies, because basement membrane-bound HS-GAGs were likely to be other kinds of proteoglycans, such as perlecan. Heparanase protein was expressed preferentially in cancer nests, and positive staining was observed at cell surfaces and in the cytoplasm. Both were evaluated separately, and cases in which the percentage of cell surface or cytoplasmic positive tumor cells was more than 50% were considered as positive for each category.

Extraction of total RNA from frozen sections From each OCT compound-mounted block, five sections serially cut at 6 μm , ten sections cut at 20 μm , and five sections cut at 6 μm , were prepared. The first and last five sections cut at 6 μm were used for H&E staining and immunohistochemistry as described above. Ten sections cut at 20 μm were used for mRNA analyses. Sections were washed in test tubes with RNAase-free water, and homogenized in 1 ml of Trizol Reagent (Gibco-BRL-Life Technology, Grand Island, NY). Total RNA was precipitated from the aqueous fraction with isopropanol, and precipitates were dissolved in RNAase-free water and assessed for purity and RNA concentration using a spectrophotometer.

Quantitative real-time RT-PCR ("TaqMan" EZ RT-PCR) To evaluate the degree of heparanase mRNA expression, quantitative real-time PCR based on the 5'-exonuclease activity of the *Taq* polymerase was used in the present study.³⁰⁾ All reagents, including the enzyme, primers and fluorescent dye-labeled probes, were purchased from PE Applied Biosystems (Tokyo). RNA was reverse-transcribed to cDNA and sequentially PCR-amplified in a single tube. rTth DNA polymerase, has the functions of both reverse transcriptase and DNA polymerase, and provides the 5'-3' nuclease activity necessary for the cleavage of the fluorogenic probe,³¹⁾ making analysis in a single tube possible. For PCR amplification of cDNA, a non-extendable oligonucleotide probe with a 5' fluorescent reporter dye (6FAM: 6-carboxyfluorescein) and 3' quencher dye (TAMRA: 6-carboxytetramethylrhodamine) hybridized downstream of the forward primer to the target gene was added to the forward and reverse primers. During the extension phase the rTth DNA polymerase hydrolyzes this probe, thereby generating a fluorescence signal, which is directly proportional to the amount of PCR product synthesized.³²⁾ This fluorescence signal was monitored with an ABI prism 5700 sequence detector (PE Applied Biosystems). The reverse transcription and PCR amplification were performed using a 96-well tray and optical caps

with a 50 μl final reaction mixture containing 1 \times "TaqMan" EZ buffer, 3 mM manganese acetate, 300 μM dATP, dCTP, dGTP, 600 μM dUTP, 200 nM of each primer, 100 nM of each probe, 0.1 U/ μl rTth DNA polymerase, 0.01 U/ μl Amp Erase UNG, and 0.5 μg total RNA sample. The reaction mixture was preheated at 50°C for 2 min, incubated at 60°C for 30 min (reverse transcription), heated at 95°C for 1 min, and then subjected to 50 cycles of 94°C for 2 min, and 50°C for 1 min. Additional reactions were performed on each 96-well plate with serial dilutions of total RNA isolated from the HSC3 cell line (originally established from a squamous cell carcinoma of the tongue and obtained from the Health Science Research Resources Bank (HSRRB)) to allow construction of a standard curve (Fig. 1). "TaqMan" primer and probe sets for heparanase, syndecan-1 and GAPDH were designed from sequences in the GenBank database using Primer Express software (PE Applied Biosystems). Primers and "TaqMan" probe sequences for heparanase were: forward, 5'-AGCCTC-GAAGAAAGACGG-3'; reverse, 5'-GTAGTGATGCCAT-GTAACTGAATC-3'; and probe, 5'-AAGGCTGGTGA-GAAGTGAT-3'; Sequences for syndecan-1 were: forward, 5'-GGAAAGAGGTGCTGGGAGGG-3'; reverse, 5'-TTG-GTGGGCTTCTGGTAGGC-3'; and probe, 5'-AGCCGA-AACAAGCCAACGGCGG-3'. Sequences for GAPDH were: forward, 5'-GAAGGTGAAGGTCGGAGT-3'; reverse, 5'-GAAGATGGTGATGGGATTTTC-3'; and probe, 5'-CCGACTCTTGCCCTTCGAAC-3'. To avoid contamination, RNA isolation and the PCR were performed in separate laboratories under sterile conditions. In every run, a negative control for each probe was included to rule out false-positive results. Experiments were repeated several times, and essentially the same for calibration curves and relative quantities of mRNA expression were detected each time.

Statistical analysis To evaluate the significance of correlations between syndecan-1 or heparanase protein expression and tumor invasion or other clinicopathological parameters, the Fisher exact test was applied. For the significance of correlations between immunohistochemical results for syndecan-1 (core protein or HS-GAGs) or heparanase protein and expression of heparanase or syndecan-1 mRNA, simple regression analysis was employed. *P* values equal to or smaller than 0.05 were considered statistically significant.

RESULTS

Immunolocalization of syndecan-1 in normal esophagus In normal esophageal epithelium, syndecan-1 core protein was localized on cell surfaces from the parabasal layer through the lower half, with expression markedly decreased in the upper half (Fig. 2A). It was also found in the cytoplasm of plasma cells. Localization of cell-surface

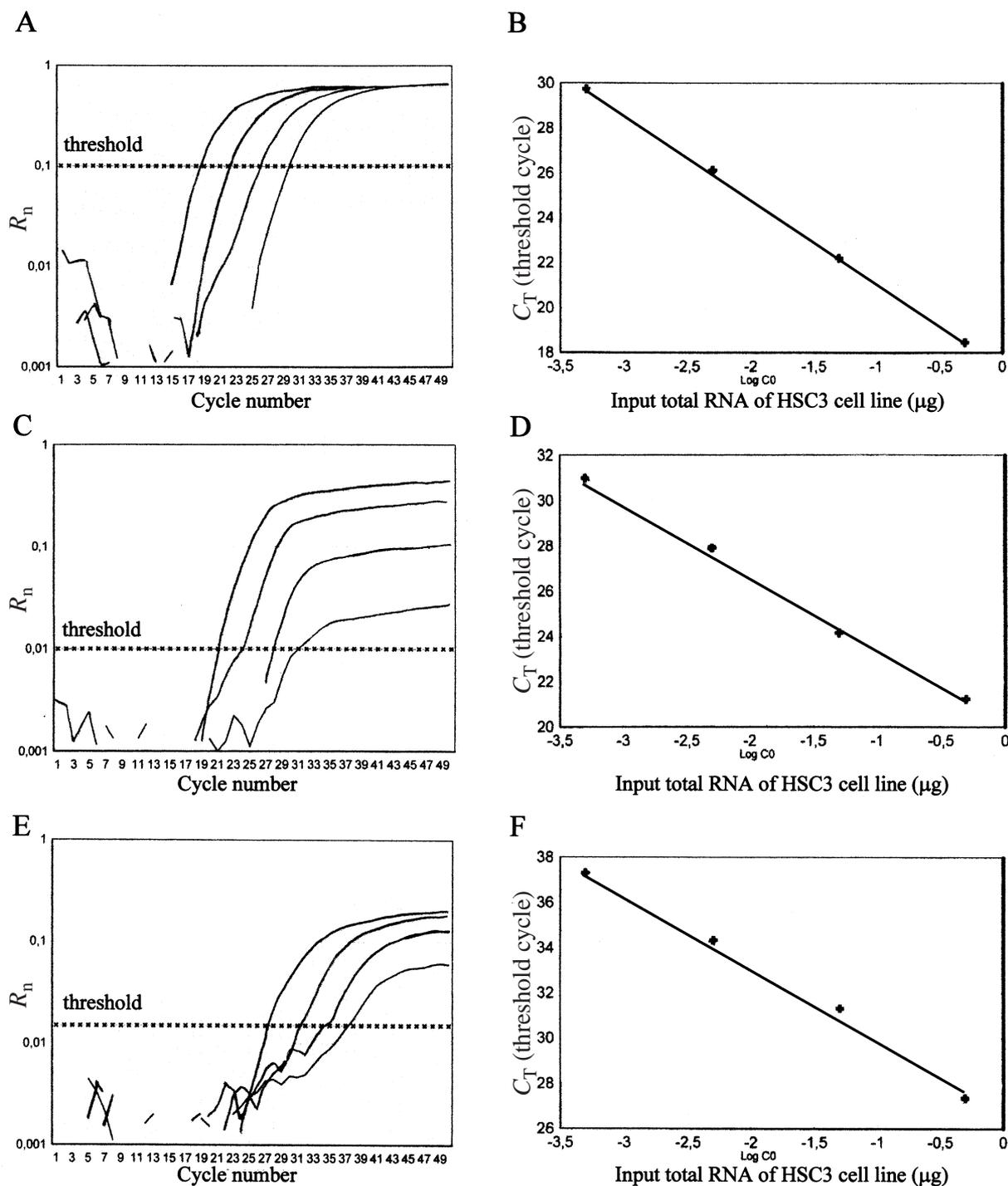


Fig. 1. Amplification plots of GAPDH mRNA (A), syndecan-1 mRNA (B), heparanase mRNA (C) and calibration curve for GAPDH mRNA (D), syndecan-1 mRNA (E) and heparanase mRNA (F). Serial dilutions of HSC3 cell total RNA were amplified using a "Taq-Man" EZ RT-PCR kit and analyzed on a model 5700 Sequence Detector (PE Applied Biosystems). For each dilution, the ΔR_n , calculated by subtracting the background fluorescence, is plotted against the cycle number. Calibration curve [(D) GAPDH, (E) syndecan-1 and (F) heparanase]: Input HSC3 cell total RNA was plotted against C_T (the cycle fluorescent signal crosses the threshold). In the present experiment, the functions of the calibration curves were, for GAPDH: $C_T = -3.77 \log [\text{HSC3 } (\mu\text{g})] + 17.33$ ($r=0.999$), for syndecan-1: $C_T = -3.30 \log [\text{HSC3 } (\mu\text{g})] + 20.11$ ($r=0.998$), for heparanase: $C_T = -3.29 \log [\text{HSC3 } (\mu\text{g})] + 26.6$ ($r=0.997$).

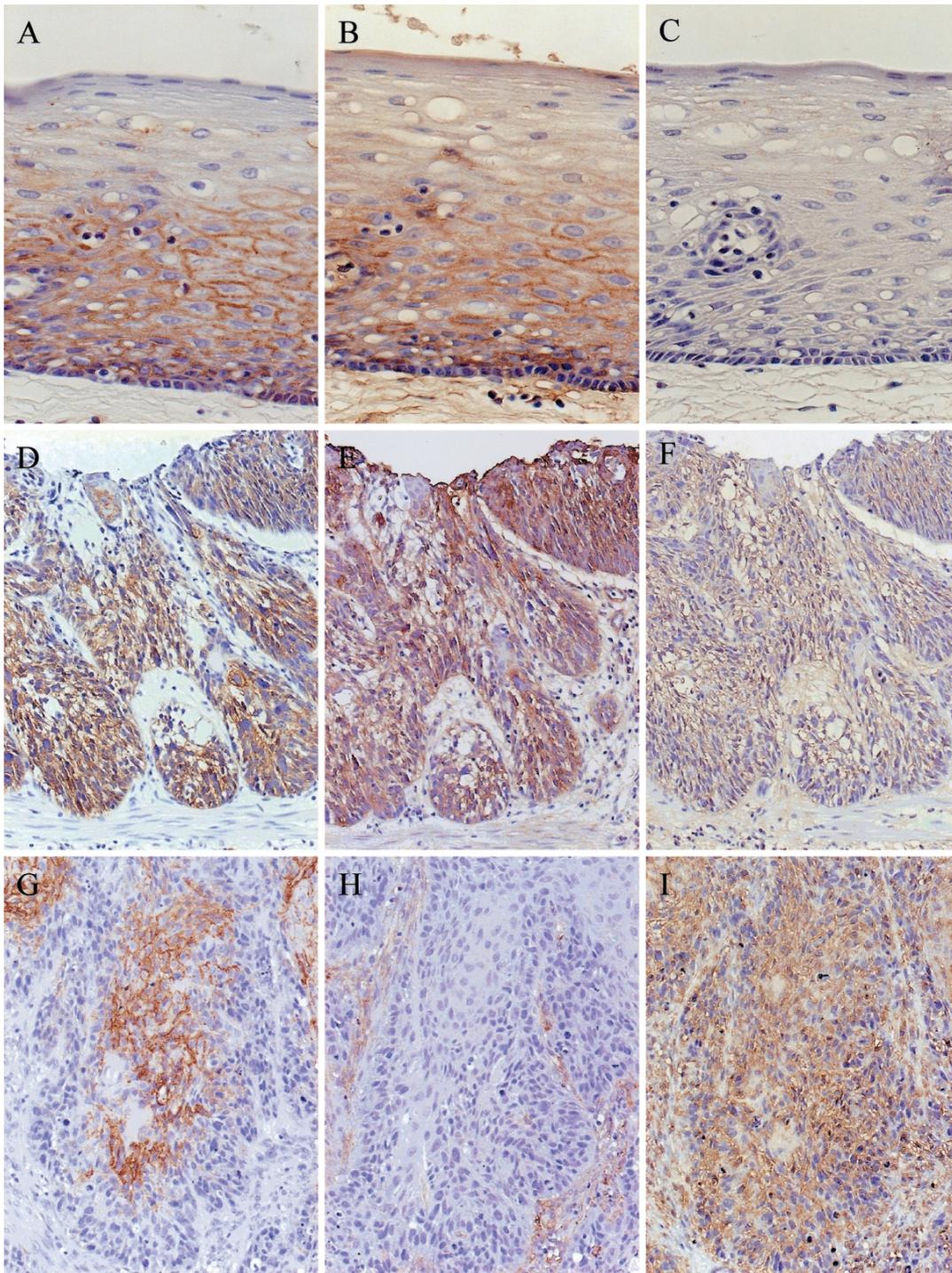


Fig. 2. Immunostaining of syndecan-1 core protein, HS-GAGs and heparanase protein in normal esophagus and squamous cell carcinomas. Original magnification $\times 150$. (A)–(C): Normal esophageal squamous epithelium. Syndecan-1 core protein (A) and HS-GAGs (B) are expressed at the cell surfaces in the lower half of the epithelium. No heparanase protein expression was observed (C). (D)–(F): Carcinoma invading the lamina propria (pT1a). Positive staining of syndecan-1 core protein (D) and HS-GAGs (E) is apparent at the cell surfaces. Weak cell surface heparanase protein expression is also evident. (G), (H): Carcinoma invading the submucosa (pT1b). Syndecan-1 core protein expression is absent in the peripheral front of a carcinoma nest. In the center, weak syndecan-1 expression is retained (G). HS-GAGs expression is completely lost in the carcinoma nest (H). In contrast, heparanase protein is expressed at the cell surfaces and in the cytoplasm in the carcinoma nest (I).

HS-GAGs was essentially the same as that of core protein (Fig. 2B), although they were also observed in the basement membrane, vessel wall, and smooth muscle. Since they were regarded as components of the basement membrane-bound HSPG, perlecan, this localization was not further investigated in the present study.

Expression of syndecan-1 in squamous cell carcinomas, and its relation to the depth of carcinoma invasion

Findings for relationships between syndecan-1 (core protein and HS-GAGs) expression and clinicopathological parameters including the depth of carcinoma invasion are summarized in Table I. In mucosal carcinomas (m, pTis and pT1a), syndecan-1 core protein expression was positive in 18 of 22 cases (82%, Fig. 2D). In submucosal carcinomas (sm, pT1b), it was lost in peripheral fronts of invading nests (Fig. 2G), and was only observed in 13 of 25 cases (52%), the percentage being significantly lower than that for m cases ($P < 0.05$). In the more advanced cases (pT2 and pT3), syndecan-1 core protein expression was lost especially in deep invasive areas (Fig. 3A), and was only observed in 21 of 56 cases (37%, $P < 0.01$, vs. m and sm cases combined). In advanced cases with well-preserved syndecan-1 expression, it was also apparent in the cytoplasm (data not shown).

Cell-surface HS-GAGs expression was positive in 16 of 22 m cases (73%, Fig. 2E), and in 13 of 25 sm cases (52%, Fig. 2H), the difference not being statistically significant. In the more advanced cases, HS-GAGs expression was only observed in 16 of 56 cases (29%, Fig. 3B, $P < 0.001$, vs. m and sm cases combined). Cell-surface HS-GAGs expression also decreased in deep invasive areas, especially in the peripheral fronts of invading nests. As in the case of core protein staining, cytoplasmic expression of HS-GAGs was also observed in some cases (data not shown).

At each depth of carcinoma invasion, the incidence of HS-GAGs-positive cases tended to be lower than that for core protein. In the peripheral fronts of invading nests, decrease of HS-GAGs was more remarkable than that of core protein, and well-preserved core protein expression was sometimes observed despite the nearly complete loss of HS-GAGs (Fig. 2, G and H). Inflammation also appeared to affect the decrease of syndecan-1 expression. In cases with marked inflammatory cells, invading carcinoma nests in contact with them showed a dramatic decrease of both core protein and HS-GAGs expression (Fig. 3, D and E).

F58-10E4 antibody (Seikagaku Corporation) was adopted to investigate HS-GAGs in the current study, since it is applicable to paraffin sections. In addition, another anti-HS-GAGs antibody (HepSS-1, Seikagaku Corporation) was applied to several frozen ESCC tissues because HS-GAGs are known to have heterogeneous structure.⁵⁾ HepSS-1 epitope was almost lost in invasive

ESCCs, and HepSS-1 and F58-10E4 epitopes showed almost the same staining pattern (Fig. 3, H and I).

Relationship between syndecan-1 expression and other clinicopathological parameters As shown in Table I, decreased expression of syndecan-1 core protein was correlated with venous involvement and lymphatic invasion ($P < 0.005$, $P < 0.05$), but not incidence of nodal metastasis, distant organ metastasis or pathological TNM stage.

Decreased expression of HS-GAGs was, however, well correlated with the incidence of nodal metastasis ($P < 0.0005$), distant organ metastasis ($P < 0.001$), pathological TNM stage ($P < 0.0005$), lymphatic permeation ($P < 0.0005$) and venous involvement ($P < 0.005$).

Expression of heparanase protein in normal esophageal epithelium and squamous cell carcinoma, and its relation to clinicopathological parameters, such as depth of carcinoma invasion In normal esophageal epithelium, no heparanase protein expression was observed (Fig. 2C)

Heparanase protein was localized in both cell membrane and cytoplasm of carcinoma cells, and was also expressed in peripheral nerves, ganglion cells (data not shown), and inflammatory cells, such as plasma cells and macrophages (Fig. 3, C and F). Carcinoma cell membranes were positive in 75 of 103 cases (73%), and even m cases demonstrated staining (Fig. 2F). Generally, cell membrane expression was weak compared with cytoplasmic expression, and there was no correlation with any clinicopathological parameter (data not shown).

Cytoplasmic heparanase protein expression was observed in 52 of 103 cases (50%). With m (pTis and pT1a) lesions, only 4 of 22 cases were positive (18%). In sm (pT1b) cases, the figure was 11 of 25 (44%, Fig. 2I), and for more advanced cases (pT2, pT3), it was 37 of 56 cases (66%, Fig. 3C), significantly higher than that for m and sm cases combined ($P < 0.001$). Heparanase protein was also expressed in fibroblasts and inflammatory cells around the invading carcinoma nests (Fig. 3, C and F). The positive reaction in fibroblasts and inflammatory cells tended to correlate with positive reaction in carcinoma cells, but the reaction was weaker than that in carcinoma cells in most cases. Cytoplasmic heparanase protein expression in carcinoma cells correlated well with lymph nodal metastasis ($P < 0.005$), pStage 3, 4 ($P < 0.0005$), lymphatic invasion ($P < 0.01$) and venous involvement ($P < 0.001$).

Relationship between syndecan-1 expression and heparanase mRNA expression Standard curves for heparanase and syndecan-1 mRNA (Fig. 1) were constructed using serial dilutions of HSC3 cell line. The initial relative quantities of mRNA templates were calculated by comparing the results with the standard curve for the HSC3 cell line, using the standard curves and C_T values at which the fluorescent signal crossed an arbitrarily determined threshold in the exponential phase of the poly-

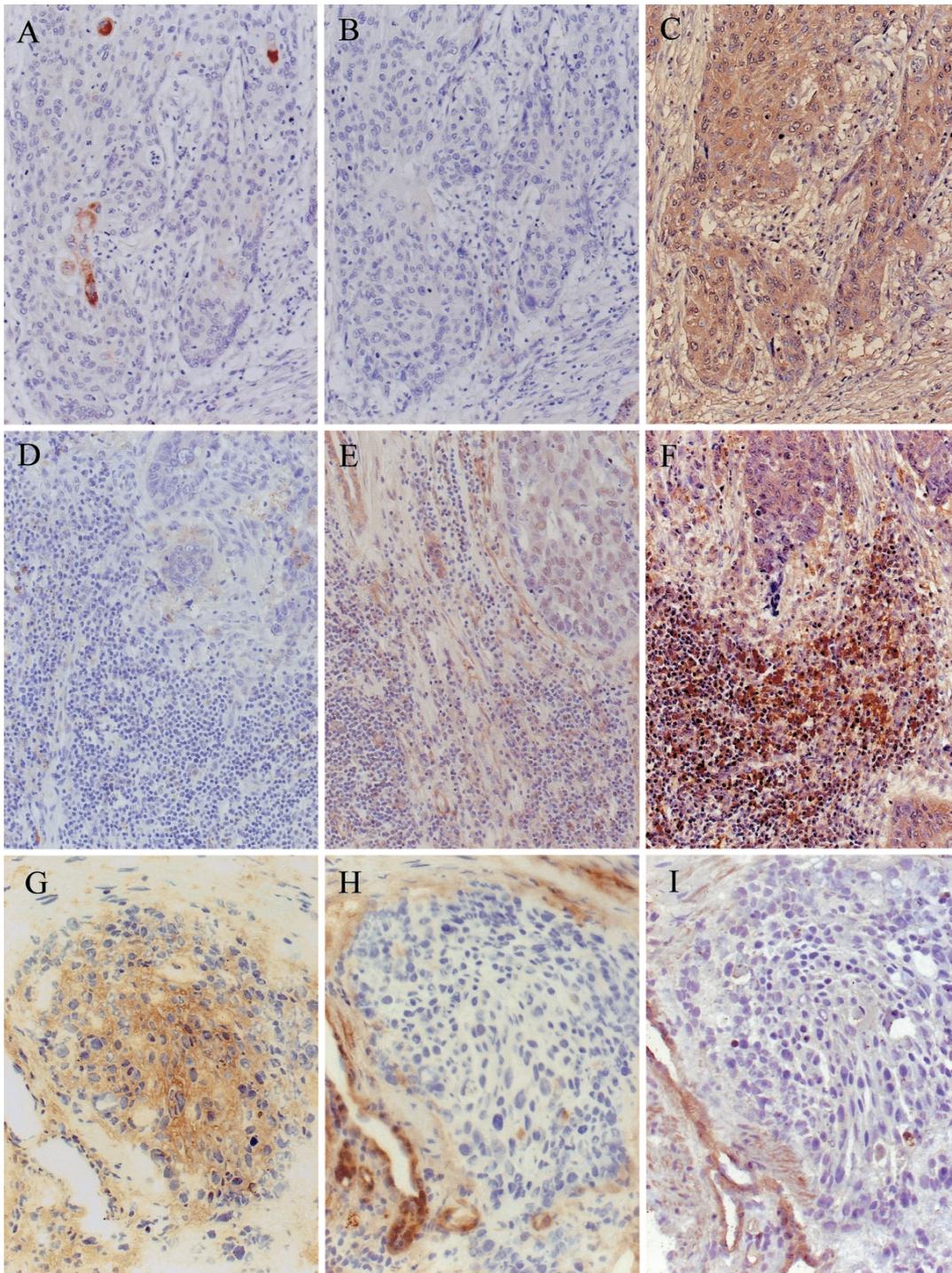


Fig. 3. Immunostaining of syndecan-1 core protein, HS-GAGs and heparanase protein in advanced esophageal squamous cell carcinomas. Original magnification $\times 150$. (A)–(C): Deep invasive area of a carcinoma invading the adventitia (pT3). Syndecan-1 core protein (A) and HS-GAGs expression (B) are almost absent in a carcinoma nest. Heparanase protein was pronounced in the cytoplasm of carcinoma cells in the nest (C). (D)–(F): Squamous cell carcinoma with marked inflammatory cell infiltration. Most carcinoma cells had neither syndecan-1 core protein (D) nor HS-GAGs expression (E). Strong heparanase protein staining is apparent in inflammatory cells in addition to the carcinoma (F). (G)–(I): Invasive squamous cell carcinoma (frozen sections). Syndecan-1 core protein expression is seen in the center of carcinoma nest, and it is absent in the peripheral front (G). F58-10E4 (H) and HepSS-1 (I) epitopes of HS-GAGs are almost lost in the carcinoma nest, and these show similar staining patterns.

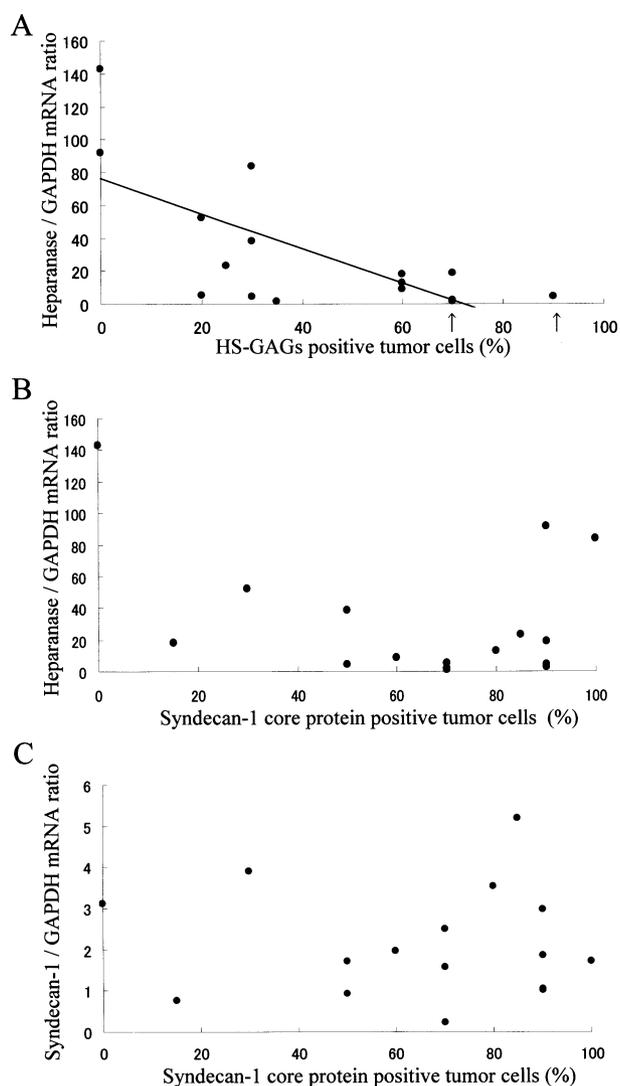


Fig. 4. Syndecan-1 or heparanase/GAPDH mRNA ratios vs. immunohistochemical findings. (A) There was a significant inverse correlation between relative heparanase mRNA expression and the HS-GAGs immunohistochemical result ($r=0.69$, $P<0.01$). Arrows indicate cases with no heparanase protein expression. In contrast, there was no significant inverse correlation between heparanase mRNA level and syndecan-1 core protein expression (B), and no apparent correlation between the latter and relative syndecan-1 mRNA expression (C).

merase chain reaction. Because the RT-PCR method is particularly sensitive to RNA quality, heparanase/GAPDH or syndecan-1/GAPDH ratios were calculated for each case to give relative heparanase or *syndecan-1* gene expression. Relationships between heparanase or syndecan-1/GAPDH mRNA ratios and percentages of tumor cells positive for syndecan-1 core protein or cell surface HS-GAGs are shown in Fig. 4. There was a significant

inverse correlation between relative heparanase mRNA expression and the HS-GAGs immunohistochemical results (Fig. 4A, $r=0.69$, $P<0.01$). In cases with high heparanase expression, the cell surface HS-GAGs positive staining rate tended to be low. In contrast, there was no significant inverse correlation between heparanase mRNA level and syndecan-1 core protein expression (Fig. 4B), or between the latter and the syndecan-1/GAPDH mRNA ratio (Fig. 4C).

Out of 16 cases examined, 14 cases were positive for heparanase protein expression. In these cases, positive reaction was observed diffusely in carcinoma cells, and quantification of the positive reaction in these cases was difficult. The average heparanase mRNA/GAPDH mRNA ratio of 14 heparanase protein-positive cases was 36.0, which was higher than that in two negative cases (average; 3.3, dots with arrows in Fig. 4A).

DISCUSSION

Invasion and secondary spread through the blood and lymphatics are characteristic features of malignant tumors and one of the greatest impediments to cancer cure. Many mechanisms are involved, and cell adhesion molecules, such as integrins, cadherins and cell surface HSPGs are especially important in the regulation of cell differentiation, morphology and migration.^{4, 6, 33-37} Syndecan-1, a cell-surface HSPG, presents at cell-cell contact sites of squamous epithelium, and functions in cell adhesion.^{6, 7, 10, 34} Transfection of ARH-77 cancer cells, which lack syndecan-1 expression, with syndecan-1 cDNA results in formation of multicellular aggregates in suspension culture³⁸ and reduced ability to invade type I collagen gel.³⁹ Similar transfection of malignant fusiform carcinoma cells restores their epithelial morphology and reduces their tumorigenicity in nude mice.⁴⁰ Syndecan-1 is lost during progression of epidermal dysplasia induced by UV irradiation,⁸ and in experimental SCCs induced in nude mice, its expression is reduced in inverse correlation with histological differentiation.⁹ Additionally, it is well known that cell surface syndecan binds various growth factors, such as basic fibroblast growth factor, vascular endothelial growth factor, and keratinocyte growth factor via HS-GAGs, and functions as a storage site for these,⁴⁻⁶ release due to its breakdown possibly accelerating angiogenesis, tumor cell migration and proliferation.

Syndecan-1 expression has already been reported to be reduced or lost in surgically resected human SCCs of head and neck, uterine cervix and skin.^{11-15, 17} In the first of these, this has prognostic significance,^{11, 12} and hepatocellular carcinomas with intra- or extrahepatic metastasis show considerably decreased syndecan-1 expression compared with those without metastasis.¹⁶ In most previous reports concerning surgically resected tumors, however,

only core protein expression was examined, and HS-GAGs expression has hitherto not been studied in detail. Syndecan-1 binds to various extracellular matrix components, such as collagen, fibronectin, via its HS-GAGs, and most of its biological functions are considered to be associated with this.⁴⁻⁶ In fact, it was reported that metastatic potential of murine B16 melanoma cells correlated with reduced surface HSPGs.⁴¹ In the present study of ESCCs, the reduction of HS-GAGs expression was more pronounced than that of core protein at each depth of invasion, and tended to correlate with more pathological parameters, such as lymph nodal or distant organ metastasis. The results indicate that cleavage of HS-GAGs from syndecan-1 core protein may be very important for reduced tumor cell adhesion and accelerated tumor cell invasion.

Because HS-GAGs is known to be structurally heterogeneous because of variability in the levels and distributions of sulfation and epimerization along the carbohydrate backbone,⁵ the staining patterns of the anti-HS-GAGs antibodies are not completely superimposable.^{28, 42} Therefore, loss of F58-10E4 epitope can be caused not only by a change in the amount of HS-GAGs, but also by a modification of HS-GAGs involving F58-10E4 epitope. This was the reason why another anti-HS-GAGs antibody, HepSS-1, was applied to several frozen ESCC tissues for the detection of HS-GAGs. Because HepSS-1 epitope was remarkably decreased in invasive ESCCs, like F58-10E4, it is very likely that the amount of HS-GAGs was diminished in invasive ESCCs. Detailed analysis of HS-GAGs could not be performed in the current study because of insufficient quantity of fresh specimens. Further studies are needed to assess the HS-GAGs in malignant tumors.

HS-GAGs shedding can be catalyzed by various proteases, such as thrombin or plasmin, which can recognize basic cleavage sites in the core protein near the plasma membrane,⁴ or endoglycosidic heparanase, which specifically cleaves HS-GAGs.²²⁻²⁵ In previous reports, heparanase activity correlated with metastatic potential of mouse melanoma, lymphoma and sarcoma cell lines,¹⁸⁻²⁰ and treatment of experimental animals with a heparanase inhibitor, such as heparin, considerably reduced the incidence of lung metastases by melanoma, Lewis lung carcinoma and mammary adenocarcinoma cells.^{43, 44} A mammalian heparanase has been cloned, and shown to be preferentially expressed in cell lines and surgically resected human breast, colon, liver carcinomas.^{21, 25} Transfection of low metastatic lymphoma cells with heparanase cDNA resulted in high metastatic potential.²⁵

In surgically resected colon carcinomas, both heparanase mRNA and protein are preferentially localized in carcinoma cells, and heparanase immunostaining may be weaker in cancer cells of Dukes A and B than in Dukes C and D cases, thus serving as an additional prognostic

marker.²¹ In the present study of ESCCs, heparanase expression was clearly increased in invasive carcinoma cells, and this was well correlated with many clinicopathological parameters, and marked loss of HS-GAGs. In contrast to cases having both cell surface and cytoplasmic heparanase protein expression, those with only cell surface expression demonstrated no association with clinicopathological parameters. Cytoplasmic heparanase expression might indicate overexpression and malignant potential.

In the present study, activity of heparanase was not examined in carcinoma tissues because sufficient quantities of fresh specimens could not be obtained. However, it has been reported that good agreement exists between enzyme activity and mRNA expression.⁴⁵ Because there was an inverse correlation between heparanase mRNA and HS-GAGs expression in ESCCs, loss of HS-GAGs might be due to cleavage by heparanase. In the present study, there was no clear link between syndecan-1 core protein immunostaining and its mRNA expression, which suggests that decrease might not be mainly due to decreased synthesis, but rather accelerated degradation by proteases. Heparanase protein was also expressed in the fibroblasts and inflammatory cells around the invading carcinoma nests. We consider that the positive reaction was not a false-positive reaction, because the specificity of the antibody used was confirmed by western blot analysis, and it has been reported heparanase is also secreted by activated T-lymphocytes, mast cells, platelets, peripheral nerves, fibroblasts and inflammatory cells.^{18, 20} Although the finding that the dramatic decrease of HS-GAGs in ESCCs in the areas with marked inflammatory cell infiltration suggested that heparanase secreted by fibroblasts and inflammatory cells contributed to the degradation of HS-GAGs in carcinoma cells, the precise role of heparanase secreted by stromal cells is unknown. In the present study, we evaluated the expression of heparanase protein in carcinoma cells, and it showed a good correlation with various clinicopathological parameters. We consider that heparanase secreted by carcinoma cells plays a dominant role in the degradation of HS-GAGs in carcinoma cells and in carcinoma invasion.

In the present study, mRNA was extracted from the whole tumor tissue, and the heparanase mRNA quantified included mRNA of both carcinoma cells and stromal cells, but heparanase mRNA expression of 14 protein positive cases in carcinoma cells was markedly higher than that of two negative cases. We consider that heparanase mRNA expressed in stromal cells plays little role in heparanase protein-negative cases. In order to elucidate the role of stromal cells in the degradation of HS-GAGs and carcinoma invasion, it is necessary to confirm expression of heparanase mRNA in stromal cells using new technique, such as micro-dissection, and to analyze the interactions between carcinoma cells and stromal cells.

In conclusion, loss of syndecan-1, especially of HS-GAGs, because of heparanase, may play an important role in ESCCs invasion and metastasis. These parameters could therefore be useful biomarkers for predicting prognosis. The use of "TaqMan" EZ RT-PCR allows quantification of heparanase mRNA in small biopsy specimens, and provides important information about malignant potential. If heparanase expression could be controlled, it might be possible to prevent tumor invasion and metastasis.

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