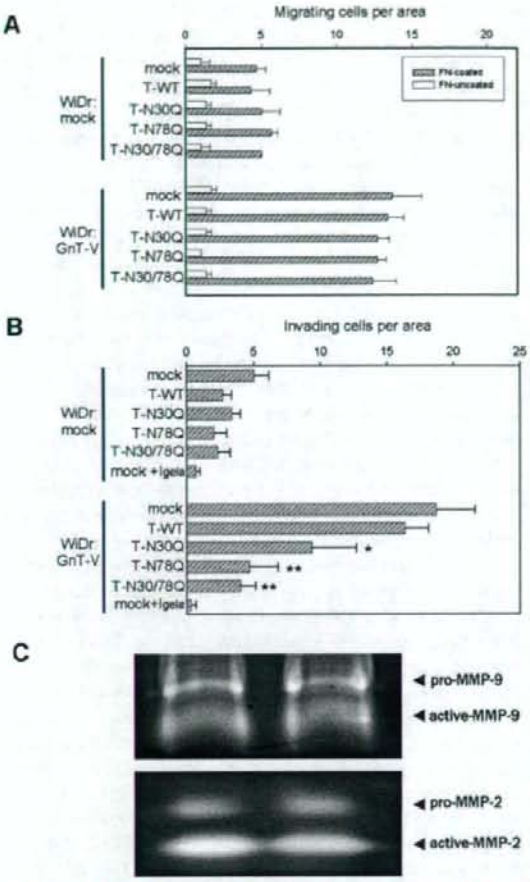

FIG. 4. **Effects of the aberrant glycosylation of TIMP-1 on cell migration and invasion.** Cells (2×10^4) were allowed to migrate in RPMI 1640 medium plus 0.1% BSA containing, if necessary, gelatinases inhibitor (*Igela*) for 4 h (*A*) and a Matrigel-coated 24-well Boyden chamber for 22 h (*B*), and cells that migrated to the lower surface of the filters, some of which were precoated with fibronectin (*FN*) (25 μg/ml), were counted. The values are the means of three independent experiments with standard deviations (*, $p < 0.05$; **, $p < 0.01$). *C*, MMP-2 and MMP-9 were retrieved from WiDr conditioned RPMI 1640 media containing 10% FBS. MMP-2 was immunoprecipitated using anti-MMP-2 monoclonal antibody, and MMP-9 was partially purified on a Con A-agarose column. Gelatin zymography was performed on 10% SDS-PAGE gel with 0.5% (w/v) gelatin copolymerized.

and T-N30Q and T-N78Q were intermediate between them (Fig. 4*B*). However, little difference was found among the TIMP-1 transfectants of WiDr:mock cells. Collectively, these data strongly suggest that aberrant TIMP-1 glycosylation, but not glycosylation itself, affects *in vitro* cell invasion.

In vitro cell invasion involves two distinct processes: the hydrolysis of basement membranes coated throughout the 8-μm pores and migration through the pores. Since no difference in cell migration itself was observed (Fig. 4*A*), we reasoned that the different cell invasion originated from differences in the hydrolysis rate of basement membrane. Treatment with gelatinase inhibitors significantly nullified the invasiveness of WiDr cells (Fig. 4*B*), indicating the involvement of hydrolysis of the basement membrane catalyzed by MMP-2 and/or MMP-9 (21, 22). In view of the result that the cell invasion rate was nearly the same among mock cells of TIMP-1 transfectant (Fig. 4*B*), the possibility that the differences in cell invasion could arise from the changes in the gelatinases-inhibitory ability of TIMP-1 induced by depletion of *N*-linked glycosylation of TIMP-1 was eliminated. Removal of the carbohydrates from human TIMP-1 by treatment with *N*-glycosidase F has been shown to have no measurable effect on the inhibitory activity (19). A difference in the migration and invasion rate between WiDr:mock and WiDr:GnT-V did not arise from a different expression of MMP-2 and MMP-9 (Fig. 4*C*); moreover, the transfection of TIMP-1 or the mutant gene did not alter the expression pattern of gelatinases (data not shown). Taken together, the results suggest that the aberration in TIMP-1 affects its inhibitory ability toward gelatinases and thus the rate in the hydrolysis of Matrigel coating materials. An increase in the hydrolysis rate through the aberration of TIMP-1 would eventually lead to the enhanced invasiveness of WiDr cells.

Effects of the Aberrant Glycosylation of TIMP-1 on Gelatinases Inhibition—To confirm that the TIMP-1 aberration results in mitigated inhibition on gelatinases, the proteolytic activities of active gelatinases were monitored in the presence of various gelatinase inhibitors. Gelatinase activities were kinetically monitored using fluorogenic substrate DABCYL-GABA-PQGL-E(EDANS)-AK-NH_2. When gelatinases were pre-incubated with equal molar ratios of TIMP-1, the steady state of the hydrolysis reaction reached directly after initiation with the pattern of nearly zero-order kinetics (inset of Fig. 5, *A* and *B*). The slope of formation of fluorescent products was used to determine the relative activity of gelatinases.

Consistent with previous reports (19, 23), our results showed that the depletion of the cognate *N*-linked glycans on TIMP-1 has little effect on gelatinase inhibition (Fig. 5, *A* and *B*). Rather, the aberrant glycans were responsible for the mitigated inhibition on gelatinases. *r*TIMP-1 and the mutant proteins purified from WiDr:mock retained wild-type levels of inhibitory activity, whereas T-WT from WiDr:GnT-V showed a significant loss of gelatinase inhibition.

The inhibition of gelatinases by TIMP-1 was confirmed by gelatin zymography (Fig. 5*C*), where the gelatinolytic ability of the latent and active forms of MMP-2 and MMP-9 were measured in the presence of various inhibitors. We reasoned that, if TIMP-1 is covalently incorporated in gelatin copolymerized gels, it could tether gelatinases in the vicinity, thereby inhibiting the gelatinolytic activity. The results show that *r*TIMP-1 isolated from WiDr:mock cells retained an inhibitory effect on

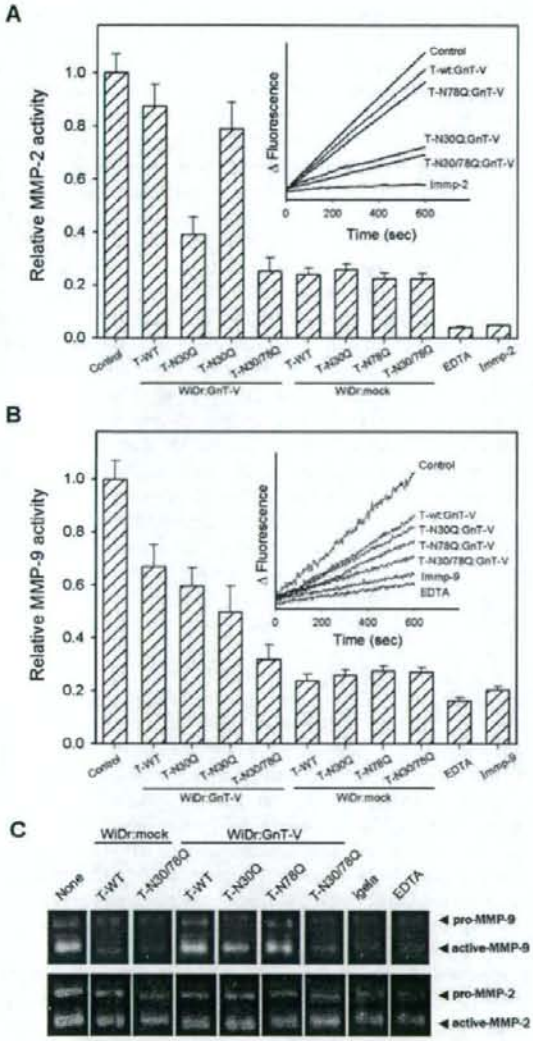

FIG. 5. **Effects of the aberrant glycosylation of TIMP-1 on gelatinase inhibition.** Following the incubation of gelatinases with the various inhibitors at 4 °C for 24 h, hydrolytic reactions of gelatinase A/MMP-2 (*A*) and gelatinase B/MMP-9 (*B*) were initiated at 37 °C by adding a fluorogenic substrate. Time courses of the activities were traced, and the slope at steady state was used for the relative activity. Immp-2 and Imp-9 refer to specific inhibitor for MMP-2 and MMP-9, respectively. *C*, effects of aberrant glycans of TIMP-1 on gelatinolytic activity of gelatinases were investigated by gelatin-zymography as described under "Experimental Procedures."

both the latent and active forms of MMP-9. rTIMP-1 from T-WT:GnT-V failed to tightly inhibit the gelatinolytic activity by MMP-9. The pattern of inhibition of MMP-2 by TIMP-1 was not identical to the invasion assays and the fluorogenic assays, which might be due to restricted interactions of TIMP-1 and MMP-2 in gels. Nevertheless, rTIMP-1 from T-WT:GnT-V showed mitigated inhibition on MMP-2 compared with rTIMP-1 from T-Q30/78G:mock. These results suggest that the aberrantly attached glycans play a role as interferences in the TIMP-1 inhibition on the gelatinases.

Effect of Aberrant Glycosylation of TIMP-1 on Interaction with Gelatinases—To address the question of why the aberrant glycosylation of TIMP-1 results in a significant loss of gelatinase inhibition, we investigated the binding properties of TIMP-1 and gelatinases. The binding shows slow, tight-binding competitive inhibition and exhibits time-dependent inhibition (16) and, as a result, gelatinase activities over time showed a curvilinear function on the progress curves. Supplemental Fig. 3, *A* and *B* show the time courses for MMP-2 activity in the presence of various concentrations of TIMP-1: mock and TIMP-1:GnT-V, respectively. The kinetic pattern for MMP-9 was similar to that for MMP-2 (supplemental Fig. 4). Kinetic parameters (k_{on}, k_{off}, K_i) for gelatinase inhibition by TIMP-1 were calculated as described in "Experimental Procedures" and are compiled in Table II.

Kinetic parameters (k_{on}, k_{off}, K_i) for gelatinase inhibition by TIMP-1 were previously reported with some variations (16, 24–26). Olson *et al.* (16) attributed the variations to differences in the concentration of proteins and substrate. This taken into account, our data are not significantly different from previously reported values. As expected, the aberrantly glycosylated TIMP-1 was found to loosely bind to active gelatinases with a lower k_{on} and to dissociate more efficiently as assessed by the higher k_{off}. K_i for aberrant TIMP-1/gelatinases interactions was found to show 7.2-fold higher value than that for wild-type TIMP-1/MMP-2. The increase in inhibition constant was more dramatic for the TIMP-1/MMP-9 interaction, with the K_i being increased by 11.4-fold. These results indicate that the TIMP-1 aberration leads to a shift in equilibria for the TIMP-1/gelatinases interactions toward the dissociation process of the complex molecules. As a result, the tight binding and control of active gelatinases by TIMP-1 is loosened, resulting in a higher bioavailability of uncomplexed, uninhibited, free gelatinases.

Relations of TIMP-1 Aberration with Colon Cancer Invasion and Metastasis—The relationships between TIMP-1 aberration and cancer progressions has not been defined yet, but both *in vitro* experiments provided sufficient circumstantial evidence for an association of the aberration in cancer progression of colon cancer patients. To deduce the involvement of the aberrant glycosylation of TIMP-1 in colon cancer, both normal and tumor tissues of colon cancer cases were analyzed in terms of expression level and TIMP-1 glycosylation.

As is seen in Fig. 6*A*, the expression level of TIMP-1 did not reflect the progression of colon cancer. TIMP-1 expression was elevated in almost all colon cancers compared with its paired normal tissues consistent with previous reports (18,

TABLE II
Inhibition constants for active gelatinase/TIMP-1 interactions

TIMP-1	Gelatinases	k_{on} ($M^{-1}s^{-1}\times10^5$)	k_{off} ($s^{-1}\times10^3$)	K_i (nM)
TIMP-1: mock	62kDaMMP-2	4.99 ± 0.72	2.71 ± 0.47	5.43 ± 1.39
	82kDaMMP-9	7.07 ± 0.64	2.26 ± 0.43	3.20 ± 1.23
TIMP-1: GnT-V	62kDaMMP-2	0.98 ± 0.06	3.83 ± 0.24	39.1 ± 8.38
	82kDaMMP-9	0.75 ± 0.07	2.74 ± 0.39	36.5 ± 6.54

27–30) and Fig. 2*B*, but the extent of the elevation was independent of Astler-Coller colon cancer stages, an index of colon cancer progression and commonly a metastatic parameter. Rather, at the stages where cell invasion and spreading to near tissues is prosperous, *i.e.* Astler-Coller grade C and D, an aberrantly glycosylated TIMP-1 was found in more than 70% of the colon cancer tissues tested, which is quite different from the case of *Group I*. Interestingly, the transcription level of GnT-V was estimated by RT-PCR to be increased in a cancer stage-dependent manner and concomitantly with the promotion of TIMP-1 aberration. When GnT-V overflows by a signal under cancerous conditions, the transferase is likely to promote TIMP-1 aberration and thus cancer malignancy.

Fig. 6*B* shows the representative results for 10 cases. Cases *1*, *4*, and *5* showed a marked increase in β1,6-GlcNAc-attached aberration in TIMP-1 glycosylation and, in agreement with Fig. 2*C*, a slight increase in molecular mass. Those cases showed an elevated transcription of GnT-V (Fig. 6*C*), a relatively high cancer stage and a clinically high tumor invasion to remote sites, especially metastasizing to regional lymph nodes (data not shown).

To our knowledge, the aberrant glycosylation of TIMP-1 has not been reported to correlate with the cancer invasion and metastasis *in vivo* or *in vitro*. Our data strongly suggest that the aberrant glycosylation of TIMP-1 induced by GnT-V is closely associated with the elevated invasion/metastasis potential in colon cancer cells.

DISCUSSION

A tumor-associated biomarker serves as an index that enables us to predict ongoing cancerous conditions and the progression of cancer by showing a difference in a quantitative or qualitative pattern. It is obvious that the current techniques for the biomarker discovery have focused on the quantitative aspects, that is, a differential expression level in cancer. A large body of data describes the correlation of a change in the quality of proteins, *i.e.* protein glycosylation, phosphorylation, and acetylation, with various diseases. In this report, we suggest that a consideration of the qualitative aspects of TIMP-1 provides the more accurate diagnostic and prognostic information on colon cancer when integrated with the data on the expression level. One of the intriguing features is that TIMP-1 has seemingly discordant, dual functions; TIMP-1 not only inhibits cancer progression by abrogating MMPs but also has effects on cancer cell growth and survival in an MMP-dependent or -independent manner. What would happen if the level of TIMP-1 is maintained at a high level? It would control MMPs quite efficiently so that cancer progression might never occur or be retarded. Actually, the overexpression of TIMP-1 inhibits tumor growth and metastasis of melanoma (31) and suppresses the metastatic potential of human gastric cells (32) and oral squamous cell carcinoma (33). However, this is quite contradictory to reports that TIMP-1 is up-regulated in many cancer types (18, 27–30) and the reports that a high level of TIMP-1 correlates with a poor prognosis (34, 35). Moreover, high preoperative plasma TIMP-1 levels are associated with a short survival of patients with colorectal cancer (36), lung cancer (37), and gastric cancer (38). If the effects of TIMP-1 on cancer development and progression are taken into account only in terms of "quantity" without consideration of "quality," incessant debates over the genuine role of TIMP-1 in biological systems would occur and, although both aspects are relevant, it would fail to clearly explain whether TIMP-1 is pro-oncogenic or not. Here we suggest a plausible compromise to this "paradox." Tumor onset occurs in the early stage, and cell growth and anti-apoptotic activity are required at that stage. The higher level of TIMP-1 meets this requirement, and *N*-glycosylation of TIMP-1 would exert no effect on this tumor onset. Consistent with this suggestion, it has been demonstrated that TIMP-1 has a significant tumor stimulating effect during tumor onset (39) but suppresses tumor growth during the late state of tumor progression (40). However, it is likely that the "reins" that have sequestered tumor progression are slackened via an acquired aberrancy of TIMP-1 glycosylation upon the onset of the communication with GnT-V (supplemental Fig. 5). This implies that, although TIMP-1 levels are maintained at a high level or increase further, TIMP-1 levels were elevated by GnT-V, the net direction of cancer phase would be oriented toward cancer progression, and the turning point toward cancer progression may be associated with the interplay of TIMP-1 and GnT-V. An overexpression of TIMP-1 inhibits invasion and metastasis ((31, 32, 41); Fig. 4*B*), but the coexpression of TIMP-1 with GnT-V, previously unreported, nullified such inhibitory effects (Figs. 4 and 5). It is likely that GnT-V affects tumor progression mainly through TIMP-1 aberration, although another collateral mechanism is also possible (5–7).

TIMP-1 binds noncovalently to catalytically active MMP-2 and MMP-9 with a 1:1 stoichiometry and a dissociation constant ~10^{-8} M. Indeed, the values are true for the interactions of normal TIMP-1 with gelatinases. Our biochemical data indicate that the aberration of TIMP-1 leads to a decrease in

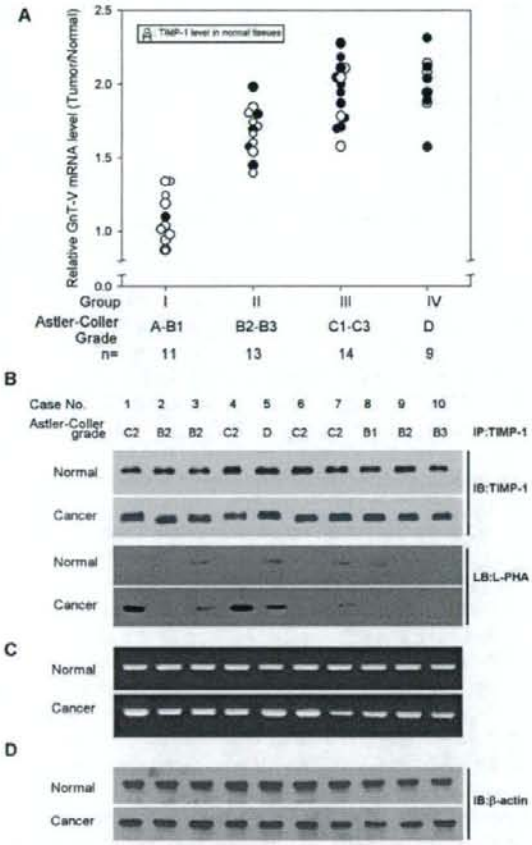

FIG. 6. **Correlation of TIMP-1 aberration with colon cancer progression.** *A*, the normal tissues and cancer tissues from colon cancer cases of each stage were compared with respect to TIMP-1 expression levels, aberrant glycosylation, and transcription levels of the GnT-V gene. *Closed circle* indicates an acquisition of β1,6-GlcNAc moiety on TIMP-1 molecule; *open circle* indicates a normal glycosylation of TIMP-1; diameters of *circles* indicate a relative TIMP-1 level in cancerous tissues compared with that in normal tissues. *B*, proteins were extracted from normal and cancer tissues from resection materials of colon cancer cases and precleaned with anti-mouse IgM-agarose beads. Immunoprecipitation was performed using the conjugates of an anti-TIMP-1 monoclonal antibody. The immunoprecipitates were subjected to both immunoblot and lectin blot using L-PHA. *C*, transcription levels of GnT-V were monitored by RT-PCR using two primers: 5′-tgtgtatggcaaagtggata-3′ (forward) and 5′-accatggtttt-tcacgtaac-3′ (backward). *D*, actin from normal and cancer tissues were compared by immunoblot analysis.

the binding affinity for active gelatinases and the inhibition ability of gelatinases (Table II). The complete structure of TIMP-1 and the inhibition mechanism was determined by X-ray crystallographic studies of the TIMP-1-MMP-3 complex (42). A wedge shaped TIMP-1 slots into the active site cleft of an MMP in a manner similar to that of the substrate. In this study, unglycosylated TIMP-1 was crystallized to elucidate the MMP inhibition mechanism. However, several lines of evidence suggest that the glycan moiety of a glycoprotein has a decisive influence on protein-protein interactions and that a change in glycan structure of a single protein could lead to significant alterations in behavior, development, or the physiology of cells. A Fringe-catalyzed addition of a GlcNAc moiety to the fucose on Notch confers a significant strength in Notch-Delta interactions during the development of Drosophila wing (43, 44), and the hypoglycosylation of dystroglycan is related to the mitigated interaction with the substrate protein laminin, resulting in weakened cell-extracellular matrix adhesion (45). Our results (Figs. 4 and 5) and the previous report (19) taken together, the aberrant glycans on TIMP-1 may affect the properties of binding with gelatinases, presumably by conferring a steric hindrance arising from the massiveness of glycosylation and an electrostatic repulsion arising from the attachment of acidic residues to the binding to gelatinases. The structural properties for the mitigated inhibition of the aberrant TIMP-1 ($TIMP\text{-}1_{ab}$) on gelatinases await the resolution of the gelatinase-$TIMP\text{-}1_{ab}$ complex structure.

One important event necessarily accompanied in the development and progression of colon cancer is the stromal invasion and traverse of the basement membrane. This process facilitates the progression of adenomas to carcinomas and the metastasis of carcinomas. A special class of enzyme implicated in this process is MMP, which constitutes a large family, including collagenases, gelatinases, stromelysins, and membrane type-MMPs. The expression and gelatinolytic activity of MMP-9 and active MMP-2 are closely associated with cancer progression and metastasis in various types of cancer, such as cervical (46), renal (47), and ovarian (48) cancer as well as colon cancer (41). Considering the important role of gelatinases in cancer progression, the notion that an aberration of TIMP-1, a major inhibitor of gelatinases, could provide the driving force for cancer progression is likely to be pertinent.

Our knowledge of the biological behavior of MMPs and TIMPs and the roles in various diseases have an eventual goal of therapeutic uses and clinical trials of anti-cancer agents. The current trend for blocking cancer progression involves either the inhibition of MMP activity using synthetic MP inhibitors or increasing the local concentration of TIMPs by the administration of a recombinant protein or gene transfer. However, lack of efficacy and untoward side effects have made the clinical trials disappointing. Here, we suggest the relevance of the "quality control" of TIMP-1 for preventing cancer progression and malignant transformation. From this viewpoint, the development of a specific inhibitor of GnT-V and attempts to block the elongation of polylactosamine may be pertinent to such quality control. Besides, it is suggestive that, when integrated with the data of TIMP-1 expression level, the glycosyl pattern of TIMP-1 could provide important information on the

diagnostic/prognostic estimation of colon cancer.

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RESEARCH ARTICLE

N-acetylglucosaminyltransferase III expression is regulated by cell-cell adhesion *via* the E-cadherin–catenin–actin complex

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Recently, our research group investigated the effects of cell–cell interactions on *N*-linked oligosaccharides (*N*-glycans). We found that *N*-acetylglucosaminyltransferase III (GnT-III) activity, and thus, the enzyme product-bisected *N*-glycans were induced in cells cultured under dense condition in an E-cadherin-dependent manner [26]. To further explore the underlying molecular mechanism, we examined the effects of α-catenin, which is a component of the E-cadherin–catenin complex that can bind to actin cytoskeleton, on the regulation of GnT-III expression in the human colon carcinoma DLD-1 cells. GnT-III activity was not substantially increased in cells cultured under dense conditions, compared with those cultured under sparse conditions. However, restoration of α-catenin gene to DLD-1 cells resulted in a significant increase in GnT-III activity and in production of the bisected *N*-glycans, which were detected by E_4-PHA, suggesting that the E-cadherin–catenin complex is required for the induction. Moreover, treatment with cytochalasin D, an inhibitor of F-actin polymerization, completely blocked the upregulation of GnT-III expression in the dense culture. Taken together, these results strongly suggest that GnT-III expression is tightly regulated by cell–cell adhesion *via* the E-cadherin–catenin complex and actin cytoskeleton formation.

Keywords:
Cell adhesion / E-cadherin / Glycosylation / GnT-III / Regulation

1 Introduction

A large body of experimental evidence indicates that complex oligosaccharides play a crucial role in many recognition, signaling, and adhesion events that take place at the cell surface [1, 2]. Modified oligosaccharides can interfere with carbohydrate–protein, or protein/glycoprotein–glycoprotein interactions, and as a result, modulate these interactions and cellular signaling [3]. In fact, changes in oligosaccharides structure are associated with many physiological and pathological events, including cell growth, migration, differentiation, and tumor invasion [4, 5]. Production of sugar chains of glycoproteins is catalyzed by various glycosyltransferases [6]. It is well-known that the structures of glycan components such as sialyl Lewis antigens [7, 8], mucin-type *O*-glycans [9], and *N*-glycans [4, 10], which are controlled by glycosyltransferases, greatly contribute to cell adhesion, cell invasion, and cancer metastasis.

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Abbreviations: **GlcNAc**, *N*-acetylglucosamine; **GnT-III**, *N*-acetylglucosaminyltransferase III; **GnT-V**, *N*-acetylglucosaminyltransferase V

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N-acetylglucosaminyltransferase III (GnT-III) transfers *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to a β1,4 mannose in *N*-glycans to form a "bisecting" GlcNAc linkage, as shown in Fig. 2A. Bisecting GlcNAc linkages are found in various hybrid and complex *N*-glycans. GnT-III is generally regarded as a key glycosyltransferase in *N*-glycan biosynthetic pathways. Introduction of a bisecting GlcNAc suppresses further processing and elongation of *N*-glycans catalyzed by *N*-acetylglucosaminyltransferase V (GnT-V), which is strongly associated with cancer metastasis, since GnT-V cannot utilize the bisected oligosaccharide as a substrate [11–13]. It has also been reported that GnT-V activity and β1,6 branched *N*-glycans levels are increased in highly metastatic tumor cell lines [14, 15]. Consistently, cancer metastasis is greatly suppressed in GnT-V knockout mice [16]. Therefore, GnT-III has been proposed as an antagonistic of GnT-V, thereby contributing to the suppression of cancer metastasis. In fact, overexpression of GnT-III in highly metastatic melanoma cells reduced β1,6 branching in cell-surface *N*-glycans and increased bisected *N*-glycans [17]. Interestingly, cell–cell adhesion was enhanced due to prolonged turnover of E-cadherin on the cell surface in these GnT-III transfectants [18]. In contrast to GnT-III, overexpression of GnT-V resulted in decreased N-cadherin clustering on the cell surface and downregulation of cadherin-associated cell–cell adhesion [19]. Therefore, the molecular mechanism for the suppression of cancer metastasis by GnT-III is partly explained by an increase in E-cadherin-mediated homotypic adhesion. The opposing effects of GnT-III and GnT-V have been observed for the same target protein [20]. Integrin α3β1, which is believed to be highly associated with tumor metastasis, can be modified by either GnT-III or GnT-V. GnT-V stimulates α3β1 integrin-mediated cell migration, while overexpression of GnT-III inhibits GnT-V-induced cell migration. The modification of the α3 subunit by GnT-III supersedes modification by GnT-V, as a result GnT-III inhibits GnT-V-induced cell migration. These results further suggest that remodeling of glycosyltransferase-modified *N*-glycan structures modulates cell adhesion and cancer metastasis.

The cadherins are a super family of adhesion molecules that function in cell recognition, tissue morphogenesis, and tumor suppression [21]. Classical cadherins, including E-cadherin, bind to either β- or γ-catenin, thereby linking the complex to α-catenin. The loss of E-cadherin expression or function in epithelial carcinoma cells has long been thought to be a primary reason for the disruption of tight epithelial cell–cell contacts and the release of invasive tumor cells from the primary tumor [22]. E-cadherin serves as a widely acting suppressor of epithelial cancer invasion and growth. Thus, its functional elimination is a key step in the acquisition of the invasive phenotype for many tumors. α-Catenin contains multiple interaction sites: actin-binding sites and binding sites for other actin-binding proteins such as α-actinin [23] and vinculin [24]. Without α-catenin, tight cell–cell associations do not form despite cadherin expression. This observation suggests that α-catenin interactions, that link cadherin–catenin complexes to the actin cytoskeleton are essential for full cadherin activity [25].

Recently, we found that GnT-III expression was significantly upregulated by cell–cell adhesion in an E-cadherin-dependent manner [26]. GnT-III activity was greatly increased when cells were cultured under dense conditions compared with sparse culture conditions. Upregulation of GnT-III was observed only in epithelial cells that express E-cadherin, and not in MDA-MB231 cells, an E-cadherin-deficient cell line. Although these results strongly suggest that E-cadherin is essential for cell–cell adhesion regulated GnT-III expression, the detailed molecular mechanisms of regulation, in particular the downstream events of E-cadherin, remain unclear. The present study focused on the effects of α-catenin on GnT-III expression. We found that GnT-III expression was tightly regulated by cell–cell adhesion *via* the E-cadherin–catenin–actin complex.

2 Materials and methods

2.1 Cell line and cell culture

DLD-1/Δα cell lacking of α-catenin expression, is a subclone of the human colon carcinoma DLD-1 cell line. DLD-1/Δα cells were kindly provided by Dr. Shintaro T. Suzuki (Kwansei Gakuin University). DLD-1 cells were cultured in DMEM with high glucose (Sigma) supplemented with 10% FCS and 100 units/mL penicillin G, and 0.1 mg/mL streptomycin under a humidified atmosphere containing 5% CO_2. The expression vector encoding the wild-type α-catenin was transfected into DLD-1 cells and selected using G418 to obtain a stable expression cell line (α-cat) [25, 27]. Cells were plated at 5×10^6 and 5×10^5 on 150-mm dishes for dense culture and sparse culture, respectively followed by incubation for 3 days [26]. Cell densities were confirmed using phase-contrast observations as shown in Fig. 1.

2.2 Western blot and lectin blot analyses

Cells were cultured under different conditions as indicated. After washing three times with ice-cold PBS, cells were solubilized in lysis buffer (10 mM Tris-HCl, 1% Triton-X, 150 mM NaCl, aprotinin, leupeptin, and 1 mM phenylmetylsulfonyl fluoride). Equal amounts of protein were separated using 8% SDS-PAGE, transferred to NC membrane and probed with the appropriate antibodies, as indicated, or with biotinylated E_4-PHA (Seikagaku kogyo, Japan). Immunoreactive bands were visualized using a Vectastain ABC kit (Vector Laboratories, CA) and an ECL kit (Amersham, UK). mAb against E-cadherin, α-catenin, β-catenin, and γ-catenin were purchased from Becton Dickinson (Franklin Lakes, NJ), and anti-α-tubulin antibody was from Sigma. HRP-labeled anti-mouse IgG was obtained from Cell Signaling (Danvers, MA).

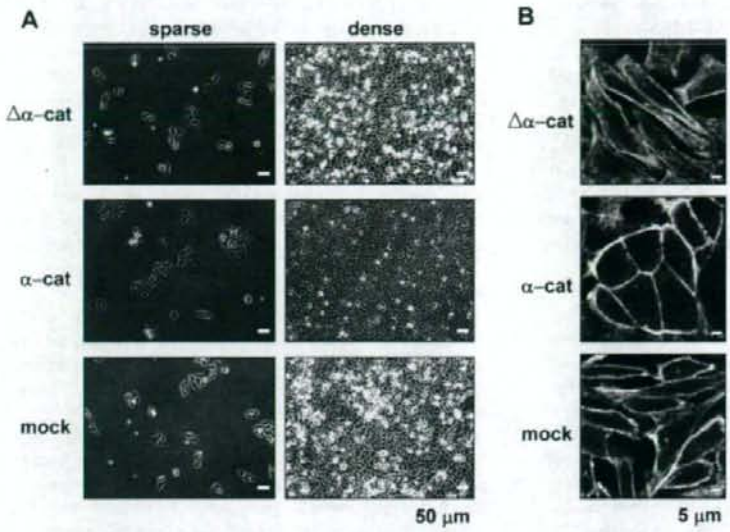

Figure 1. Different phenotypes of DLD-1 cells. (A) Phase contrast micrographs of human colon carcinoma DLD-1 cells lacking expression of α-catenin (DLD-1/Δα) and cell restored with α-catenin (α-cat) or vector only (mock) cultured under sparse or dense conditions. (B) Localization of F-actin was examined by immunofluorescence microscopy for actin.

2.3 Cell surface biotinylation and immunoprecipitation

Cell surface biotinylation was performed as described previously [28]. Briefly, cells were rinsed twice with ice-cold PBS and then were incubated with ice-cold PBS containing 0.2 mg/mL EZ-Link® Sulfo-NHS-Biotin (Pierce) for 1 h at 4°C. After incubation, 50 mM Tris-HCl (pH 8.0) was used for the initial wash to quench any unreacted biotinylation reagent. The cells were then washed three times with ice-cold PBS, and solubilized in lysis buffer. Insoluble material was removed by centrifugation at 15 000 rpm for 10 min at 4°C. The supernatant (2.5 mg protein) was incubated with anti-E-cadherin mAb (3 μg/mL) (BD Biosciences) and either protein G beads (20 μL in 50% slurry) (Amersham) or Streptavidin–agarose (10 μL in 50% slurry) (Upstate Biotechnology) for another 2 h at 4°C with roration. After washing three times with lysis buffer, the immunoprecipitates were subjected to 8% SDS-PAGE, and the separated proteins were transferred to a NC membrane. The membrane was incubated using either streptavidin or anti-E-cadherin mAb for immunoblot analysis. Immunoreactive bands were visualized using a Vectastain ABC kit and an ECL kit.

2.4 Microscopy and cell image

Cells were seeded on glass bottom dishes for 48 h before fixation. After washing two times with PBS, cells were fixed for 30 min in 3.7% paraformaldehyde solution at 37°C, and then blocked with 5% FCS in PBS for 1 h at room temperature. Following three washes in PBS, the cells were incubated with 1:300 dilution of Alexa Fluor® 488 phalloidin (Invitrogen) in blocking buffer for 1 h at room temperature. After washing three times with PBS, cells were analyzed using an Olympus fluorescence microscope (FV1000 system).

2.5 GnT-III activity assay

Cells were cultured under sparse or dense conditions, as described above. After washing with PBS, the cells were lysed by sonication. The cell lysate protein concentration was determined using a BCA protein assay kit (Pierce). Equal amounts of protein were used in the GnT-III activity assays, as described previously [29]. The substrate, pyridylaminated GlcNAcβ1-2Manα1-6(GlcNAcβ1-2 Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (GnGn-PA), was produced according to Hase *et al.* [30]. Each assay used 5 μM substrate (in 10 μL of total reaction solution). The activity of endogenous GnT-III was measured by HPLC, expressed as pmol of GlcNAc transferred/h/mg of proteins [26].

3 Results

Recently, we investigated the effects of cell–cell interactions on *N*-glycan biosynthesis using the dense *versus* sparse culture conditions model. We found that GnT-III expression was markedly induced in cells cultured under dense conditions in an E-cadherin-dependent manner. To investigate whether downstream components of E-cadherin, such as cytoplasmic complexes of E-cadherin–catenin–actin, are also involved in the regulation of GnT-III expression, we focused on α-catenin, which links E-cadherin–catenin complexes to actin filaments.

3.1 Typical epithelial actin organization was rescued by restoration of α-catenin in DLD-1/Δα

α-Catenin is a 102-kDa multifunctional protein with multiple interaction sites, including an amino-terminal β-/γ-catenin binding site, a central region for α-actinin binding, and carboxyl-terminal actin-binding sites. DLD-1/Δα is a subclone of the DLD-1 cell line, which is a colon carcinoma line that exhibits characteristics typical of epithelial cell. DLD-1/Δα expresses normal levels of E-cadherin and β-catenin, but not α-catenin. Lack of α-catenin expression results in loss of cell–cell adhesion and desmosome formation. This phenomenon was also observed in cells cultured under sparse culture conditions, as shown in Fig. 1A. Introduction of α-catenin into DLD-1/Δα cells induced cell clusters, in which their intercellular associations appeared stronger than those of either wild type DLD-1/Δα or mock transfectants. To determine if the loss of α-catenin affects actin cytoskeletal organization, we stained cells cultured under dense conditions with anti-actin antibody. It is well-known that epithelial cells cultured under dense conditions usually exhibit cortical actin skeletons localized at cell–cell boundaries. However, the actin staining pattern of DLD-1/Δα cells was diffuse (Fig.1B). Interestingly, the cortical actin staining pattern of DLD-1/Δα cells, was completely rescued by restoration of the α-catenin gene, but not by mock transfection. These results further confirm that α-catenin plays an essential role in cell–cell adhesion and actin cytoskeletal organization.

3.2 Effects of α-catenin on GnT-III expression

In previous studies, we found that cell–cell adhesion specifically regulates activation of GnT-III, but not of other glycosyltransferases such as GnT-V and α1,6 fucosyltransferase. To examine the effects of α-catenin on GnT-III expression, we compared GnT-III activity in lysates obtained from cells cultured under either dense or sparse culture conditions. As shown in Fig. 1, cells cultured under sparse conditions had adequate space for cell spreading. In contrast, cell cultured under dense conditions have no space for full cell spreading, resulting in a reduction in cell size, as confirmed by phase contrast microscopy. Then the GnT-III activity of parent cells, mock transfectants, and α-catenin transfectants was investigated under both sparse and dense growth conditions. As shown in Figs. 2B and C, parent and mock transfected cells showed slight increases in GnT-III activity when cultured

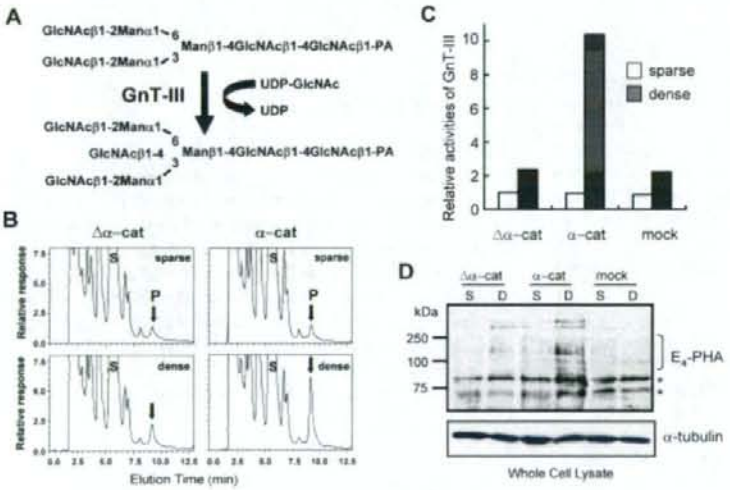

Figure 2. Induction of GnT-III expression under dense culture conditions could be rescued by reintroduction of α-catenin into DLD-1/Δα cells. (A) The reaction pathway for biosynthesis of bisecting GlcNAc by GnT-III. (B, C) A representative HPLC elution pattern for assaying of GnT-III activities expressed as pmol of *N*-acetylglucosamine transferred/h/mg of proteins (B) and relative activities of GnT-III expressed as the -fold increase in dense culture coditions, compared to the activities at sparse culture conditions, which was set equal to 1 (C). DLD-1 cells were plated at 5×10^6 and 5×10^5 on 150-mm dishes for the dense culture and sparse culture, respectively, followed by incubation for 3 days. Cells were then harvested and equal amounts of proteins in cell lysates were used as the enzymatic source for GnT-III as described under "Section 2". S: substrate; P: product. (D) Enhanced expression of bisected *N*-glycans in α-catenin-restored cells cultured under dense culture conditions. Cells were grown to different cell confluences as described above. Equal amounts of cell lysate proteins (20 μg) were subjected to 8% SDS-PAGE. The membranes were probed with E_4-PHA (upper panel), or with anti-α-tubulin antibody as a control for equal loading (lower panel). The asterisks indicate nonspecific staining of E_4-PHA, since those bands did not disappear after treatment with 100 mM acetic acid.

under dense conditions. Interestingly, GnT-III activity dramatically increased in DLD-1/Δα cells restored with the α-catenin gene in a cell density-dependent manner. GnT-III activity of cells cultured under dense conditions was ~8-fold higher than the activity of cells cultured under sparse conditions (Fig. 2B). To determine if the products of GnT-III are increased in cells cultured under dense conditions, total cell lysates were analyzed using E_4-PHA lectin, which specifically recognizes bisected *N*-glycans. As shown in Fig. 2D, the intensity of E_4-PHA staining at approximately 150 kDa was clearly increased in α-catenin-restored cells cultured under dense conditions. To further reinforce our conclusion, we gathered quantitative data of real-time PCR (qPCR) for detection of mRNA of GnT-III, but could not obtain significant Ct (cycles to the threshold) values because the GnT-III mRNA level was quite low, especially in DLD-1 cells. qPCR seems to reflect a cell-type dependence for detection of the mRNA of GnT-III, since it could be detected in GE11 epithelial cells, but not Madin–Darby Canine Kidney (MDCK) cells, as previously described [26]. However, the data shown in Fig. 2 strongly suggests that GnT-III activity is significantly increased in dense conditions compared to sparse conditions in DLD-1 cells restored with α-catenin gene.

3.3 Levels of E-cadherin expression and E-cadherin-associated proteins in α-catenin-deficient cells

It is well-known that the level of E-cadherin expression on the cell surface is upregulated by cell–cell adhesion in most epithelial cells [31]. A loss of, or decrease in E-cadherin expression is associated with the tumor invasion and metastasis. The E-cadherin–catenin complex is devoted to maintain cell formation and cell–cell adhesion. The E-cadherin intracellular domain binds β-catenin, which in turn binds α-catenin. E-cadherin is required for upregulation of GnT-III under dense culture conditions, as previously reported; therefore, we examined the effects of α-catenin deficiency on cell-surface E-cadherin expression. As shown in Fig. 3A, there were significant differences neither in total E-cadherin expression patterns, nor in expression of E-cadherin-associated molecules such as β- and γ-catenin, among mock transfectants, wild type, and restored cells, as confirmed by Western blot analysis. On the other hand, the levels of total E-cadherin (Fig. 3A) and cell surface E-cadherin expression detected by biotinylation (Fig. 3B) were enhanced to a similar degree in all three cell types cultured under dense conditions. These results further support our previous conclusion that E-cadherin is exposed on the cell surface independent of α-catenin [32]. Furthermore, α-catenin did not affect E-cadherin and β-catenin complex formation (Fig. 3C). Taken together, these findings strongly suggest that α-catenin is required for up regulation of GnT-III under dense growing conditions.

3.4 Importance of actin cytoskeletal formation in the regulation of GnT-III

α-Catenin is best known as an important cytoplasmic component of the classical cadherin complex that is responsible for cell–cell adhesion. By virtue of its F-actin-binding capaci-

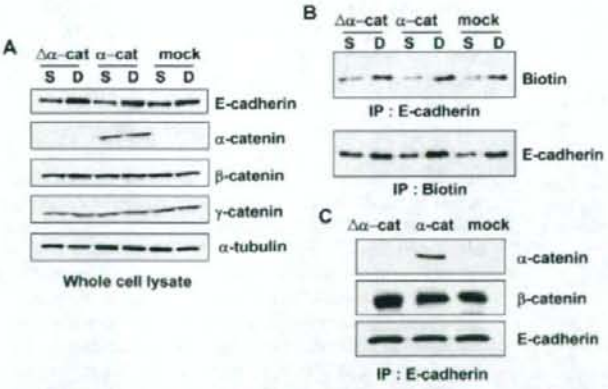

Figure 3. Overexpression of α-catenin did not affect expression levels of E-cadherin nor of its associated proteins in α-catenin-deficient cells. (A) DLD-1 cells were grown to different cell confluences as described in Fig. 2. Equal amounts of proteins (10 μg) were subjected to 8% SDS-PAGE. The membranes were probed with anti-E-cadherin, α-catenin, β-catenin, γ-catenin or with anti-α-tubulin antibody as a control for equal loading. (B) Cell surface biotinylation was performed as described under "Section 2". Equal amounts of the cell lysates were immunoprecipitated with anti-E-cadherin antibody or biotin. The immunoprecipitates were subjected to 8% SDS-PAGE. The membrane was incubated with streptavidin (upper panel) or anti-E-cadherin mAb (lower panel) for immunoblot analysis. (C) DLD-1 cells were cultured under dense conditions as described above. Equal amounts of the cell lysates (100 μg) were immunoprecipitated with anti-E-cadherin antibody. The immunoprecipitates were subjected to 8% SDS-PAGE. The membranes were probed with anti-E-cadherin, β-catenin and α-catenin for immunoblot analysis.

ty, α-catenin is generally thought to support cadherin function by coupling the adhesion receptor to the actin cytoskeleton. To examine if actin reorganization induced by α-catenin generates signals that affect induction of GnT-III expression, cells were treated with cytochalasin D. Cytochalasin D is a cell-permeable fungal toxin that binds to the barbed end of actin filaments and inhibits both the association and dissociation of subunits, causing disruption of actin filaments and inhibition of actin polymerization. Interestingly, cytochalasin D treatment abolished the enhancement of GnT-III expression in α-catenin-restored cells cultured under dense conditions (Fig. 4). The cytotoxic effects of DMSO and cytochalasin D treatment to cells were checked. Trypan blue staining and phase contrast microscopy observation revealed no dead cells in either condition (data not shown), indicating that altered GnT-III activity was due to neither to cytotoxic effects of DMSO nor from cytochalasin D treatment. These results suggest that actin cytoskeletal formation also plays an important role in GnT-III induction.

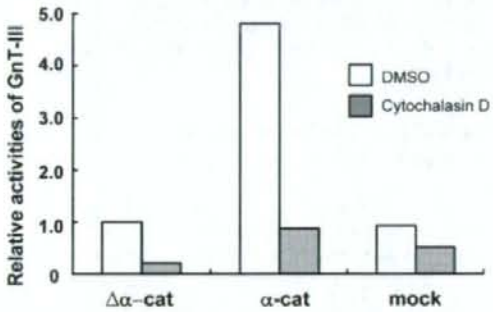

Figure 4. Cytochalasin D treatment inhibited GnT-III activation under dense culture conditions. Cells were plated at 5×10^6 and 5×10^5 on 150-mm dishes for the dense cultures and sparse cultures, respectively. After a 2-day-incubation, cells were incubated with cytochalasin D at a final concentration of 10 μg/mL, followed by another incubation for 1 day. Cells were then harvested. Equal amounts of cell lysate proteins (20 μg) were used as the enzymatic source for GnT-III. GnT-III activity is expressed as fold-decrease relative to the activity in the presence of DMSO without cytochalasin D in Δα-cat cells, which was set equal to 1. The final concentration of DMSO was adjusted to 0.2% of the cell culture medium.

4 Discussion

In the present study, we found that GnT-III and its products, the bisected *N*-glycans, are upregulated by cell–cell interaction *via* the E-cadherin–catenin–actin complex. Disruption of actin polymerization by treatment with cytochalasin D or lack of α-catenin expression, interferes with regulation of GnT-III. Interestingly, reintroduction of α-catenin into α-catenin-deficient cells rescued enhanced GnT-III expression under dense culture conditions. Given the previously described important biological functions of GnT-III [33, 34], the present study provides new insight into the molecular mechanism of relationships among cell–cell interaction, normal development and cancer metastasis. The E-cadherin–catenin–actin complex formation is very important for regulation of GnT-III expression, but not exclusive, since the modest increase in GnT-III expression under dense culture conditions could be observed in α-catenin-deficient DLD-1 cells (this study) as well as in E-cadherin-deficient MDA-MB231 cells [26], which may be modulated by an E-cadherin–catenin-independent pathway (weak signal) as shown in Fig. 5.

Regulation of cadherin-mediated adhesion and associated adherence junctions is thought to underlie the dynamics of intercellular adhesive interactions, which are regulated during tissue development and homeostasis, as well as during tumor cell progression. During normal development, E-cadherin-mediated cell adhesion is vital to gastrulation, which recognizes embryonic germ layers, as well as to the development of other migratory cell types such as the neural crest [35]. E-cadherin engagement at cell–cell contacts is known to suppress proliferation; this effect has been best described in the context of tumorigenesis [36]. Conversely, the disruption of E-cadherin-mediated cell adhesion appears to be a central event in the transition from non-invasive to invasive carcinomas. Therefore, most studies have focused on the identification and characterization of transcriptional repressors of E-cadherin expression in epithelial tumor cells. The most prominent factors identified in these studies included the related factors, Slug, Snail, SIP1, and Twist, which are best known for their roles in early embryogenesis and tumor progression [37]. Interestingly, our earlier study showed that E-cadherin-mediated cell–cell adhesion is regulated by post-transcriptional modification of *N*-glycans. Overexpression of GnT-III slowed E-cadherin turnover, resulting in increased E-cadherin expression on the surface of B16 melanoma cells [18]. Thus, expression of E-cadherin may be regulated, not only by transcriptional factors, but also by post-transcriptional modifications. Conversely, in our studies, E-cadherin-mediated cell–cell interaction upregulated GnT-III expression, suggesting that regulation of GnT-III and E-cadherin expression may exist as a positive feedback loop.

In contrast, cell–cell interaction due to interaction among members of the cadherin superfamily in the assembly of epithelia, initiates a molecular cascade that leads to the formation of additional junctions such as desmosomes and tight junctions [38]. It is generally accepted that the cytoplasmic regions of cadherins must bind catenins inside the cell for the extracellular domains to mediate adhesion. Formation of this molecular complex confers adhesive strength by linking cadherins to the actin cytoskeleton [39, 40]. Some existing evidences suggest, however, that the extracellular regions of cadherins retain some biological activity, even in the absence of catenin linkages. For example, cells expres-

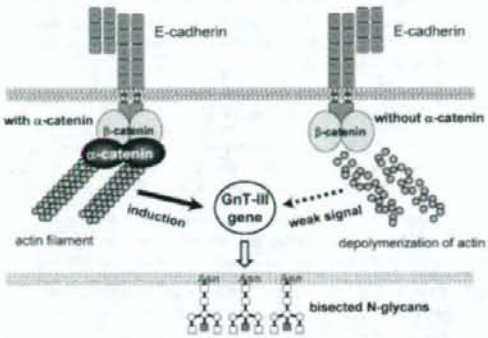

Figure 5. A working model for cell–cell adhesion and regulation of GnT-III expression. Recently, it has been reported that GnT-III expression was significantly upregulated by cell–cell adhesion in an E-cadherin-dependent manner [26]. In the present study, we found that GnT-III and the bisected *N*-glycans, were upregulated by cell–cell interaction *via* the E-cadherin–catenin–actin complex. The weak signal could be considered that GnT-III expression is regulated by an E-cadherin–catenin-independent mediated cell–cell adhesion.

sing E-cadherin without a cytoplasmic domain, still exhibit E-cadherin-dependent cell aggregation and cell–cell adhesion [41, 42]. Interestingly, α-catenin-deficient cells retain the capacity for cadherin-mediated cell adhesion. However, restoration of α-catenin to the cells increases resistance to sphingosine-induced apoptosis and reduces proliferation in 3-D culture [27, 43]. In the present study, α-catenin was essential for induction of GnT-III expression regulated by cell–cell adhesion. Taken together, these results clearly indicate a putative signal-transduction role for α-catenin.

In light of GnT-III upregulation in the dense culture model, cells grown under sparse and dense culture conditions can be viewed, to a certain extent, as cells in the proliferative and differentiative states, respectively. GnT-III expression can be significantly upregulated by cell–cell interactions *via* the E-cadherin–catenin–actin complex, which is reasonable for the maintenance of cell differentiation rather than cell proliferation, since growth factor-mediated activation can be suppressed by GnT-III upregulation [44]. In fact, the results of several studies suggest that E-cadherin has the ability to induce a ligand-independent activation of the EGFR and subsequent activation of Rac1 and MAP kinase, which appear to be involved in cell migration and proliferation [45, 46]. Thus, it is possible that upregulation of GnT-III by cell–cell interactions neutralizes the signals responsible for maintenance of differentiative phenotypes. The possibility is required for further study.

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RAPID COMMUNICATION

Lectin precipitation using phytohemagglutinin-L_4 coupled to avidin–agarose for serological biomarker discovery in colorectal cancer

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N-acetylglucosaminyltransferase V (GnT-V) has been reported to be upregulated in malignant cancer cells, and its targets have been sought after with regard to biomarker identification. The low capacity and high false positive rates of 2-DE gel-based lectin blots using phytohemagglutinin-L_4 (L-PHA) prompted us to develop a novel protocol for identifying GnT-V targets, in which serum proteins were subjected to immunodepletion, alkylation, and lectin precipitation using L-PHA coupled to avidin–agarose bead complexes, and tryptic digestion. Proteins captured by L-PHA conjugates were analyzed by a nano-LC-FT-ICR/LTQ MS. Here, we report 26 candidate biomarkers for colorectal cancer (CRC) that show 100% specificity and sensitivities of greater than 50%. Not only can these candidate proteins be used as analytes for validation, but the novel protocol described herein can be applied to biomarker discovery in nonCRCs.

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Nearly 800 000 new CRC cases are believed to develop each year globally, which accounts for approximately 10% of all incident cancers. In addition, the mortality from CRC is estimated to reach nearly 450 000 deaths annually [1]. The MLH1 and MSH genes are associated with hereditary non-polyposis CRC [2], and the APC gene is associated with familial adenomatous polyposis [3], but these factors fail to account for the occurrence of a wide range of CRC. Moreover, CRC is just one of many epithelium-derived cancers in which circumstantial factors govern over hereditary genetic factors. These attributes necessitate a clear marker that serves as a tracer molecule for the efficacious treatment of CRC, but unfortunately, a discrete biomarker for CRC has yet to be discovered.

Traditional approaches have stressed the dynamics of protein expression levels associated with the biochemical processes of cancer. In contrast, many lines of evidence have demonstrated the role of various glycosyltransferases in the pathogenicity of cancer cells, wherein changes in protein glycosylation have been reported to be associated with the

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Abbreviations: AFP, α-fetoprotein; **CRC,** colorectal cancer; **GlcNAc,** *N*-acetylglucosamine; **GnT-V,** *N*-acetylglucosaminyltransferase V; **ID,** immunodepletion; **L-PHA,** phytohemagglutinin-L_4

* Both authors contributed equally to this work.

pathogenic processes of cells [4]. One of the best-characterized glycosyltransferases is *N*-acetylglucosaminyltransferase V (GnT-V), which catalyzes the formation of a β1,6-*N*-acetylglucosamine (GlcNAc) moiety in an N-linked core glycan. An increase in β1,6-branching on N-linked glycans is associated with the metastatic potential of cancer cells [5]. Several target molecules for GnT-V have been proposed to be involved in cancer progression, including matriptase [6], β_1 integrin [7], N-cadherin [8], and TIMP-1 [9]. Although these proteins were found to be relevant to the biological and pathological processes in cancers, none of them has demonstrated any use as a serum biomarker, which may be due to the fact that molecular fluctuations observed at the cellular level are buffered by the complexity of blood composition, or that current proteomics technologies are unable to trace changes in minute expression level of the proteins in blood.

Despite the strong implication of GnT-V in cancer pathology, no targets of GnT-V have been identified in serum as biomarker candidates. Thus, in this report, we attempted to identify colorectal cancer (CRC)-related GnT-V targets in serum using phytohemagglutinin-L_4 (L-PHA), a lectin that recognizes β1,6-GlcNAc moiety attached to an N-linked core glycan. Though L-PHA binds rather specifically to β1,6-GlcNAc moiety of N-glycans, a method of detachment for L-PHA-based lectin affinity chromatography is not available, which has limited use of the lectin to the gel-based lectin blot analysis for search for GnT-V targets. However, the gel-based approach innately has several drawbacks: (i) the 2-DE gel has limited protein-loading capacity, and (ii) despite the development of cutting-edge, high-sensitivity mass spectrometers, a ostensibly single protein spot can contain several scores of proteins, in which case selection of a true positive is often difficult. These two drawbacks make it even more difficult to mine candidate proteins for GnT-V by lectin blot analysis in serum that shows high dynamic range of proteins. In this regard, we developed a simple and reliable protocol wherein GnT-V targets are captured, precipitated, tryptic-digested, and identified using an FT-ICR-LTQ mass spectrometer. Here, we report our optimized protocol, as well as 26 candidate biomarkers for CRC discovered through this method.

Serum samples were prepared from CRC patients at Our Lady of Mercy Hospital at The Catholic University of Korea (Inchon, Korea) and from healthy volunteers at KRIBB (Daejeon, Korea), with an agreement of participation from all subjects. Serum samples were diluted two to three-fold with an appropriate buffer and subjected to partial purification performed with a ProteomPrep 20 plasma immunodepletion (ID) LC column kit (Sigma) or by Con A-affinity column chromatography (GE Healthcare). With a ProteomPrep 20 plasma ID LC column (Sigma), 20 highly abundant serum proteins were removed according to the manufacturer's instructions. For Con A-affinity column chromatography, diluted sera were loaded onto a Con A-agarose column pre-equilibrated with 50 mM Tris-HCl (pH 7.4); the column was then washed with equilibration buffer, and bound proteins were eluted with 0.4 M β-D-methylmanno pyranoside in 50 mM Tris-HCl (pH 7.4). The partially purified samples were reduced by treatment with 1% v/v β-mercaptoethanol and alkylated with excess iodoacetamide at room temperature for 1 h. The modified samples were desalted on a HiPrep 26/10 desalting column (GE Healthcare) and concentrated to a final volume of 1 mL. Protein samples were precleared with avidin–agarose beads for 1 h at room temperature, and the precleared proteins were allowed to bind to L-PHA–avidin–agarose or L-PHA–agarose complexes overnight at 4°C. After extensive washing with PBS, the bound proteins were separated from the bead complexes by adding 1 × SDS-PAGE denaturation buffer or 6 M urea. Protein preparations that were denatured by addition of denaturation buffer were in-gel digested as described previously [10]. Protein preparations denatured in 6 M urea were diluted to 0.6 M for tryptic digestion. The digested peptides were lyophilized in a SpeedVac system and analyzed by MS.

The peptide mixtures were loaded onto a C18 trap column (5 μm, 100μm, 300 μm id × 5 mm) by an autosampler (Surveyor) at a flow of 20 μL/min for desalting and concentration. The trapped peptides were then back-flushed and separated on a homemade column (length 100 mm) packed with C18 resin (Aqua, 5 μm, Phenomenex) in 75 mm silica tubing (8 mm id orifice). The mobile phases A and B were composed of 0 and 80% ACN, respectively, each containing 0.5% acetic acid and 0.02% formic acid. The gradient began at 5% B for 15 min; was ramped to 20% B for 3 min, 50% for 47 min, and 95% for 2 min; and held at 95% for 5 min, then 5% for 2 min. The column was equilibrated with 5% B for 6 min before the next run. The eluted peptides were directly electrosprayed into a mass spectrometer, which was controlled by Xcalibur software (ThermoElectron Corporation, Home Page Version 2.0 SR1). During the gradient elution, three IT MS/MS spectra were acquired *per* data-dependent cycle from a high-resolution (*R* set at 100 000) FT-ICR master spectrum. Ions selected for MS/MS were dynamically excluded for 60 s. Peak lists were generated, and the resulting .raw files were converted to .xml files in Bioworks software (ThermoElectron, ver. 3.3). The.xml files were used for protein identification using MASCOT search engine version 2.0 (Matrix Science) against the IPI human database 20070905 (67 524 sequences; 28 722 560 residues). MASCOT was used with monoisotopic mass selected, a precursor mass tolerance of ±1.5 Da, and a fragment mass tolerance of ±0.8 Da. Trypsin was selected as the enzyme, with one potential missed cleavage. Oxidized methionine, pyro-glutamate (N-term Q), propioamide cystein and carbamidomethylated cystein was chosen as variable modifications. With regard to acceptance criteria for protein identification, proteins that were identified with more than two peptides among which at least one peptide shows MASCOT individual ion score more than 42 ($p<0.05$) were considered to be candidates. Criteria that proteins must be a known glycoprotein or otherwise have at least one *N*-glycosylation consensus motif (N-X-S/T, where X is any amino acid except proline) were also considered.

Our experiments constituted the following steps: partial purification of serum proteins, lectin capture and precipitation, tryptic digestion, and protein identification *via* MS and database searches. As diagrammed in Fig. 1A, the overall workflow consisted of two separate strategies: one for optimizing protocols for L-PHA-based lectin capture and another for discovery of CRC biomarkers based on the optimized protocol. Protocol optimization included selection of enrichment or fractionation methods, selection of the form of L-PHA conjugates to be used for capturing β1,6-GlcNAc-containing *N*-glycoproteins, and determination of the steps for tryptic digestion. Each step that improved the end-result was chosen and combined to generate an "optimized protocol" for biomarker discovery. A primary reason as to why the use of L-PHA is inappropriate for chromatography originates from the unavailability of a nondenaturation-based elution method, as opposed to other widely used lectins such as Con A, *Lens culinaris* agglutinin, and wheat germ agglutinin (WGA), which has necessitated of the development of a method to detach L-PHA-bound proteins under denaturing conditions. To this end, L-PHA bound proteins were denatured in either 6 M urea or SDS-PAGE denaturation buffer. In the solution digestion method, protein preparations denatured in 6 M urea were diluted ten-fold and subjected to tryptic digestion. Meanwhile, protein preparations denatured in denaturation buffer were resolved on an 8% SDS-PAGE gel, after which the entire gel was cut into pieces and in-gel tryptic digestion was performed. We established criteria to determine which digestion method was more effective for protein identification, prioritized by the following criteria: (i) the number of proteins identified from at least two nonoverlapped peptides among which at least one peptide has a significant MASCOT score ($p<0.05$), (ii) sequence coverage, and (iii) total score values. As is seen in Figs. 1B and C, we identified 28 and 41 proteins satisfying these criteria from the in-gel and solution digestion methods, respectively. In addition, proteins identified through the solution digestion method had higher total scores and sequence coverage compared with those identified through in-gel digestion. Thus, we chose the solution digestion method as described above for subsequent protocol optimization and biomarker discovery.

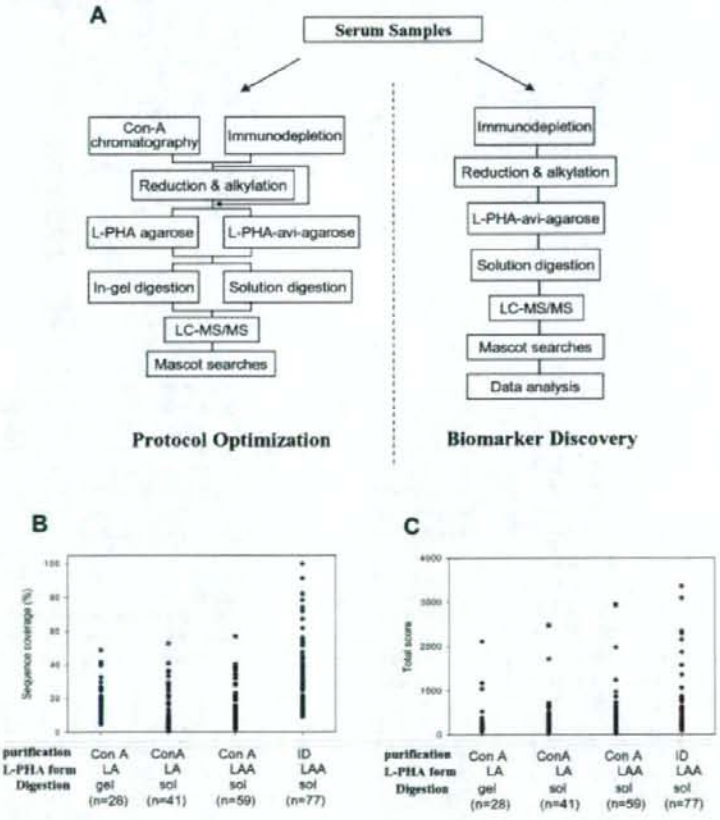

Figure 1. Strategy for protocol optimization of biomarker discovery using serum. (A) Partial purification methods (Con A chromatography or ID), L-PHA bound forms (L-PHA agarose or L-PHA-avidine-agarose), and tryptic digestion methods (in-gel digestion or solution digestion) were varied to monitor the effects on protein identification (left panel). Methods that produced better results were selected and combined for biomarker discovery, as outlined in the right panel. (B, C) Partial purification, L-PHA bound form, and tryptic digestion methods were varied, and the effects on sequence coverage (B) and MASCOT score (C) of the identified proteins from a normal serum sample were investigated. Based on the result, ID as a partial purification method, L-PHA-avidine-agarose, and solution digestion methods (sol) were selected and comprised the optimized protocol for biomarker discovery. A combination of these methods allowed for a ~2.8-fold increase in the number of proteins identified with more than one significant ion score ($p<0.05$). The results were derived from an experiment showing the best result among three independent experiments.

Another issue related to optimization was deciding which form of L-PHA conjugates – direct conjugation of L-PHA to glycan-based beads such as agarose or sepharose, or indirect conjugation of L-PHA to beads with an intermediate protein – would yield better results. For direct conjugation of L-PHA to beads, reactive group-activated conjugation of the lectin to beads was possible. Instead of performing the conjugation in our laboratory, we purchased commercially available L-PHA-bound agarose beads (L-PHA–agarose) (Sigma) for consistency and reproducibility of results. Indirect conjugation was performed by incubating biotin-labeled L-PHA with avidin-coupled agarose beads (L-PHA–avidine–agarose). Following enrichment of glycoproteins on a Con A-agarose column and preclearing with agarose or avidin–agarose beads, the partially purified serum samples were subjected to lectin precipitation using either L-PHA–agarose or L-PHA–avidine–agarose. The bound proteins were tryptic-digested in solution, and peptide sequencing was performed using an FT-ICR/LTQ mass analyzer. Figures 1B and C show that L-PHA–avidine–agarose was much more effective in capturing target proteins in sera. More proteins were captured by L-PHA–avidine–agarose compared with L-PHA–agarose, most of which were identified by higher total scores and sequence coverage. Steric hindrance and restricted flexibility may be an obstacle for biomolecular interactions, especially for ligand–receptor interactions. Thus, spacer arm-appended matrices are often used to overcome these restrictions in affinity chromatography. Avidin can act as a spacer arm in L-PHA–avidine-agarose complexes and lessen the hindrances that are presumably generated between agarose beads and serum glycoproteins.

Biomarker discovery using plasma or serum is hampered primarily because of the high dynamic range of serological proteins. Albumin accounts for ~45% of total serum proteins, and when combined with Ig, accounts for ~60% of them. In addition, since almost all highly abundant proteins are glycoproteins, depletion of such proteins is demanding, precluding the identification of low-abundance proteins with high accuracy. To the best of our knowledge, the ProteomPrep 20 LC column (Sigma), a commercially available ID column, removes the highest number of high-abundance proteins. When run on the column, high-abundance proteins were effectively resolved from the remaining serological proteins (Fig. 2A). The pass-through fractions contained mostly low-abundance proteins, from which most of the 20 high-abundance proteins were separated. When 50 μg of proteins were loaded on an SDS-PAGE gel and stained with CBB, albumin was notably visible together with Igs. However, equal amounts of the depleted proteins shows little trace of the high-abundance proteins (Fig. 2B). More importantly, the depleted protein samples were found to be more responsive to L-PHA compared with the undepleted batches (Fig. 2C). Though the amounts of L-PHA-captured proteins are so small that they are invisible in the coomassie stained gel (Fig. 2D), several proteins were differentially displayed between normal and cancer serum in an L-PHA blot analysis

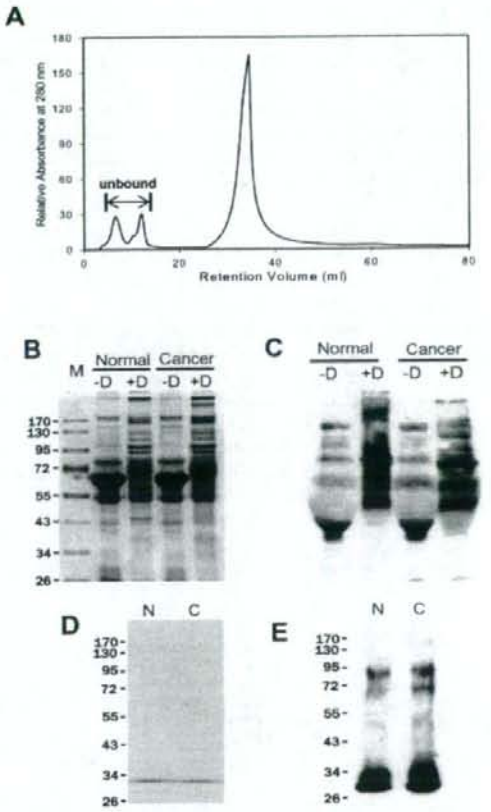

Figure 2. ID of 20 high-abundance proteins from serum. (A) Twenty high-abundance serum proteins were separated from low-abundance proteins (unbound) on a ProteomPrep 20 plasma ID LC column, and the unbound proteins were used for subsequent procedures. (B) Depleted serum samples (+D) were compared with the undepleted ones (−D) on an SDS-PAGE gel following CBB staining. High-abundance proteins such as albumin (~67 kDa) and Ig (~56 kDa) were almost depleted. (C) Equal amounts of serum samples (50 μg proteins) were separated on an SDS-PAGE gel and blotted against L-PHA. After removal of high-abundance proteins, the remaining proteins were more responsive to L-PHA, suggesting that more expansive identification of GnT-V targets is facilitated by ID. (D, E) The immunodepleted protein preparations of normal (N) and cancer (C) sera were captured by L-PHA-aviding-agarose bead complexes, the captured proteins were run on 10% SDS-PAGE gel, stained by CBB (D) and lectin-blotted against L-PHA (E), in which an X-ray film was exposed to chemiluminiscence for sufficient time.

(Fig. 2E). In addition, this ID step enabled us to identify more proteins with higher total score values and sequence coverages (Figs. 1B and C). Thus, we implemented ID as a first step in our discovery protocol, which was compatible

with the following step, obviating the need to insert an additional procedure such as buffer exchange that might cause further loss of proteins. Taken together, our results rendered an optimization protocol in which serum samples are immunodepleted and then subjected to denaturation and alkylation steps. These pretreated proteins are bound to L-PHA–avidine–agarose conjugates, and the bound form proteins are denatured in 6 M urea and subjected to tryptic digestion and protein identification.

Clinical serum samples used in our study were grouped into "normal" and "patient" samples. Normal blood samples were taken from six volunteers (mean age, 44 years) who had no previous record of occurrence of any cancer and showed no abnormal signs in medical tests. Patient samples were taken from ten colorectal patients whose demographics and clinical parameters were as follows: mean age of 49 years, regional lymph node metastasis (pN0: 40%, pN1: 50%, pN2: 10%), depth of invasion (pT2: 10%, pT3: 80%, pT4: 10%), and TNM stage (stage I: 10%, stage II: 20%, stage III: 70%). In this study, we adopted a semiquantitative approach: proteins that were captured and detected in patient samples, but not in normal ones, were screened using Microsoft Excel. Among those proteins, proteins identified by only one peptide were eliminated. More than a hundred of proteins satisfied both criteria, from which we narrowed down to 26 candidate biomarkers by screening (i) glycoproteins or proteins that had at least one *N*-glycosylation consensus sequence (N-X-S/T, where X is any amino acid except proline), and (ii) proteins that were identified with a sensitivity of more than 50%. Proteins that passed these filters are listed in Table 1. Because these proteins were not identified in any normal samples, they showed a specificity of 100% in this study.

Oncological processes accompany bilateral changes of biomolecules *i.e.*, qualitative and quantitative. A quantitative change refers to an alteration in expression, especially of proteins, and accordingly, the majority of biomarker discovery studies has relied on the expressional disturbance or fluctuation of proteins throughout a pathological process. Qualitative changes include phosphorylation/dephosphorylation, proteolytic cleavage, glycosylation, and other

Table 1. Lists of biomarker candidates for CRC

Accession no.	Identities	MASCOT score[a]	No. of unique peptides	Sequence coverage (%)	Sensitivity (%)	Specificity (%)
IPI00013835	Isoform long of diacylglycerol kinase zeta	87–196	4–8	7.0–12.2	90	100
IPI00166020	JmjC domain-containing histone demethylation protein 2A	88–135	5–9	7.1–15.2	90	100
IPI00001516	Isoform long of protocadherin α C2 precursor	99–187	2–5	2.3–9.5	80	100
IPI00718805	Brain-rescue-factor-1	86–159	5–10	12.9–35.0	80	100
IPI00002469	Host cell factor 2	96–142	3–6	6.0–13.0	70	100
IPI00021733	Bifunctional heparan sulfate *N*-deacetylase/*N*-sulfotransferase 4	88–141	3–5	5.8–10.1	70	100
IPI00235481	Polyamine modulated factor 1-binding protein 1	96–147	7–11	10.3–18.4	70	100
IPI00293057	Isoform 2 of carboxypeptidase B2 precursor	92–301	2–6	15.8–41.9	70	100
IPI00418544	FLJ00268 protein (fragment)	86–143	4–7	9.8–20.3	70	100
IPI00739954	Similar to α 3 type VI collagen isoform 1 precursor	86–150	7–14	23.6–48.7	70	100
IPI00786924	Similar to B0416.5a	85–134	7–10	24.3–30.2	70	100
IPI00787145	OTU domain containing 1	98–191	6–14	17.2–46.4	70	100
IPI00009793	Complement C1r-like protein	90–263	2–4	9.4–16.2	60	100
IPI00019223	Isoform 1 of A-kinase anchor protein 9	101–158	8–15	4.4–7.7	60	100
IPI00164623	187 kDa protein	154–875	5–8	6.2–10.2	60	100
IPI00169307	Rho-GTPase activating protein 10	86–207	4–8	3.4–7.8	60	100
IPI00218823	Isoform 1 of WW domain-binding protein 7	136–232	20–27	19.8–30.1	60	100
IPI00291638	183 kDa protein	86–117	5–9	5.7–10.5	60	100
IPI00452247	Isoform 2 of kinesin-like motor protein C20orf23	90–138	5–8	6.0–11.2	60	100
IPI00477931	361 kDa protein	91–160	4–10	2.0–6.5	60	100
IPI00553067	Coiled–coil domain containing 132 isoform a	88–152	4–7	7.7–14.4	60	100
IPI00746049	Similar to breast cancer antigen NY-BR-1.1	105–152	7–13	10.5–34.7	60	100
IPI00854651	Isoform 1 of formin-1	101–140	4–7	3.5–10.7	60	100
IPI00021274	Ephrin type-A receptor 8 precursor	104–165	6–9	10.9–15.8	50	100
IPI00163493	Isoform 1 of aminopeptidase O	86–106	5–7	17.8–24.3	50	100
IPI00299059	Isoform 2 of neural cell adhesion molecule L1-like protein precursor	135–390	2–7	3.0–13.8	50	100

a) Score is $-10 \times \log(p)$, where p is the probability that the observed match is a random event.

PTMs. In particular, glycosylation changes have been reported to be associated with malignant transformation, invasion, and metastasis [4]. In this regard, a concomitant consideration of qualitative and quantitative changes in biomarkers can provide us with more useful information for cancer prediction and diagnosis. For example, α-fetoprotein (AFP) has been used as the sole biomarker for hepatocellular carcinoma, but its use is limited by its low sensitivity (~50%). However, when α1,6-fucosylation on AFP is considered together with the titer elevation, the sensitivity increases to 69% and the specificity was 96% in benign liver diseases [11]. Use of L-PHA as a capture lectin enables us to measure both qualitative and quantitative changes in proteins, because an increase in capture by L-PHA reflects a net increase in the β1,6-GlcNAc glycan moiety without any significant change in expression level. Such a signal, however, can also result simply from the upregulation of otherwise undetectable glycoproteins with a cognate β1,6-GlcNAc linkage or from simultaneous increases in β1,6-GlcNAc glycan moiety and protein levels.

Most glycoproteins either are retained on the membrane fraction (*e.g.*, plasma membrane and ER membrane) or flow out of cells by secretion and shedding. For this reason, serum is a depository for glycoproteins originating from multiple tissues, and many useful biomarkers such as PSA and AFP are in fact glycoproteins. This utility of glycoproteins as potential biomarkers has led to the use of various lectins for up-front capture of glycoproteins, and as such, lectin capture has been recognized as a robust step for identifying serological glycoprotein biomarkers when combined with MS [12]. *Aleuria aurantia* lectin (AAL), *Anguilla anguilla* agglutinin (AAA), and *Sambucus nigra* (SNA1) belong to a lectin group that recognizes a specific glycan moiety. Con A and WGA are two examples of lectins with broad coverage of glycoproteins. The aforementioned lectins may be suitable for unbiased biomarker discovery, but an additional fractionation step would be needed for *de novo* biomarker discovery using serum because evidence indicating that the glycan branches recognized by the lectins are implicated in the oncopathology is scanty. Moreover, Con A catches nearly all *N*-glycosylated proteins, and if the lectin is used, much more lectin is needed to capture glycoproteins in a specified volume of serum, and high amounts of lectin surely interfere with identification of proteins of interest during MS analysis. The restrictions of these lectins when used in glycoprotein enrichment propelled us to conduct "pathology-implicated targeted capture" using L-PHA, which characteristically binds with high affinity to β1,6-GlcNAc on *N*-glycoproteins generated by GnT-V activity [13].

Table 1 lists 26 candidate biomarkers for CRC, most of which have not been reported as biomarkers except for NY-BR-1.1. NY-BR-1.1 is a homolog of NY-BR-1, which was previously identified by the SEREX method [14] and is sporadically expressed in 32% of breast tumors [15]. To test if the candidate proteins are specific as biomarkers for CRC patients, we performed biomarker discovery using sera of liver cancer patients following the same protocol developed in this study. Liver cancer is chosen because it is a representative cancer that shows high expression of GnT-V [16, 17]. As a result, we could obtain a list of candidate biomarkers for liver cancer comprising 24 proteins with 100% specificity and more than 50% sensitivity (data not shown). Interestingly, most of biomarker candidates for liver cancer did not overlap with those for CRC except for polyamine modulated factor 1-binding protein 1 (IPI00235481) and isoform 1 of formin-1 (IPI00854651), which probably reflect that each of the candidate proteins is specific biomarkers for the corresponding cancer type. As biomarker discovery innately generates a panel of proteins with a high false discovery rate and low credentialing [18], our list of proteins should be tentatively considered "candidate biomarkers" for CRC. These proteins await further validation steps in order to distinguish the true positives. Additionally, our optimized protocol may be directly applicable to the discovery of candidate biomarkers for other, nonCRCs.

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