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N-Acetylglucosamine 6-*O*-sulfotransferase-2 as a tumor marker for uterine cervical and corpus cancer

Akira Seko · Fumio Kataoka · Daisuke Aoki · Masaru Sakamoto · Toshiaki Nakamura · Masayuki Hatae · Suguru Yonezawa · Katsuko Yamashita

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Abstract *N*-Acetylglucosamine 6-*O*-sulfotransferase-2 (GlcNAc6ST2) is ectopically expressed in ovarian mucinous and clear cell adenocarcinoma [Kanoh *et al.*, Glycoconj J 23:453–460, 2006]. Here we studied whether GlcNAc6ST2 protein can be detected in sera from patients with gynecological cancers and could serve as a tumor marker. First, we created a monoclonal antibody and polyclonal antiserum against GlcNAc6ST2. These antibodies were specific for GlcNAc6ST2, as shown by Western blot analysis and immunoprecipitation. Using these antibodies, we constructed a sandwich ELISA method for detecting GlcNAc6ST2 in the serum. GlcNAc6ST2 provided lower positive rates for ovarian cancer than CA125, but higher positive rates for uterine cervical and corpus cancer than SCC antigens and CA125, respectively. A significantly higher percentage of stage I uterine cervical and corpus cancers were positive for GlcNAc6ST2 than for SCC antigens and CA125, respectively. GlcNAc6ST2 could therefore be a good serological marker for detecting early-stage uterine cervical and corpus cancers.

Keywords Sulfotransferase · Ovarian cancer · Tumor marker · Sandwich ELISA · Uterine corpus cancer · Uterine cervical cancer

Abbreviations

GlcNAc	*N*-Acetylglucosamine
HRP	Horseradish peroxidase
GlcNAc6ST	GlcNAc 6-*O*-sulfotransferase
SCC	Squamous cell carcinoma
mAb	Monoclonal antibody

Introduction

N-Acetylglucosamine (GlcNAc) 6-*O*-sulfotransferase (GlcNAc6ST) catalyzes 6-*O*-sulfation of GlcNAc residues in various glycoconjugates. The GlcNAc6ST family includes seven members in humans: GlcNAc6ST1, 2, 3, 4, and 5, Gal6ST, and Ch6ST1 [1]. We previously found that GlcNAc6ST2 is ectopically expressed in colonic mucinous adenocarcinomas [2] and in ovarian mucinous and clear cell adenocarcinomas [3]. In normal human adults, expression of GlcNAc6ST2 is highly restricted to high endothelial cells in lymph nodes [4], where specific

A. Seko · K. Yamashita (✉)
Innovative Research Initiatives, Tokyo Institute of Technology,
Yokohama, Japan
e-mail: kyamashi@bio.titech.ac.jp

F. Kataoka · D. Aoki
Department of Obstetrics and Gynecology, School of Medicine,
Keio University,
Tokyo, Japan

M. Sakamoto
Department of Gynecology,
Sasaki Foundation Kyoundo Hospital,
Tokyo, Japan

T. Nakamura · M. Hatae
Department of Gynecology, Kagoshima City Hospital,
Kagoshima, Japan

S. Yonezawa
Department of Human Pathology,
Kagoshima University Graduate School
of Medical and Dental Sciences,
Kagoshima, Japan

sulfated glycans are synthesized and function as homing ligands for L-selectin-expressing lymphocytes [5, 6]. Because of its limited expression in normal tissues and ectopic expression in specific types of cancer tissues, GlcNAc6ST2 could be a good candidate for a tumor marker. Ideally, such a marker could be identified in a blood sample, however, it has never been reported whether GlcNAc6ST2 protein is shed into the sera from patients with such types of colonic or ovarian adenocarcinomas.

Ovarian cancer, uterine cervical cancer, and uterine corpus cancer are the major malignant gynecologic diseases. Ovarian epithelial cancer is classified mainly into four types: serous, endometrioid, mucinous, and clear cell adenocarcinomas. Generally speaking, the former two types are chemotherapy-sensitive, whereas the latter two types are chemotherapy-resistant, resulting in poor prognosis. Several tumor markers have been explored for use in evaluating medical treatment against ovarian cancer. Among them, CA125 [7–9] has been the most extensively utilized to date. CA125, also known as MUC16, is a mucinous glycoprotein that is composed of an *N*-terminal domain, Ser/Thr-rich tandem repeat domain, transmembrane region, and short cytoplasmic tail [10, 11]. CA125 is expressed in a membrane-bound form on the surface of tumor cells and is cleaved off from the cells via an unidentified proteolytic process. Squamous cell carcinoma (SCC) antigens have been developed as tumor markers for uterine cervical cancer [12, 13]. The two isoforms of SCC antigens, SCCA1 and SCCA2, belong to the serine protease inhibitors (serpin) superfamily [14, 15] and are expressed in various types of squamous epithelium [16]. In contrast to ovarian and uterine cervical cancer, the sensitivities of CA125 and SCC antigens are relatively low for uterine corpus and cervical cancers [17], and no effective tumor marker for this cancer has yet been developed.

In this study, we prepared monoclonal and polyclonal antibodies against GlcNAc6ST2 protein and examined whether this protein can be detected in sera from patients with ovarian or uterine cancer by the sandwich ELISA method. GlcNAc6ST2 was frequently present in sera from patients with uterine cervical and corpus cancer, suggesting that it could serve as a tumor marker for detecting these cancers, especially in the early stages.

Materials and methods

Sera

The sera used in this study were obtained from Keio University Hospital, Tokyo (76 specimens from patients with ovarian cancer [ages 19–85; mean ± SD 55.5±12.3], 26 from patients with uterine corpus cancer [33–81, 53.1±11.8], 16 from patients with uterine cervical cancer [27–79, 47.2±14.1], and 55 from normal women [23–76, 51.3±16.2]); Sasaki Foundation Kyoundo Hospital, Tokyo (four from patients with ovarian cancer [35–60, 46.5±10.3] and four from patients with uterine corpus cancer [55–71, 60.8±7.3]); and Kagoshima City Hospital, Kagoshima, Japan (six from patients with ovarian cancer [45–73, 55.7±11.2], six from patients with uterine corpus cancer [54–64, 60.2±4.1], and one from a patient with uterine cervical cancer [55 years old]). Of the 86 ovarian cancers, there were 26 cases of ovarian clear cell adenocarcinoma (18 and eight at stages I and III, respectively), 11 cases of ovarian mucinous adenocarcinoma (seven, one, two, and one at stages I, II, III, and IV, respectively), 28 cases of ovarian serous adenocarcinoma (five, three, 18, and two at stages I, II, III, and IV, respectively), and 21 cases of ovarian endometrioid adenocarcinoma (six, eight, and seven at stages I, II, and III, respectively). Of the 17 uterine cervical carcinomas, there were 13 cases of squamous cell carcinoma (nine and four at stages I and II, respectively), two cases of mucinous adenocarcinoma (stage I), one case of adenoid basal carcinoma (stage I), and one case of endometrioid adenocarcinoma (stage I). Of the 36 uterine corpus carcinomas, there were 30 cases of endometrioid adenocarcinoma (20, four, and six at stage I, II, and III, respectively), three cases of carcinosarcoma (stage III), one case of spindle cell sarcoma (stage IV), one case of smooth muscle tumor (stage I), and one case of serous adenocarcinoma (stage III). The 55 normal specimens included 21, four, two, one, and one sera from patients cured of uterine corpus cancer, uterine cervical cancer, choriocarcinoma, cystadenoma, and uterine adenomyosis, respectively. For the experimental use of the sera and surgical specimens, written informed consent was obtained from the patients according to the hospital ethical guidelines.

Preparation of soluble forms of the seven members of the GlcNAc6ST family

cDNA fragments of truncated forms of the five GlcNAc6STs, Gal6ST, and Ch6ST1 lacking the cytoplasmic and transmembrane domains were amplified by PCR using corresponding full-length cDNAs prepared previously [2]. The oligonucleotide primers used were 5′-tttgtcgacTACAA GTGGCACAAG-3′ (forward primer for GlcNAc6ST1), 5′-tttgtcgacCCCTTTTAGAGACGG-3′ (reverse primer for GlcNAc6ST1), 5′-tttgtcgacAGCCACAACATCAGCT-3′ (forward primer for GlcNAc6ST2), 5′-tttaagcttAGTG GATTTGCTCAG-3′ (reverse primer for GlcNAc6ST2), 5′-tttgtcgacTCCCGGCCAGGGCCCTC-3′ (forward primer for GlcNAc6ST3), 5′-tttaagcttCAGTCAGGCGATGCCCA-3′ (reverse primer for GlcNAc6ST3), 5′-tttgtcgacGGCGGC CGCGACGG-3′ (forward primer for GlcNAc6ST4), 5′-

tttaagcttGGGAGGCTACGTGGCGC-3′ (reverse primer for GlcNAc6ST4), 5′-tttgtcgacTCCCGGCCAGGGCCCTC-3′ (forward primer for GlcNAc6ST5), 5′-tttaagcttGCTACAA CTGTGGCCTC-3′ (reverse primer for GlcNAc6ST5), 5′-tttgtcgacACCTTCACCGCCAAGTC-3′ (forward primer for Gal6ST), 5′-tttaagcttCACGAGAAGGGGCGGAA-3′ (reverse primer for Gal6ST), 5′-tttgtcgacATATCAAGGG TCTCAGA-3′ (forward primer for Ch6ST1), and 5′-tttaagcttCTACGTGACCCAGAAGGT-3′ (reverse primer for Ch6ST1). Sequences in lowercase letters contain appropriate restriction sites. The cDNAs were cloned into pQE9 (QIAGEN GmbH, Hilden, Germany) between the appropriate restriction sites and introduced into *E. coli* M15 cells. The plasmids were sequenced with a Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Recombinant GlcNAc6ST2 was prepared as follows. An overnight culture of transformed M15 cells was diluted 1:100 in 1.25 l of LB broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin, and grown at 30°C for 3 h with vigorous shaking. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.1 mM, and expression was induced at 18°C for 16 h. The cells were collected by centrifugation and sonicated in 200 ml of buffer A (6 M guanidine–HCl, 20 mM potassium phosphate buffer [pH 8.0], 10 mM 2-mercaptoethanol, and 20 mM imidazole). The homogenate was kept at 25°C for 16 h. After centrifugation at 5,000×*g* for 20 m, 3 ml of Ni-NTA agarose (QIAGEN GmbH) was added to the supernatant, which was allowed to stand for 0.5 h with occasional mixing. The resin was packed into a plastic column and washed with 60 ml of buffer A. Thereafter, the recombinant protein was eluted with buffer A containing 0.25 M imidazole. The eluate was dialyzed against 8 M urea in PBS and concentrated with a Microcon YM-10 centrifugal filter (Millipore Corp., Bedford, MA). The yield was 3.5 mg of the recombinant protein.

Recombinant proteins for Western blotting analysis were prepared in almost the same manner as above, except for a few conditions. The overnight cultures of M15 cells were diluted 1:100 in 15 ml of LB broth containing the antibiotics and grown at 30°C for 3 h. IPTG was added to the cultures at a final concentration of 0.1 mM, and the induction was performed at 18°C for 16 h. The cells were collected and sonicated in 10 ml of buffer A, and the homogenates were allowed to stand at 25°C for 16 h. After centrifugation, 0.5 ml of Ni-NTA agarose (QIAGEN GmbH) was added to the supernatants, which were then incubated for 0.5 h. The resins were washed with 10 ml of buffer A, and the recombinant proteins were eluted with buffer A containing 0.25 M imidazole. The eluates were concentrated and used for Western blotting analysis. The yields of GlcNAc6ST1, -3, -4, -5, Gal6ST, and Ch6ST1 were 0.20, 1.2, 0.26, 0.83, 0.26, and 0.13 mg, respectively.

Preparation of a soluble form of GlcNAc6ST2 in *Pichia pastoris*

A cDNA fragment of a truncated form of GlcNAc6ST2 was amplified by PCR using a full-length cDNA prepared previously [2]. The oligonucleotide primers used were 5′-tttcctaggTACAGCCACAACATCAG-3′ (forward primer) and 5′-tttgcggccgcTTAGTGGATTTGCTCAGG-3′ (reverse primer). The cDNA was cloned into pPIC9-His [18] between the appropriate restriction sites, and the resulting plasmids were sequenced with a Prism 310 Genetic Analyzer.

Production and purification of recombinant GlcNAc6ST2 was performed as previously described [18]. From a 500-ml culture in buffered methanol-complex medium, 300 μg of the recombinant protein was obtained. This fraction contained substantial GlcNAc6ST2 activity.

The enzymatic activity of GlcNAc6ST-2 was assayed using GlcNAcβ1→3GalNAcα1→*p*-nitrophenyl (core 3) as an acceptor substrate, as described previously [2].

Preparation of a monoclonal antibody (6S2-49) and polyclonal antiserum

Antibodies against GlcNAc6ST2 were commercially prepared by Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). Briefly, to generate monoclonal antibodies (mAbs), the antigen was injected into the footbat of four mice three times with 3 day intervals. Hybridomas were selected based on binding of their secreted antibodies against enzymatically active GlcNAc6ST2 fraction prepared by *P. pastoris*. To generate polyclonal antiserum, the antigen was injected into a rabbit six times at 1-week intervals.

Abdominal fluids were prepared using five mice obtained from Medical and Biological Laboratories Co., Ltd. Thirteen milliliters of the abdominal fluid was applied to a protein A-Sepharose affinity chromatography column (0.9×7.9 cm; equilibrated with PBS; GE Healthcare, Buckinghamshire, UK). After washes with PBS, the antibodies were eluted with 0.1 M sodium citrate buffer (pH 4.0) and immediately neutralized. The antibodies were dialyzed against PBS and concentrated. This procedure yielded 12.6 mg of the monoclonal antibody 6S2-49. Its isotype was determined to be IgG_3 by using an Isostrip mouse monoclonal antibody isotyping kit (Roche Diagnostics GmbH, Mannheim, Germany).

Immunoprecipitation of GlcNAc6STs by the antibodies

Expression vectors for C-terminally FLAG-tagged GlcNAc6STs were prepared by using the p3XFLAG-CMV™-14 vector (Sigma-Aldrich, St. Louis, MO) and cDNA fragments encoding full-length GlcNAc6STs without a stop codon.

The fragments were amplified by PCR as described previously [2].

The plasmids (5 μg) were transfected into semiconfluent COS-7 cells on 10-cm dishes by using 20 μg Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were harvested after 24 h and washed twice with PBS. After adding 1 ml of lysis buffer (50 mM Tris–HCl [pH 7.5], 0.15 M NaCl, 1% [*v*/*v*] NP-40, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml pepstatin, and 1 μg/ml leupeptin), the cell pellets were suspended and lysed on ice for 20 m. After centrifugation, the supernatants were collected and incubated with 3 μg 6S2-49 mAb or 3 μl of the antiserum and 15 μl Protein G-Sepharose™ 4 Fast Flow (GE Healthcare) at 4°C for 2 h. The resins were washed four times with lysis buffer. Equal aliquots of the resins were used for SDS-PAGE and the proteins were transferred onto a nitrocellulose membrane (Trans-Blot® Transfer Medium, Bio-Rad, Hercules, CA). The FLAG-conjugated proteins on the membranes were treated with an HRP-conjugated anti-FLAG-M2 antibody (0.3 μg/ml; Sigma-Aldrich) and detected with ECL Western Blotting Detection Reagents (GE Healthcare).

Assay of the amounts of GlcNAc6ST2 protein in the sera of ovarian and uterine cancer patients

GlcNAc6ST2 protein in the sera was quantified by sandwich ELISA using chemiluminescent substrates. Fifty microliters of 3 μg/ml 6S2-49 in PBS was added to 96-well black, high-binding plates (Corning Inc., Corning, NY) and incubated at 4°C for 16 h. The surface was blocked by 1% BSA in PBS at 25°C for 1 h. After washes with PBS, 50 μl of sera diluted 10-fold with PBS–0.1% Tween 20 (PBS-T) was added, and the plates were incubated at 25°C for 2 h. After three washes with PBS-T, 50 μl of 3,000-fold-diluted polyclonal antiserum was added and incubated at 25°C for 1.5 h. After three washes, 50 μl of HRP-labeled donkey anti-rabbit Ig antibody (0.3 μg/ml in PBS-T; GE Healthcare) was added and incubated at 25°C for 1 h. The wells were washed with PBS-T 4 times, and 100 μl of SuperSignal ELISA Pico Chemiluminescent Substrate (PIERCE, Rockford, IL) was added. Chemiluminescent quantification was performed with a Plate CHAMELEON V (HIDEX Oy, Turku, Finland).

Preparation of 6S2-49-conjugated resin

CNBr-activated Sepharose 4B (1.5 ml; GE Healthcare) was mixed with 5 mg of 6S2-49 monoclonal antibody in 6 ml of 0.1 M $NaHCO_3$–0.5 M NaCl (pH 8.3) and rotated at 4°C for 60 h. Thereafter, 0.6 ml of 1 M Tris–HCl (pH 8.0) was added and the mixture was rotated at 4°C for 18 h. The resin was washed sequentially with 10 ml of 0.1 M $NaHCO_3$–0.5 M NaCl (pH 8.3), 10 ml of 0.1 M sodium citrate buffer (pH 4.0), and 20 ml of PBS. The 6S2-49-conjugated resin (3.6 mg antibody/ml gel) was kept at 4°C in the presence of 0.1% NaN_3.

Results

Construction of a sandwich-ELISA method for detecting GlcNAc6ST2 protein

To investigate the utility of GlcNAc6ST2 as a marker for gynecologic cancers, we first prepared a monoclonal antibody and polyclonal antiserum. We used denatured GlcNAc6ST2 as an immunogen, because we were not able to obtain a sufficient amount of enzymatically active, natively folded GlcNAc6ST2 protein from *P. pastoris* for immunization. Hybridoma clones that could bind to denatured GlcNAc6ST2 were isolated, of which only one clone (6S2-49) produced antibodies capable of binding to native GlcNAc6ST2. The specificities of the 6S2-49 mAb and rabbit polyclonal antiserum were examined by Western blotting analysis (Fig. 1). The GlcNAc6ST family consists of seven members: GlcNAc6ST1-5, Gal6ST, and Ch6ST1 (Fig. 1 A). The 6S2-49 mAb bound strongly only to GlcNAc6ST2 (Fig. 1 B), whereas the antiserum bound to all seven proteins (Fig. 1 C). To assess whether the antiserum also has broad specificity for native GlcNAc6STs, we performed immunoprecipitation experiments. GlcNAc6STs tagged with the FLAG-sequence at their C termini were expressed in COS-7 cells, and lysates of the cells were immunoprecipitated using the antiserum. Both the 6S2-49 mAb (Fig. 1 D) and the antiserum (Fig. 1 E) bound to and immunoprecipitated only GlcNAc6ST2, indicating that both are highly specific for the native form of GlcNAc6ST2.

Using these antibodies, we constructed a sandwich ELISA; the 6S2-49 mAb was absorbed on the well surface and diluted sera from patients were added to the wells. GlcNAc6ST2 trapped in the well was sandwiched by the polyclonal antibody. A standard curve for detecting GlcNAc6ST2 in serum from *three* normal adults (diluted 1:10) is shown in Fig. 2. The curve was linear at least up to the concentration of 34 ng/ml. Diluted serum without exogenous GlcNAc6ST2 exhibited substantial chemiluminescence (data not shown). It was unlikely that the anti-GlcNAc6ST2 antibodies could recognize uncertain serum proteins other than GlcNAc6ST2 protein, because the same level of the chemiluminescence appeared even in the absence of the normal sera. Furthermore, to assess whether this serum contains intrinsic GlcNAc6ST2, we treated the serum with 6S2-49-conjugated resin and repeated the assay. The treatment did not change the chemiluminescence units (RU); therefore we considered the RU value in the absence of exogenous GlcNAc6ST2 to

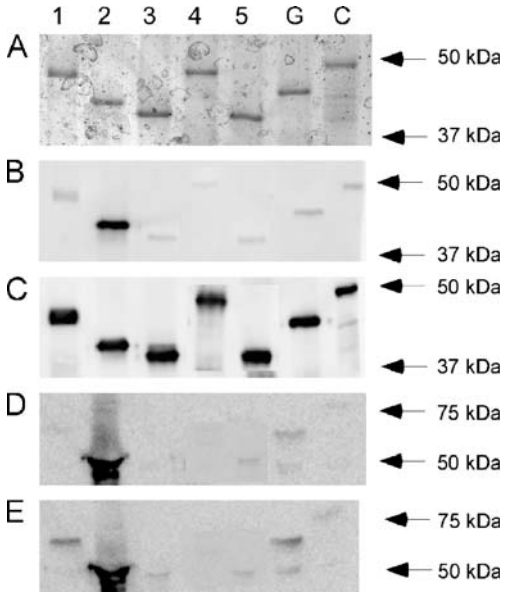

Fig. 1 Binding of the anti-GlcNAc6ST2 monoclonal antibody 6S2-49 and the polyclonal antiserum to recombinant GlcNAc6STs. *A–C*, Soluble recombinant forms of GlcNAc6STs generated in *E. coli* were loaded onto a polyacrylamide gel (300 ng each lane for *A* and 200 ng each lane for *B* and *C*). *1*, *2*, *3*, *4*, *5*, *G*, and *C* indicate GlcNAc6ST1 (48 kDa), GlcNAc6ST2 (42 kDa), GlcNAc6ST3 (40 kDa), GlcNAc6ST4 (48 kDa), GlcNAc6ST5 (39 kDa), Gal6ST (44 kDa), and chondroitin 6-*O*-ST1 (49 kDa), respectively. *A*, Sypro Orange (Invitrogen Corp., Carlsbad, CA) staining of the gel. *B* and *C*, Western blotting using 6S2-49 (*B*) and the polyclonal antiserum (*C*). After transfer, the membrane was incubated with 1 μg/ml 6S2-49 (*B*) or the 1,000-fold-diluted polyclonal antiserum (*C*) and then with 0.3 μg/ml HRP-labeled rabbit anti-mouse IgG/IgM antibody (Jackson ImmunoResearch Lab. Inc., West Grove, PA) (*B*) or 0.3 μg/ml HRP-labeled donkey anti-rabbit IgG antibody (*C*). Chemiluminescent detection was performed by using ECL Western Blotting Detection Reagents. *D* and *E*, Immunoprecipitation of natively folded, FLAG-tagged, full-length GlcNAc6STs expressed in COS-7 cells by 6S2-49 (*D*) and the polyclonal antiserum (*E*). The immunoprecipitates were subjected to Western blotting analysis and detected with an HRP-conjugated anti-FLAG-M2 antibody as described in the "Materials and methods"

be background and subtracted the background value from observed RU values.

The amount of GlcNAc6ST2 in sera from ovarian cancer patients

We analyzed the amounts of GlcNAc6ST2 protein in sera from normal adult women and patients with each type of ovarian epithelial adenocarcinoma. The cut-off value was set at 5.5 ng/ml, which approximately corresponded to the average (2.8 ng/ml) plus half of the standard deviation value (4.9 ng/ml) for the data from normal women. The false positivity rate for normal women was 11% (Fig. 3). There were no common clinical characteristics among the six GlcNAc6ST2-positive specimens from normal women. The rates of GlcNAc6ST2 positivity for serous, mucinous, endometrioid, and clear cell adenocarcinomas were 36%, 64%, 52%, and 54%, respectively. These rates were lower than those of CA125 positivity (93%, 78%, 75%, and 75%, respectively; data not shown). We previously demonstrated by immunohistochemistry that GlcNAc6ST2 is frequently expressed in mucinous, serous papillary, and clear cell adenocarcinoma tissues, whereas the immunostaining was not detected in serous solid-type and endometrioid adenocarcinomas [3]. Our current results seem to be at odds with this previous study. However, in the current study 14%, 55%, 10%, and 31% of serous, mucinous, endometrioid, and clear cell adenocarcinomas, respectively, exhibited over 10 ng/ml of GlcNAc6ST2. It is therefore possible that serous and endometrioid adenocarcinomas express GlcNAc6ST2 at a level below the threshold of immunohistochemical detection, and that mucinous and clear cell adenocarcinomas actively express GlcNAc6ST2 and release more of the enzyme into body fluids than the other two types of adenocarcinoma.

A higher percentage of stage I ovarian cancers (56%) than stage II–IV ovarian cancers (44%) were positive for GlcNAc6ST2 (Fig. 4). The CA125-positive rates for the same specimens were 68% for stage I and 91% for stages II–IV (data not shown). This result suggests that the levels of GlcNAc6ST2 release are not related to the progression of ovarian cancer. GlcNAc6ST2 proteins might be unstable in the serum, because we could not detect the enzymatic activity of GlcNAc6ST2 in the GlcNAc6ST2-positive sera (data not shown). The combination of GlcNAc6ST2 and CA125 yielded a 92% positive rate (n=24) for stage I ovarian cancer (data not shown).

In contrast, the rates of GlcNAc6ST2 and CA125 positivity were comparable for stage I ovarian clear cell and mucinous adenocarcinomas. Although the rate of

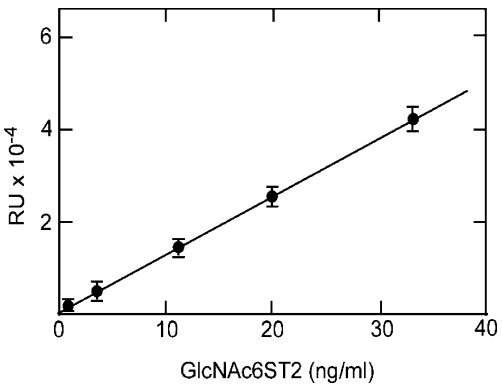

Fig. 2 Standard curve generated by using different denatured GlcNAc6ST2 protein concentrations in the sandwich-ELISA method developed in this study. The *y*-axis indicates the chemiluminescence units (*RU*). The RU values were determined by subtracting the background value observed with 10% human normal serum from the value obtained with each sample

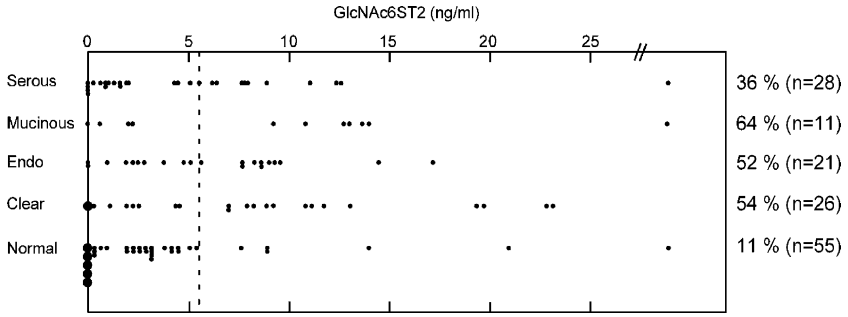

Fig. 3 GlcNAc6ST2 levels in the sera from patients with various types of ovarian cancer. *Serous*, serous adenocarcinoma (n=28); *Mucinous*, mucinous adenocarcinoma (n=11); *Endo*, endometrioid adenocarcinoma (n=21); *Clear*, clear cell adenocarcinoma (n=26); *Normal*, normal women (n=55). The *dotted line* indicates the cut-off value (5.5 ng/ml). *Small and large closed circles* indicate one and five persons, respectively. *The values on the right side* indicate the positive rates for the respective groups

GlcNAc6ST2 positivity (69%, n=13) was slightly lower than that of CA125 positivity (75%, n=12) for the stage I clear cell adenocarcinomas, a slightly higher percentage of stage I mucinous adenocarcinomas were positive for GlcNAc6ST2 (71%, n=7) than for CA125 (60%, n=5).

The amount of GlcNAc6ST2 in sera from uterine cancer patients

Next, we assayed GlcNAc6ST2 levels in the sera of patients with uterine cervical and corpus cancer (Fig. 5 and Table 1). It is accepted that SCC antigens are good serum markers for uterine cervical cancer [12, 13], whereas there is no effective marker for uterine corpus cancer. In this study, the positive rate of GlcNAc6ST2 for cervical cancer (82%) was higher than that of SCC antigens (53%). In particular, the positive rate of GlcNAc6ST2 for stage I cervical cancer (85%, n=13) was significantly higher ($p<0.05$) than that of SCC antigens (42%). The combination of GlcNAc6ST2 and SCC antigens yielded 92% and 100% positive rates for stage I (n=12) and stages II–III (n=4) cervical cancer, respectively. Similarly, a higher percentage of corpus cancers were positive for GlcNAc6ST2 (44%) than for CA125 (37%), and GlcNAc6ST2 positivity (52%) was significantly higher ($p<0.01$) than CA125 positivity (15%) for stage I corpus cancer. The positive rates of GlcNAc6ST2 and CA125 for stages II–IV were 33% and 67%, respectively. The combination of GlcNAc6ST2 and CA125 yielded rates of 52% and 80% positive for stage I (n=21) and stages II–IV (n=15) corpus cancer, respectively. These results indicate that GlcNAc6ST2 could be a good marker for early-stage uterine cancer.

Discussion

In this report, we constructed a sandwich-ELISA method for measuring the amounts of GlcNAc6ST2 in the sera using new monoclonal antibody. GlcNAc6ST2 exhibited high positivities for the sera from the patients with ovarian cancer, and uterine cervical and corpus cancers, and especially, in the cases of uterine cancers, the positive rates of GlcNAc6ST2 were higher than those of conventional tumor markers including SCC antigen and CA125. These results indicate that GlcNAc6ST2 would be a candidate for tumor markers detecting uterine cancers.

Generally, positive rates of tumor markers in the early stage of cancer are lower than those in the advanced stages, however, GlcNAc6ST2 exhibited slightly higher positive rates in the stage I of ovarian and corpus cancers than those

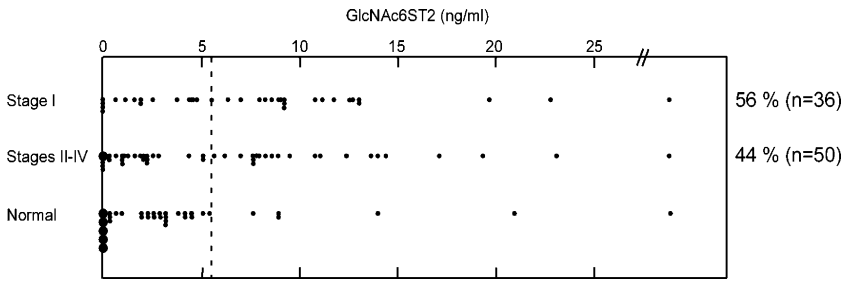

Fig. 4 GlcNAc6ST2 levels in the sera from patients with stage I (n=36) and stage II–IV (n=50) ovarian cancer. *Normal*, normal women (n=55). The *dotted line* indicates the cut-off value (5.5 ng/ml). *Small* and *large closed circles* indicate one and five persons, respectively. *The values on the right side* indicate the positive rates for the respective groups

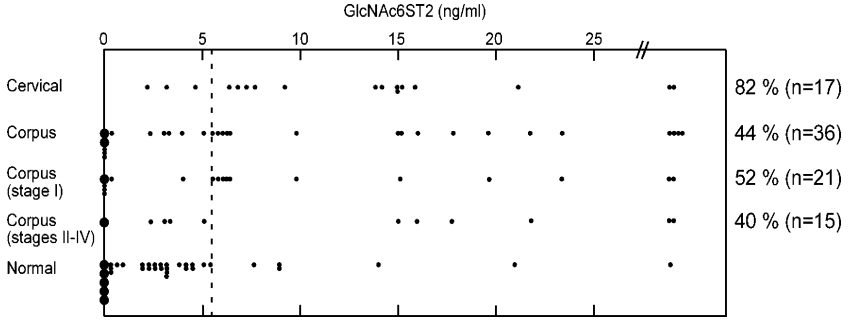

Fig. 5 GlcNAc6ST2 levels in the sera from patients with various types of uterine cancer. *Cervical*, uterine cervical cancer (n=17); *Endometrial*, uterine corpus cancer (n=36); *Normal*, normal women (n=55). The *dotted line* indicates the cut-off value (5.5 ng/ml). The *small and large closed circles* indicate one and five persons, respectively. *The values on the right side* indicate the positive rates for the respective groups

in the stages II–IV. The reason for this event is unclear now, but it could be speculated that expression levels of some proteases in the Golgi apparatus and the serum may increase in the advanced stages of cancer cells, and that GlcNAc6ST2 protein is susceptive to the action of such proteases.

As shown in Fig. 3, six normal specimens were GlcNAc6ST2-positive. At present, there were no apparent clinical characteristics among the six specimens. We cannot explain their positivities now, but we should be careful to monitor the six women in the future with regard to recurrence and metastases

Many glycosyl/sulfotransferases responsible for the biosynthesis of *N*-linked and *O*-linked glycans are localized in intracellular vesicles. They are synthesized in membrane-bound form, but can be cleaved off from the membranes and released into body fluids. It has been shown that the amounts of some glycosyltransferases are elevated in the sera of cancer patients [19–21], and that β1,4-galactosyltransferase-I can serve as a tumor marker for ovarian cancer [22, 23]. Recently, we developed a sandwich-ELISA method for detecting β1,3-galactosyltransferase-4/-5 and showed that it can serve as a tumor marker for uterine corpus cancer (unpublished data). If glycosyl/sulfotransferases are ectopically expressed in certain cancers, they would be promising candidates for tumor markers.

Table 1 Positive rates of SCC antigens, CA125, and GlcNAc6ST2 for uterine cervical and corpus cancers

	SCC antigens	CA125	GlcNAc6ST2
Cervical	53% (15)[a]	ND	82% (17)
Stage I	42% (11)	ND	85% (13)[b]
Corpus	ND	37% (35)	44% (36)
Stage I	ND	15% (20)	52% (21)[c]

ND not determined
[a] Values in parentheses indicate the number of specimens
[b] Significantly higher than SCC antigens (p<0.05)
[c] Significantly higher than CA125 (p<0.01)

Although it is well known that several glycosyl/sulfotransferases flow in the blood stream as soluble forms, the biological significance remains unclear. It is unlikely that they work as 'transferases', because their donor substrates, sugar-nucleotides or PAPS, are not available in the blood. One possibility is that they act as lectins, which bind to specific sugar chains similar to acceptor substrates. Shur *et al.*, [24] have showed that sperm cell surface β1,4-galactosyltransferase functions as cell-recognition lectin by binding to non-reducing terminal GlcNAc residues on zona pellucida glycoprotein, ZP3. Ma and Colley [25] reported that α2,6-sialyltransferase-I can form a disulfide-bonded dimer, which exhibits no enzymatic activity but can bind to non-reducing terminal Gal residues. Generally, binding abilities of glycosyl/sulfotransferases to acceptor glycans are weaker than those of plant lectins, however, it would be probable that the oligomerization could increase the binding abilities. Uemura *et al.*, [22] reported that β1,4-galactosyltransferase-I forms oligomer in the serum, although it is unclear that the oligomer exhibits lectin-like ability. On the other hand, Saito *et al.*, [26] showed that soluble β1,6-*N*-acetylglucosaminyltransferase V can non-enzymatically induce tumor angiogenesis, probably by binding to heparan sulfate proteoglycan with its polybasic domain and then causing the release of fibroblast growth factor-2. It is unclear whether soluble GlcNAc6ST2 in the blood has certain function or not, but it is interesting to analyze binding molecules to soluble GlcNAc6ST2 in the serum or the endothelial cells of the vein.

Expression profiles of glycan chains possessing GlcNAc 6-*O*-sulfate residues in the uterus largely remain unclear, but Lai *et al.*, [27] showed that carbohydrate antigen(s) recognized by MECA-79 mAb is expressed in the endometrium depending on menstrual cycle. Minimal structure of the carbohydrate antigen is an *O*-linked oligosaccharide, Galβ1→4(SO_3^-→6)GlcNAcβ1→3Galβ1→3GalNAc [28],

which is partly identical with L-selectin ligand, 6-sulfo sialyl Lewis X [29]. It is unknown to date which GlcNc6STs are responsible for the biosynthesis of the MECA-79-epitope in the endometrium. If that would be GlcNAc6ST2, a possibility could be raised that serum levels of GlcNAc6ST2 are altered in relation to menstrual cycle. This is an important issue to be resolved in the future.

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