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Cytoplasmic expression of the JM403 antigen GlcA-$GlcNH_3^+$ on heparan sulfate glycosaminoglycan in mammary carcinomas—a novel proliferative biomarker for breast cancers with high malignancy

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Abstract The expressions of heparan sulfate glycosaminoglycans (HSGAGs) in breast carcinoma specimens from 60 patients were immunohistochemically investigated using monoclonal antibodies (mAbs) that recognized different epitopes of the glycan structure. Cytoplasmic expression of GlcA-$GlcNH_3^+$ on HSGAG was detected in carcinomas at high frequency (58.3%) using mAb JM403, whereas it was almost undetectable in normal breast ducts. This cytoplasmic expression was confirmed using confocal laser scanning microscopy. The expression of JM403 antigen in invasive carcinomas significantly correlated with nuclear atypia score (p=0.0004), mitotic counts score (p=0.0018), nuclear grade (p=0.0061) and the incidence of metastasis to axillary lymph nodes (p=0.0061). Furthermore, its expression was significantly correlated with the Ki67-labeling index in 55 invasive carcinomas ($p<0.05$) as well as in 26 non-invasive carcinomas (5 non-invasive carcinomas and 21 non-invasive carcinomas that were observed in individual invasive

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carcinomas) ($p<0.005$). Interestingly, the JM403 antigen GlcA-$GlcNH_3^+$ was also expressed in the cytoplasm of normal crypt epithelial cells where Ki67 protein was expressed in the cell nuclei in the proliferative compartment of the human small intestines. To date, HSGAGs have generally been found to exist on cell surface membranes and in extracellular matrices as components of HS proteoglycans, and the negatively-charged sulfated domains on HSGAGs are considered to be important for their functions. However, our present findings indicate that the cytoplasmic expression of the JM403 antigen GlcA-$GlcNH_3^+$ on positively charged, non-sulfated HSGAG may be involved in cell proliferation and associated with increased degrees of malignancy. The unordinary carbohydrate antigen of GlcA-$GlcNH_3^+$ on HSGAGs recognized by mAb JM403 may represent a novel proliferative biomarker for highly malignant mammary carcinomas.

Keywords Breast cancer · Heparan sulfate glycosaminoglycan · JM403 · Biomarker · Ki67-labeling index

Abbreviations

CLSM	confocal laser scanning microscopy/microscope
ER	estrogen receptor
PgR	progesterone receptor
HER2	Human epidermal growth factor receptor type 2

Introduction

It is well known that the glycans on the cell surface dramatically change according to the stage of malignancy. These cell surface glycans influence many biological behaviors such as cell proliferation, differentiation, apoptosis, adhesion, invasion, metastasis, and/or angiogenesis and can also act as biomarkers for the prognosis and/or as molecular targets for drugs [1, 2]. However, compared with studies on the glycans in glycolipids and glycoproteins, the number of studies about the glycans in heparan sulfate proteoglycans (HSPGs), called HS glycosaminoglycans (HSGAGs), is limited [3, 4], In particular, although studies on the core proteins of HSPGs [5–8] and heparanase [9, 10], which endogenously digests HSGAGs, have been reported, few studies have investigated human specimens [3, 4]. HSGAGs are linear polysaccharides composed of repeated units of disaccharides formed by hexuronic acids and glucosamines. The hexuronic acid is either glucuronic acid (GlcA) or iduronic acid (IdoA), and the glucosamine is *N*-acetylated glucosamine (GlcNAc), *N*-sulfated glucosamine (GlcNS) or the rarer unsubstituted glucosamine ($GlcNH_3^+$). These sugars are variously *O*- and *N*-sulfated and the chain lengths of the glycans differ significantly depending on cells and tissues [3, 11, 12]. Consequently, the myriad of the heterogeneity of HSGAGs has hindered analyses, which are still at a developmental stage even though current glycomics techniques for analyzing biological materials including mass spectrometry [13] and lectin arrays [14] are now available. To overcome these limitations, we employed three monoclonal antibodies (mAbs) that recognize different parts of HSGAGs to immunohistochemically investigate the expressions of specific structures of HSGAG antigens in mammary carcinomas. HSPGs are normally expressed on cell surfaces and in extracellular matrices [11, 12], and the sulfated domains on HSGAGs are believed to be important because many signaling molecules such as growth factors, cytokines and morphogens are transduced into cells through binding to these domains together with their own receptors [3, 11, 12]. Indeed, a targeted therapy against vascular endothelial growth factor (VEGF), to which HSGAGs bind [15], is utilized for breast cancer [16, 17]. Therefore, we hypothesized that these heavily sulfated binding domains on HSGAGs in breast cancer may influence the tumor malignancy. Surprisingly, however, we uncovered unordinary expression of mAb JM403 antigen GlcA-$GlcNH_3^+$ on HSGAGs in the cytoplasm of mammary carcinomas with high malignant characteristics.

Materials and methods

Materials

A total of 55 invasive and 5 non-invasive mammary carcinomas surgically resected from Japanese women were collected at Nagoya University Hospital and Kamiiida Daiichi General Hospital. Histological classification was performed according to the guideline of the Japanese Breast Cancer Society [18]. The nuclear atypia, the mitotic counts and the nuclear grade that rank histological degrees of malignancy were scored as previously defined [19]. Normal human small intestine specimens were ethically obtained from surgical specimens from patients who underwent digestive surgeries. The current study was approved by the Research Ethics Committees of both Nagoya University Hospital and Kamiiida Daiichi General Hospital.

Immunohistochemistry

Three mAbs against HSGAGs were obtained from Seikagaku Biobusiness Corp. (Tokyo, Japan). Specifically, these mAbs were JM403, 10E4, and NAH 46 that were previously reported to recognize $GlcNH_3^+$ [20], GlcA-GlcNS(+/-6S)/GlcNAc [21] and GlcA-GlcNAc [22], respectively. All specimens were embedded in paraffin and cut at a thickness of 4 μm for immunohistochemical analyses. After deparaffi-

nization, endogenous peroxidase activity was quenched with H_2O_2 (0.3%) containing 0.1% sodium azide, and endogenous avidin and biotin binding sites were blocked using an Avidin/Biotin Blocking Kit SP-2001 (Vector Laboratories Inc., Burlingame, CA). After non-specific binding sites were blocked with PBS containing 0.05% casein and 10% goat serum for 0.5 h, the sections were incubated with the primary antibodies (0.5–5 μg/ml) in PBS containing 0.1% casein, overnight at 4°C. After washing with PBS containing 0.05% casein, the sections were incubated with biotinylated $F(ab')_2$ fragment goat anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, PA) for 0.5 h at room temperature. After washing with PBS containing 0.05% casein, the sections were treated with horseradish peroxidase-streptavidin (Vectastain Elite ABC Kit PK6100; Vector Laboratories Inc.). The sections were washed with PBS containing 0.05% casein, and stained with 3-3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO). The sections were counterstained with methyl green solution for 1 h at room temperature. Some sections were pretreated with a mixture of the HSGAG-degrading enzymes, heparitinase I, II and heparinase from *Flavobacterium heparinum* (Seikagaku Biobusiness Corp.) as previously described [22]. The sections were counterstained with 5% methyl green solution. The Ki67 protein labeling index was scored using mAb MIB-1 (Abcam Inc, Cambridge, UK) as previously described [23]. Immunohistochemical staining was performed with mAb 6F11 for the estrogen receptor (ER) and mAb 16 for the progesterone receptor (PgR) using Benchmark XT (Ventana, Tucson, AZ). Human epidermal growth factor receptor type 2 (HER2) was immunostained using HercepTest II with an Autostainer (Dako, Glostrup, Denmark).

Immunohistochemical scores determined for the HSGAG mAbs

The scores for positive signals in the cytoplasm were determined according to the following criteria: negative (-), less than 10% of cancer cells in the section were immunostained; one positive (+), ≥10 to <50% of the cells were immunostained; two positive (++), ≥50% of the cells were immunostained.

Statistical analysis

$\chi 2$ tests for independence were performed to assess the correlations between the immunohistochemical scores based on the HSGAG mAbs, and nuclear atypia score, mitotic counts score, nuclear grade, incidence of metastasis to axillary lymph nodes and expressions of ER, PgR and HER2. The Kruskal-Wallis test and the Wilcoxon rank-sum test were performed to assess the correlations between the Ki67-labeling index and the immunohistochemical scores based on the HSGAG mAbs.

Double immunofluorescence staining of human small intestines

For double immunofluorescence staining, a polyclonal antibody against Ki67 protein (Abcam Inc.) was used in combination with mAb JM403. After blocking with goat serum diluted 1:10 in PBS at room temperature for 1 h in a humidity chamber, the sections were rinsed with PBS containing 1% BSA (PBS-BSA) and 100 μl of the primary antibody (against Ki67) was applied. After 20 min, 100 μl of the other primary antibody (JM403) was added and the sections were incubated for 24 h at 4°C in a humidity chamber. After the exposure to the primary antibodies, the sections were rinsed with PBS–BSA, and sequentially incubated with secondary antibodies (1:20 dilutions of rhodamine-conjugated goat anti-mouse and fluorescein-conjugated donkey anti-rabbit immunoglobulins) for 1 h each at room temperature in a dark humidity chamber. The sections were washed with PBS and covered with a fluorescence mounting medium (Vectashield; Vector Laboratories, Inc.). To visualize the cell nuclei, the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Control sections in which the primary antibodies were either omitted, or replaced with normal rabbit serum or normal goat serum were included in each staining procedure. The sections were viewed and photographed with confocal laser scanning microscopy (CLSM), a LSM 5 (Carl Zeiss MicroImaging,.GmbH, Jena, Germany).

Detail analysis of JM403 antigens in mammary carcinoma cells using CLSM

All specimens were embedded in paraffin and cut at a thickness of 6 μm for immunofluorescence analyses. After deparaffinization, the specimens were retrieved with autoclave at 121°C for 10 min in 0.01 M citrate buffer pH 6.0 and then cooled. Next non-specific binding sites were blocked using PBS containing 0.1% casein for 1 h. Endogenous avidin and biotin binding sites were blocked for 15 min using a Streptavidin/Biotin Blocking Kit SP-2002 (Vector Laboratories Inc.). After washing with 0.01 M PBS, the sections were incubated with primary antibodies (either JM403, 1:200 dilution or anti-Ki67 polyclonal antibody ab833, 1:200 dilution, or both) overnight at 4°C in a dark humidity chamber. After washing, the sections were incubated with secondary antibodies Alexa Fluor 594 conjugated to goat anti-mouse IgM (1:200; Invitrogen, Carlsbad, CA, USA) or a biotinylated anti rabbit immunoglobulin, or both) in a dark humidity chamber at room temperature for 1 h. For Ki67 staining, the sections were

further incubated with Alexa Flour 488 conjugated streptavidin (1:200; Invitrogen, Carlsbad, CA, USA) for 30 min. The nuclei were then counterstained with TOTO3 (Invitrogen, Carlsbad, CA, USA). Some sections were pretreated with a mixture of the HSGAG-degrading enzymes, heparitinase I, II and heparinase from *Flavobacterium heparinum* (Seikagaku Biobusiness Corp.) as previously described [22]. Double or triple-labeled immunofluorescent signals were detected and analyzed with CLSM, using a LSM-510 META (Carl Zeiss Micro-Imaging,.GmbH). The volumetric data sets of these images taken with CLSM were digitized and subjected to image analysis manipulation and computer-assisted 3D reconstructions using ZEN2008 and IMARIS version 6.1.2 (BITPLANE, Zurich, Switzerland).

Further characterization of the JM403 antigen by enzyme-linked immunosorbent assay (ELISA)

Further characterization of the epitope recognized by mAb JM403 was performed by ELISA as previously described [22]. Briefly, a polysaccharide composed of GlcA-GlcNAc and GlcA-$GlcNH_3^+$, (GlcA-GlcNAc/GlcA-$GlcNH_3^+$)n and a polysaccharide composed of IdoA-GlcNAc and IdoA-$GlcNH_3^+$, (IdoA-GlcNAc/IdoA-$GlcNH_3^+$)n were prepared by alkaline treatment from *N*-acetyl-heparosan (GlcA-GlcNAc)n and acharan (IdoA-GlcNAc)n, respectively [22]. HSGAG from bovine kidney was biotinylated and fixed onto streptavidin-coated 96-well of plates. Next 100 μl of 0.8 μg/ml JM403 in PBS containing 5% ApplieDuo (Seikagaku Biobusiness Corp.) with or without the polysaccharides or the HSGAG as inhibitors was added to each well and incubated for 1 h. After incubation with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Dako) as a second antibody, 3,3', 5,5'-tetramethylbenzidine was added and the absorbances at 450 nm were measured with a reference wave-length of 630 nm.

Results

Clinical and pathological features

The clinical and pathological characteristics of the patients are summarized in Table 1. The invasive carcinoma specimens comprised 27 papillotubular carcinomas, 8 solid-tubular carcinomas, 16 scirrhous carcinomas, 1 mucinous carcinoma, 1 invasive lobular carcinoma, and 2 tubular carcinomas. The non-invasive carcinoma specimens comprised 5 ductal carcinomas *in situ*. Seventeen patients showed metastasis in the dissected axillary lymph nodes, while 43 patients did not.

Table 1 Profiles of the patients and their cancers

Variables		n
Patients		60
Tumors	Non-invasive	5
	Invasive	55
Age (years)	Mean	54.6 (27–87)
Histologic types	Ductal carcinoma *in situ* (non-invasive)	5
	Papillotubular carcinoma	27
	Solid-tubular carcinoma	8
	Scirrhous carcinoma	16
	Mucinous carcinoma	1
	Invasive lobular carcinoma	1
	Tubular carcinoma	2
Primary tumor statuses	Tis	5
	T1	40
	T2	13
	T3	2
Axillary lymph node metastasis	Positive	17
	Negative	43
Hormone receptors	ER-positive	37
	PgR-positive	34
HER2	Positive[a]	7
	Negative	53

[a] Specimens with immunohistochemical score 3 for HER2 were regarded as positive

ER estrogen receptor, *PgR* progesterone receptor, *HER2* human epidermal growth factor receptor type 2

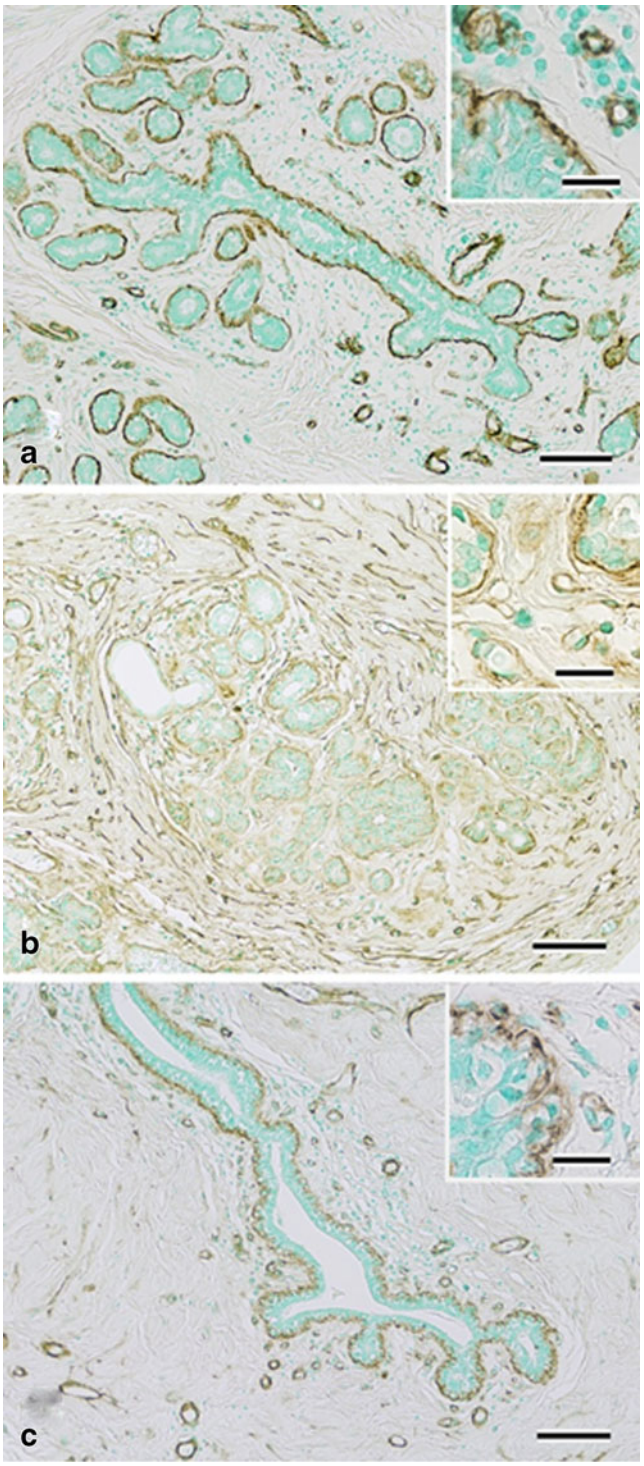

Fig. 1 Expressions of the HSGAG antigens recognized by mAbs JM403 **a**, 10E4 **b** and NAH46 **c** in normal breast tissues. All the antibodies immunostain the basement membranes and vessel walls but hardly stain the cytoplasm in cells in the non-neoplastic mammary duct epithelium, lobular epithelium and myoepithelium. Bars indicate 100 μm **a**, **b**, **c** and 20 μm (insets), respectively

Expression of HSGAG antigens in normal breast and mammary carcinomas

The expressions of HSGAG antigens were immunohistochemically examined using mAb JM403, 10E4, and

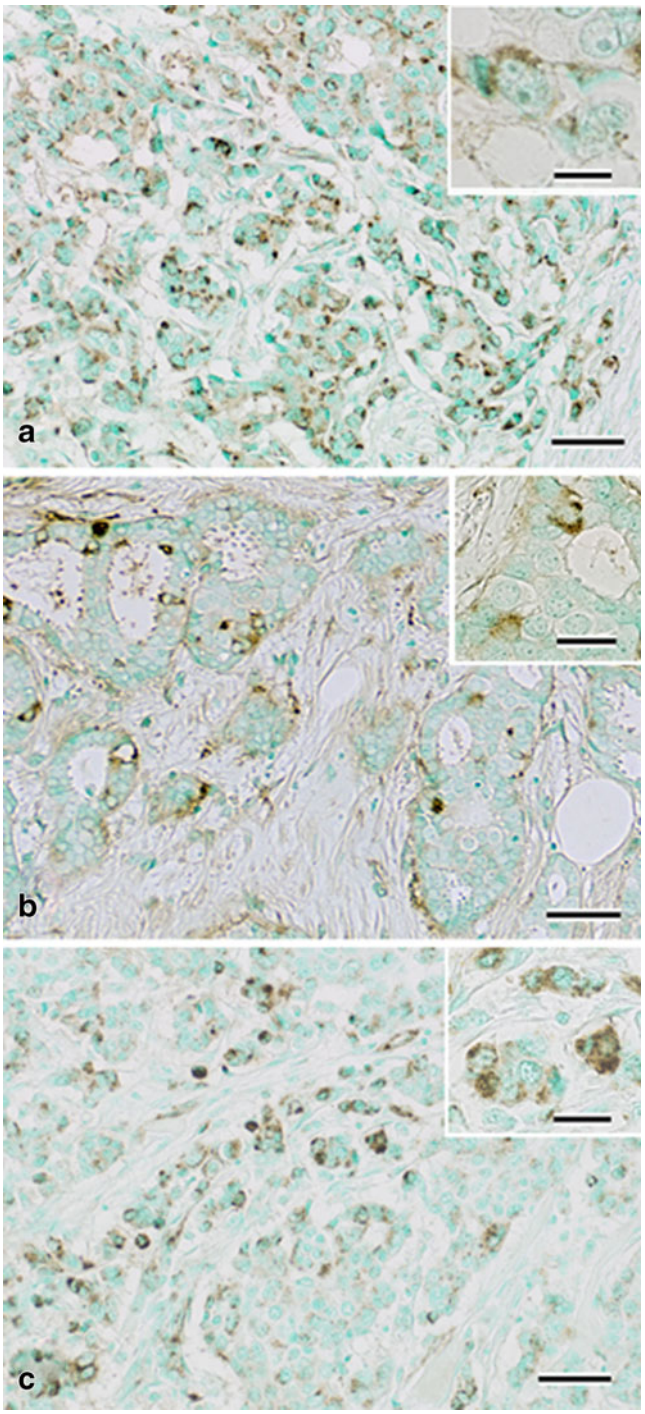

Fig. 2 Expressions of the HSGAG antigens recognized by mAbs JM403 **a**, 10E4 **b** and NAH46 **c** in mammary carcinomas. All the antibodies immunostain the cytoplasm of cells with varying degrees and patterns. Bars indicate 100 μm **a**, **b**, **c** and 20 μm (*insets*), respectively

NAH46 in mammary carcinomas and normal breast tissues resected from cancer patients (Fig. 1). Although all the mAbs immunostained the basement membranes and vessel walls, where HSPGs normally exist as a component of the extracellular matrix, little staining was observed in the cytoplasm of cells in the non-neoplastic mammary duct epithelium, lobular epithelium and myoepithelium. In contrast, immunostaining for all three mAbs was observed in the cytoplasm of cancer cells with varying patterns and extents of staining (Fig. 2). The surface membranes of some cancer cells were also immunohistochemically positive for 10E4. The cytoplasmic positivity rates are summarized in Table 2. The highest rate was observed for JM403 (58.3%) while the lowest rate was observed for NAH46 (25%).

Correlations between the immunohistochemical scores and nuclear atypia score, mitotic counts score, nuclear grade and incidences of metastasis to axillary lymph nodes

The correlations between the immunohistochemical scores and the malignant parameters were statistically analyzed for the three mAbs (Fig. 3). The immunohistochemical score for the JM403 antigen was significantly correlated with nuclear atypia score (p=0.0004), mitotic counts score (p=0.0018), nuclear grade (p=0.0001) and incidence of metastasis (p=0.0061). The score for the NAH46 antigen was significantly correlated with nuclear atypia score (p=0.001), mitotic counts score (p<0.0001), nuclear grade (p=0.0003), but not with the incidence of metastasis. The score for 10E4 antigen was not correlated with either score, grade or incidence of the metastasis.

Correlations between the immunohistochemical scores and Ki67-labeling indexes

The correlations between the Ki67-labeling indexes and the immunohistochemical scores for three mAbs were analyzed using 55 invasive carcinoma specimens and 26 non-invasive carcinoma specimens (5 non-invasive carcinomas

Table 2 Expression incidences (%) of HSGAG antigens recognized by mAbs

mAbs	Immunohistochemical scores			Positive incidences[a]
	–	+	++	
JM403	41.7	36.7	21.6	58.3
10E4	51.7	43.3	5	48.5
NAH46	75	20	5	25

[a] Positive incidences were defined as + and ++

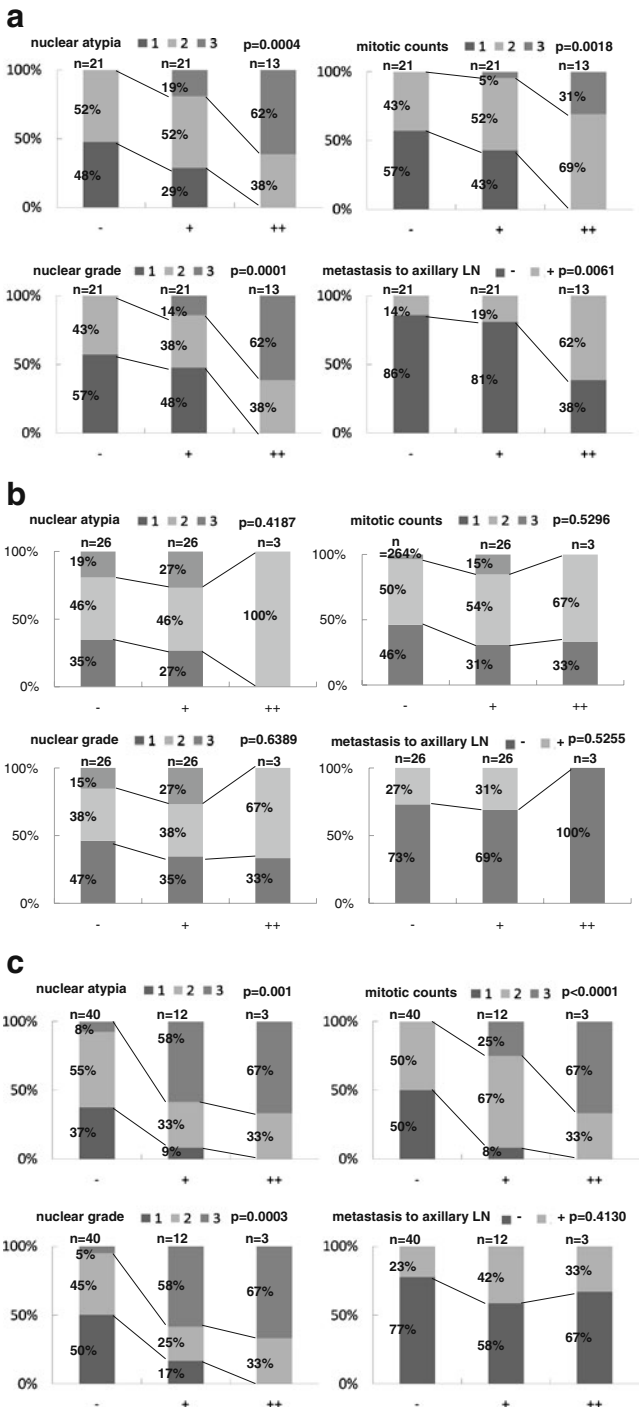

Fig. 3 Correlations between immunohistochemical scores and degrees of malignancy in invasive breast cancers. a, JM403; b, 10E4; c, NAH46. The x-axis indicates the scores of positive signals from the cytoplasm (See "Materials and Methods"). The y-axis indicates the percentage of nuclear atypia score (1, 2, 3), mitotic counts score (1, 2, 3), nuclear grade (1, 2, 3) and presence/absence of metastasis to axillary lymph nodes (LN), determined in each immunological score from each antibody. The figure (n) described on the top of each column indicates the number of the cases. $\chi 2$ test for independence using 3×3 contingency table was performed to assess the correlations with the immunohistochemical score and malignant parameters

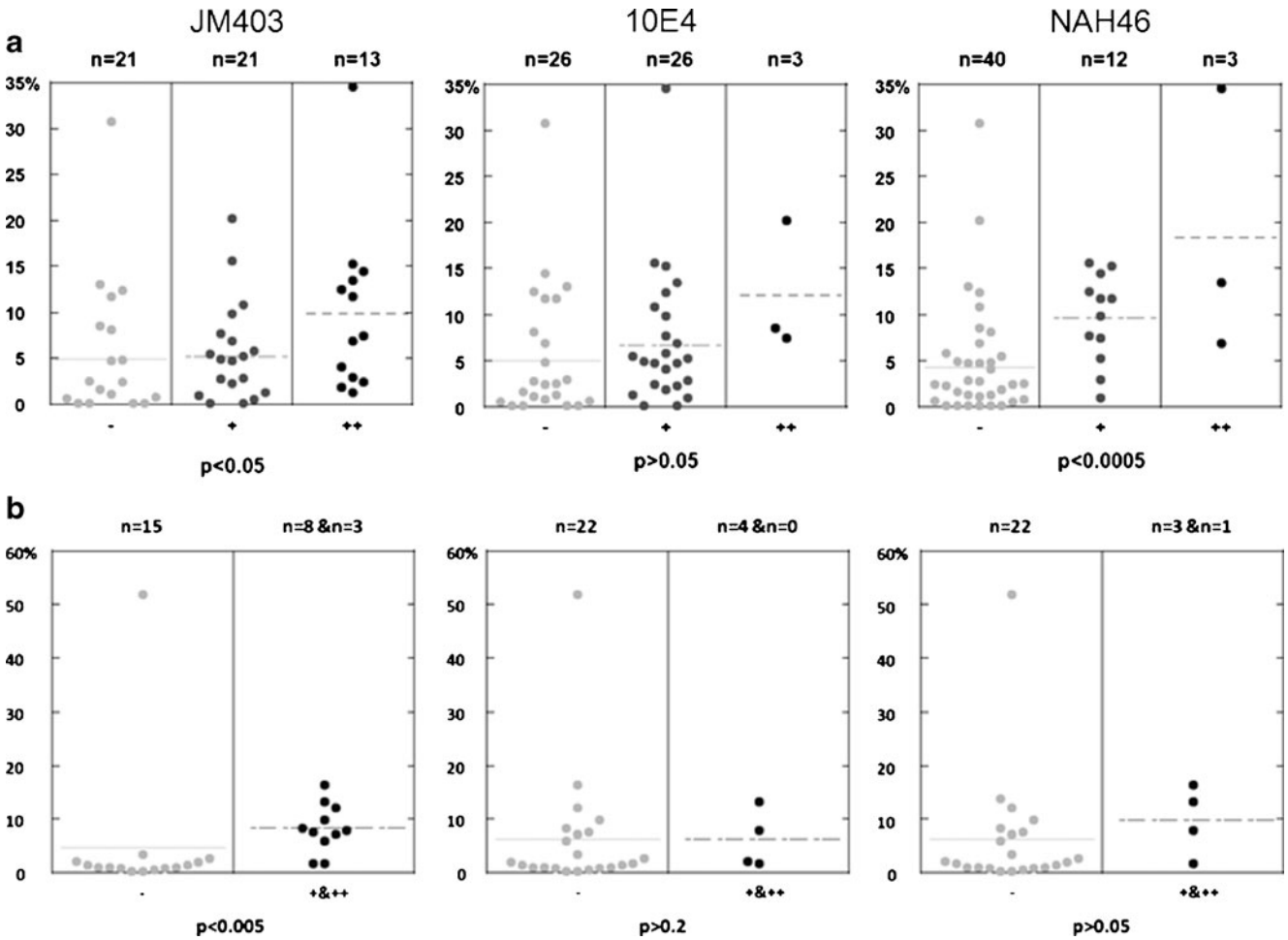

Fig. 4 Correlations between the immunohistochemical scores and the Ki67-labeling indexes. **a** The results for 55 invasive carcinomas. Kruskal-Wallis tests were performed for assessment. **b** The results for 26 non-invasive carcinomas (5 non-invasive carcinomas and 21 non-invasive carcinoma observed in individual invasive carcinomas). Wilcoxon rank-sum tests were performed for assessment

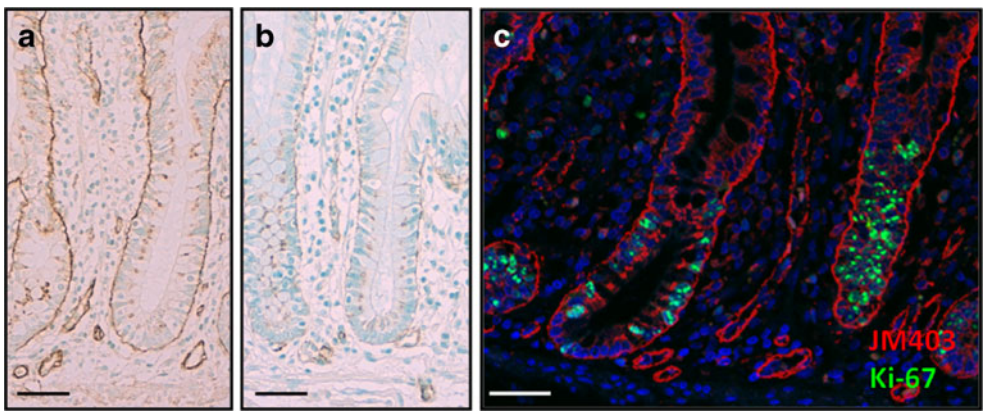

Fig. 5 Expression of the JM403 antigen in human small intestines, and comparison of the JM403 antigen expression with Ki67 protein expression. **a** Immunohistochemical staining with JM403. The JM403 antigen is expressed in the cytoplasm of normal crypt epithelial cells located in the proliferative compartment. The antigen is expressed at basement membranes of the epithelium and vessel walls. **b** Treatment with heparitinases/heparinase. The signals from the cytoplasm as well as the basement membranes and vessel walls are almost abolished after pretreatment with the enzymes. **c** Double immunofluorescence staining. The JM403 antigen is more broadly distributed than the Ki67 protein, while both the JM403 antigen and Ki67 protein are negative in the cells at the crypt base. Nuclei were stained with DAPI. Bars indicate 50 μm

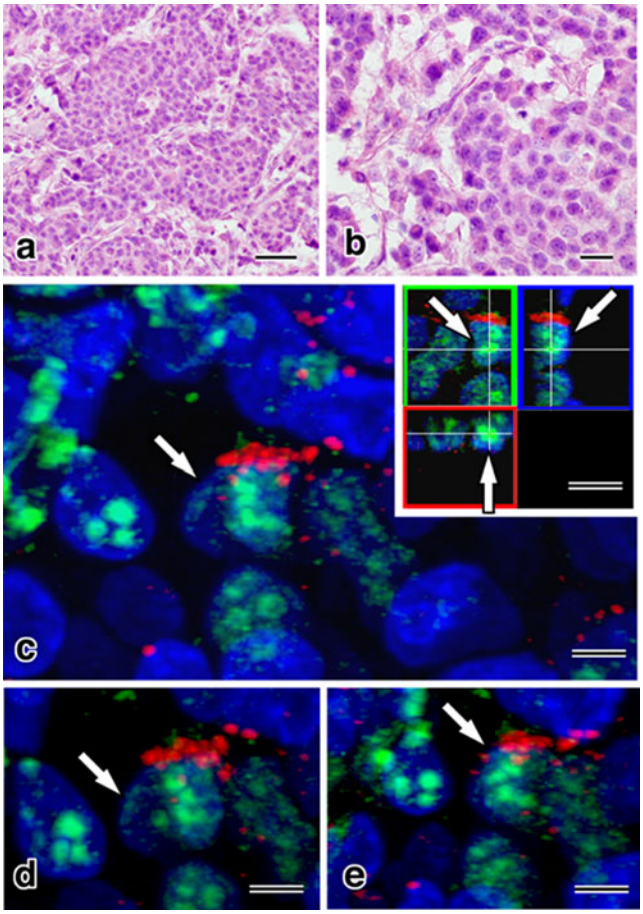

Fig. 6 Detail analyses of JM403 antigens and Ki67 proteins in mammary carcinoma using CLSM. **a** and **b** show hematoxylin-eosin staining of the carcinoma cells with atypical nuclei. This specimen was used for the following 3D analysis. **c**, **d**, **e** and inset images show three dimensional images from JM403 antigens (red signals, Alexa 594), Ki67 proteins (green signals Alexa 488) and nuclei (blue signals, TOTO3). **c** front view; **d**, view 15° turn the left; **e**, view 60° turn the left. Insets show orthogonal image of JM403 positive cells and Ki67 positive cells in 3D. Upper left, X-Y plane image, upper right, Y-Z plane image, lower left, X-Z plane image. The JM403 signals are localized in the cytoplasm but not in the nuclei of the Ki67 positive carcinoma cells. Arrows indicate the same cell. Bars indicate 50 μm **a**, 20 μm **b**, 5 μm **c**, **d**, **e** and 10 μm inset), respectively

and 21 non-invasive carcinomas observed in individual invasive carcinomas) (Fig. 4). The scores for the JM403 antigen were significantly correlated with Ki67-labeling index not only in the invasive specimens ($p<0.05$) but also in the non-invasive specimens ($p<0.005$). The scores for the NAH46 antigen were highly correlated with the Ki67-labeling index in the invasive specimens ($p<0.0005$) and tended to be correlated in the non-invasive specimens ($p=0.067$), although the positive incidence was low (17%; see Table 2). The scores for the 10E4 antigen were not correlated with the Ki67-labeling index for either the invasive specimens or the non- invasive specimens.

Expression of the JM403 antigen in human small intestines and comparison with Ki67 protein expression

Since the cytoplasmic expression of the JM403 antigen was significantly correlated with the Ki67-labeling index, we examined small intestines, which normally express the Ki67 protein. The JM403 antigen was expressed in the cytoplasm of normal crypt epithelial cells located in the proliferative compartment of the small intestine (Fig. 5a and c). Interestingly, the positive signals for the JM403 antigen were observed in an orderly fashion at the apical site of the cells, whereas the signals were randomly distributed in the cytoplasm of mammary carcinoma cells (Fig. 2a). The signals in the cytoplasm as well as in the basement membranes and vessel walls almost disappeared after pretreatment with heparitinases/heparinase (Fig. 5b), indicating that the signals were specific.

The localizations of the JM403 antigen and Ki67 protein expressions were further examined using a double immunofluorescence technique (Fig. 5c). The JM403 antigen and Ki67 protein were co-localized in some cells, although the former was more broadly distributed than the latter. Both the JM403 antigen and Ki67 protein were negative in the cells at the crypt base.

Detailed-3D analyses of JM403 antigen in human mammary carcinoma cells using CLSM

To completely rule out the possibility that JM403 antigens were present in the cytoplasm as a result of convolution of the cell surfaces, where JM403 antigens may exist, we performed 3D analyses of the carcinoma cells using high performance CSLM. The JM403 antigens appeared with a granular pattern in the cytoplasm of the cells, where Ki67 proteins also appeared in the nuclei of the same cells. 3D analysis also showed that JM403 antigens were not present

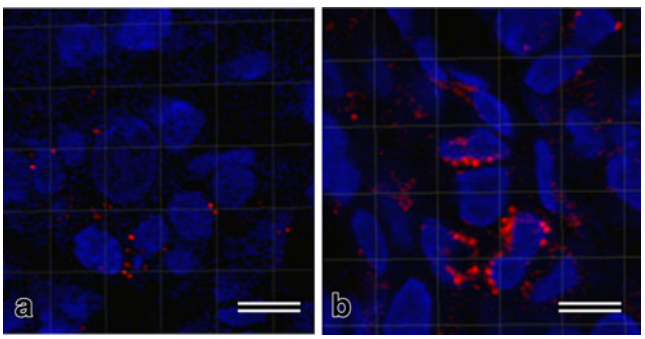

Fig. 7 The effect of heparitinases/heparinase treatment on the expression of JM403 antigens in mammary carcinoma cells. The signals were observed using CLSM. The red signals (Alexa594) were from JM403 antigens and blue signals from nuclei (TOTO3). The expression of JM403 antigen was markedky reduced with treatment **a**, compared with the control **b**. Bars indicate 10 μm

in the nuclei (Fig. 6(c), (d), (e) and inset). Heparitinases/heparinase treatment clearly diminished the intensities of the signals from JM403 antigens (Fig. 7).

Correlations between the immunohistochemical score for the JM403 antigen and the expressions of ER, PgR and HER2

The correlations between the immunohistochemical score for the JM403 antigen and the expressions of ER, PgR and HER2 were examined. The incidences of ER and PgR expressions were inversely correlated with the score (p= 0.0028 and p=0.0211, respectively), whereas the incidence of HER2 expression was correlated with the score for the JM403 antigen (p=0.045) (Fig. 8).

Further characterization of the JM403 antigen by ELISA

The JM403 epitope, which was previously reported to be $GlcNH_3^+$ on HSGAGs [20], was further characterized by ELISA using (GlcA-GlcNAc/GlcA-$GlcNH_3^+$)n and (IdoA-GlcNAc/IdoA-$GlcNH_3^+$)n. The binding of JM403 to pre-coated HSGAG was strongly and moderately inhibited by (GlcA-GlcNAc/GlcA-$GlcNH_3^+$)n and HSGAG, respectively, but was minimally inhibited by (IdoA-GlcNAc/IdoA-$GlcNH_3^+$)n and (GlcA-GlcNAc)n (Fig. 7), indicating that mAb JM403 requires a sequence of GlcA-$GlcNH_3^+$ but not IdoA-$GlcNH_3^+$on HSGAGs as an epitope. (Fig. 9)

Discussion

There have been many reports about significant correlations between high malignancy and abnormal expressions of glycans in glycoproteins and glycolipids [1, 2]. Many studies on the core proteins of HSPGs such as syndecans and glypicans [5–8] have also been reported. Indeed, high expression of glypican 3 is well known as a biomarker for hepatocellular carcinomas [7, 8]. High expression of heparanase in many cancers including breast cancers [9] has been reported to facilitate cancer metastasis and angiogenesis by degrading HSGAGs in extracellular matrices. Inhibitors of heparanase have been elucidated, some of which are currently being evaluated in various stages of clinical trials [10]. However, the number on studies on HSGAGs as glycans of HSPGs is limited, especially for human specimens from clinical settings [3, 4]. HSGAGs are composed of alternating GlcNAc or GlcNS together with very small amounts of $GlcNH_3^+$, and hexuronic acids (GlcA or IdoA). In addition, they are extremely complicated in terms of their degrees of sulfation and chain lengths [3, 11, 12]. These multiple factors have hampered analyses of HSGAGs in specimens from cancer patients. To overcome these difficulties, we employed three mAbs to profile the HSGAGs from mammary carcinomas. Unexpectedly, we observed unordinary expression of the JM403 antigen, a very rare domain of $GlcNH_3^+$ in the cytoplasm of cells in highly malignant mammary carcinomas.

HSGAGs on cell surfaces can bind to many growth factors such as fibroblast growth factors and VEGFs, cytokines/chemokines (TGF-β, IL-8, and MIP-1β), morph-

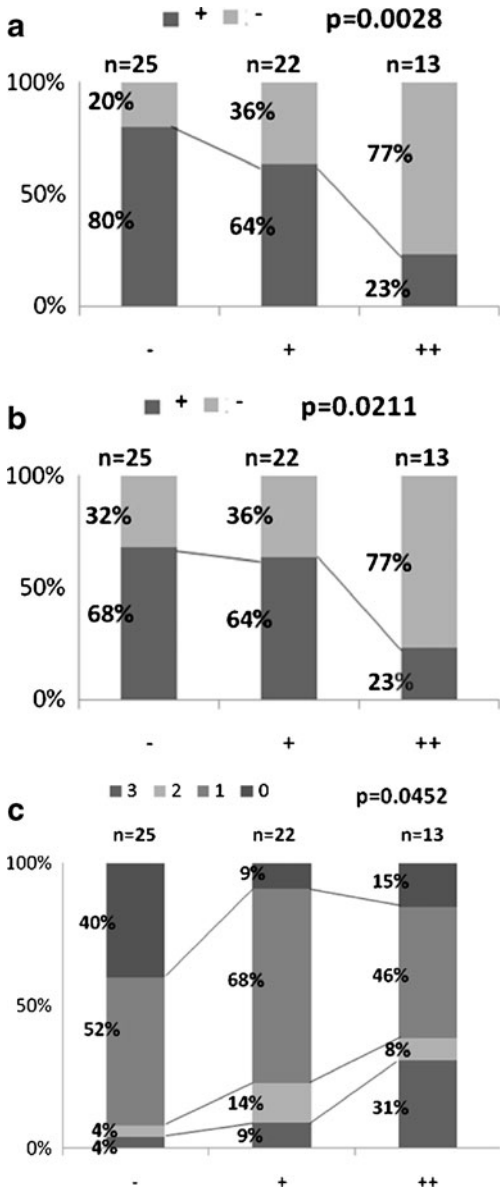

Fig. 8 The immunohistochemical score for the JM403 antigen is inversely correlated with the expressions of ER **a** and PgR **b** but correlated with the expression of HER2 **c.** The x-axis indicates the immunohistochemical scores for the JM403 antigen. The y-axis indicates the percentages of the receptor expressions **a**, **b** or the percentage of HER2 score **c**. The n numbers at the top of each column indicate the numbers of specimens. Specimens with immunohistochemical score 3 for HER2 were regarded as positive. $\chi 2$ tests for independence were performed for assessment of the correlations

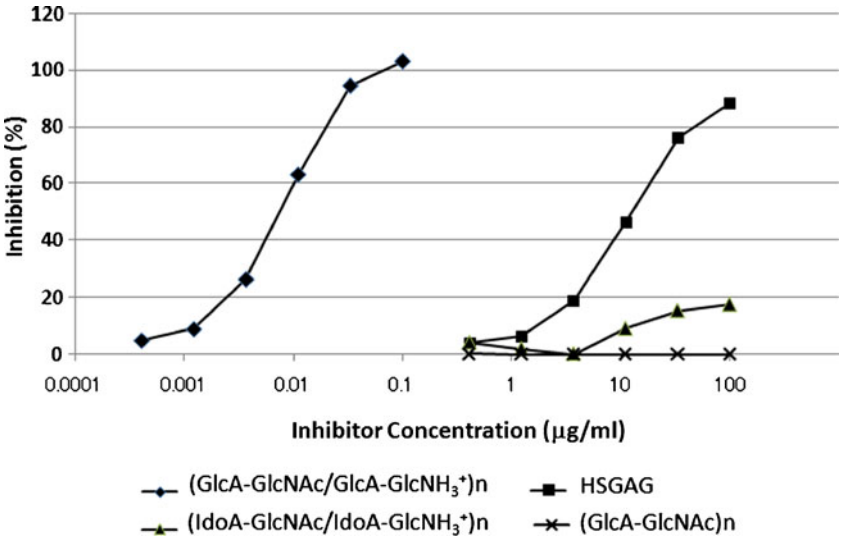

Fig. 9 Further characterization of JM403 antigen using ELISA. The binding of JM403 to HSGAG was strongly and moderately inhibited by polysaccharide of (GlcA-GlcNAc/GlcA-$GlcNH_3^+$)n and HSGAG, but hardly inhibited by (IdoA-GlcNAc/IdoA-$GlcNH_3^+$)n and (GlcA-GlcNAc)n

ogens (Hedgehog and BMP), and adhesive molecules (P-selectin, laminin, and fibronectin) through their specific glycans, to efficiently transduce their signals into the cells [3, 11, 12]. Therefore, it is rational to suppose that abnormal expressions of HSGAGs on cell surface of cancers may significantly modulate the biological behaviors of these cells. In fact, the high expression levels of VEGF isoforms have been investigated in breast cancers [16] and abnormal expressions of VEGF receptors (VEGFRs) on cancer cells have also been reported [24]. Currently VEGFs/VEGFRs are good molecular targets for chemotherapies and biologics [16, 17]. Therefore, it may also be rational to expect that abnormal HSGAGs on cancer cell surfaces, which would be highly able to bind VEGF, may modulate cancer cell behaviors and contribute to the malignancy. On this basis, expression of mAb 10E4 antigen would be expected to be correlated with malignancy because it recognizes GlcNS, which is a part of the structure of HSGAGs that binds to VEGF [15]. In the present study, however, we did not find any correlation between the cell surface expression of HSGAGs recognized by 10E4 and malignancy.

In contrast, the cytoplasmic expression of the JM403 antigen $GlcNH_3^+$ [20] was unordinary for two reasons. First, HSPGs are generally classified into extracellular matrix types, such as perlecan and agrin, and membrane bound types, such as syndecans and glypicans, according to their sites of expression [11]. The exceptional case is heparin, which is a special type of HSPG with high sulfation. Heparin exclusively exists in the cytoplasmic granules of mast cells, inhabiting connective tissues. [25]. To rule out the possibilities that the convolution of the JM403 antigens on cell surfaces into the cytoplasm were misinterpreted as the occurrence of the JM403 antigen in the cytoplasm, we performed 3D analysis with CLSM; showing the JM403 antigens in the cytoplasm of the human mammary carcinoma cells with atypical nuclei. Second, the negatively-charged sulfated domains on HSGAGs are believed to be important because many signaling molecules bind to these domains [3, 11, 12]. Nevertheless the structure of $GlcNH_3^+$ is positively charged. This structure on HSGAGs is very uncommon and was reported to exist on only 0.7–4% of GlcNAc/GlcNS/$GlcNH_3^+$ in HSGAGs. Its occurrence and biological significance have long been ignored, and the genes and enzymes responsible for generating this structure have not yet been elucidated [20, 26]. $GlcNH_3^+$ has a free NH_3^+ group, which is also a component of naturally bioactive molecules such as certain basic peptides [27], polyamines [28] and sphingosine [29]. Therefore, it is possible that mAb JM403 may non-specifically bind to such molecules in the cytoplasm. Accordingly, we carefully investigated whether JM403 only bound to the NH_3^+ on HSGAGs by using different approaches. First, we digested specimens with the HSGAG-degrading enzymes heparitinases/heparinase before immunostaining with JM403. After treatment, the signals from the cytoplasm almost disappeared to similar a extent as those from the basement membranes and vessel walls in human intestines, whereas the signals from mammary carcinoma cells were markedly diminished. These results suggested that the structures of HSGAGs surrounding JM403 antigens in mammary carcinomas may be different from those found in human intestines. Second, the epitope recognized by JM403 was further characterized using inhibitory polysaccharides and ELISA. The binding of JM403 to HSGAG was strongly inhibited by (GlcA-GlcNAc/GlcA-$GlcNH_3^+$)n but hardly inhibited by (GlcA-GlcNAc)n. This is consistent with a previous report that the JM403 strongly reacted with *N*-deacetylated heparosan but hardly with native heparosan [30]. On the other hand, the

mAb hardly inhibited by (IdoA-GlcNAc/IdoA-$GlcNH_3^+$)n. These findings clearly revealed that JM403 requires GlcA-$GlcNH_3^+$ but not IdoA-$GlcNH_3^+$ as its epitope in the sugar sequence of HSGAGs and that the signals in the immunohistochemical analysis were specific. The biological significance of the cytoplasmic expression of the JM403 antigen GlcA-$GlcNH_3^+$ on HSGAGs is currently unknown. Ki67 protein, a nonhistone protein is expressed during mitosis and its expression is currently used as a biomarker of mitotic activity [23]. Interestingly, we observed co-expression of Ki67 protein with the JM403 antigen in the cytoplasm of cells in the normal intestinal epithelium in the proliferative compartment, strongly suggesting that the expression may be involved in cell proliferation. This notion is supported by the findings that the expression of the JM403 antigen GlcA-$GlcNH_3^+$ was correlated with the mitotic counts score. The expression was inversely correlated with the positive incidences of ER and PgR, further suggesting that the expression of the JM403 antigen is correlated with malignancy. The JM403 immunohistochemical score was correlated with the Ki67-labeling index, but the correlation was relatively low ($p<0.05$ Fig. 4) in invasive carcinomas. This result may reflect the evidence that GlcA-$GlcNH_3^+$ is expressed on more differentiated cells than Ki67 protein because JM403 immunostained a broader area in the small intestine. Although the Ki67-labeling index was included as a proliferative marker in the St Gallen recommendations for breast cancer treatment in 2009 [31], there are some discrepancies between breast cancer malignancy and this index [32, 33]. The score for the JM403 antigen is expected to present qualitatively different information in terms of proliferation and the degree of malignancy.

It has long been considered that the sulfated domains, which together with hexuronic acids, provide negative charges to HSGAGs, are important in cancer cells because the domains function as binding sites for biological ligands like growth factors, cytokines and morphogens [3, 11, 12]. Higher expression of heparanase, which degrades basement membranes and other extracellular matrices, in cancers has been reported to function in invasion, angiogenesis and metastasis [9, 10]. In addition to these established aspects of HSGAGs in cancer biology, we have presented a new view that cytoplasmic expression of the JM403 antigen GlcA-$GlcNH_3^+$, which endows HSGAGs with a positive charge, is significantly correlated with highly malignant breast cancers. Although, the biological functions of GlcA-$GlcNH_3^+$are still unknown, its potential role as a biomarker could be useful for accurately predicting the clinical outcome of breast cancers. Since the JM403 antigen is a glycan, which is generally considered to be a more robust antigen than a protein, more reproducible and reliable results can be expected in the daily clinic. Large-scale clinical studies to confirm and expand this finding are currently ongoing

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