Biochemical and Biophysical Research Communications 431 (2013) 658–663

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

N-Acetylglucosaminyltransferase V triggers overexpression of MT1-MMP and reinforces the invasive/metastatic potential of cancer cells

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ARTICLE INFO

Article history:
Received 16 January 2013
Available online 26 January 2013

Keywords:
GnT-V
MMP-2
MT1-MMP
Tumor progression

ABSTRACT

N-Acetylglucosaminyltransferase V (GnT-V) is an enzyme that catalyzes the formation of a β1,6-*N*-acetylglucosamine (GlcNAc) side chain to a core mannosyl residue in N-linked glycoproteins. Besides its direct function of producing aberrant glycoproteins, it promotes cancer progression by its involvement in the stimulation of oncoproteins. Herein, we report that GnT-V guided the transcriptional activation of membrane-type matrix metalloproteinase-1 (MT1-MMP) in cancer cells. The activated MT1-MMP expression had dual effects on cancer progression. It not only promoted proteolytic activity for cancer cells *per se*, but also led to the activation of MMP-2. Consequently, the activation of the two MMPs triggered by GnT-V intensified the invasive potential. A quantitative analysis using clinical tissues revealed a relatively strong correlation between GnT-V overexpression and MT1-MMP upregulation. In this study, we report for the first time that GnT-V directs cancer progression by modulating MMPs in cancer.

1. Introduction

Cancer cells operate a variety of molecular machinery to fulfill metastasis, which is the process in which a primary tumor cell infiltrates into adjacent tissues, spreads to a secondary tissue, and finally forming a secondary tumor. Metastasis is one of the hallmarks of malignancy in cancer, being the most critical factor indicating poor prognosis [1]. Because the basement membrane and the extracellular matrix (ECM) surrounding primary tumors serve as major barriers that prevent epithelial cells from migrating, degradation of the matrix architecture in the tumor microenvironments should be accompanied during cancer invasion and metastasis [2]. The basement membrane and ECM are composed of fibrotic proteins, such as collagen, fibronectin, laminin, and so on [3], and are widely known to lose their integrity due to the action of cancer cells as well as cellular and molecular interactions during cancer invasion [4]. Tumor cells express on the cell surface or secrete various proteases that are involved in the degradation of the basement membrane and ECM, including serine/threonine proteases and matrix metalloproteinases (MMPs). As far as MMPs are concerned, cumulative evidence indicates that several MMP members including MMP-2, MMP-9, and MMP-13 are overexpressed in various tumors and are associated with metastasis and poor prognosis [5].

MT1-MMP, also known as MMP14, is a prototypical membrane-type MMP, which is tethered to the cell surface by a transmembrane segment [6]. MT1-MMP exerts its pro-invasive activity not only by participating in the degradation of ECM but also by activating MMP-2. MT1-MMP displays an unusually broad substrate specificity for ECM proteins and is responsible for hydrolysis of diverse ECM proteins. The enzyme also has an intracellular juxtamembranous sequence, interacts with the intracellular transduction machinery and plays a role as a transducer of extracellular signals [7]. Although MT1-MMP activity is stringently regulated during development and under normal conditions [8], an elevated expression of the protease is observed in a wide variety of human cancers [9–11]. Multidimensional regulation of MT1-MMP activity has been reported, including inhibition by TIMPs [12], degradation by autocleavage [13], internalization/recycling [14], and modulation by glycosylation [15]. However, little is known about how MT1-MMP expression is perturbed during tumor progression.

N-Acetylglucosaminyltransferase V (GnT-V) is an enzyme that catalyzes the branching of the β1,6-GlcNAc side chain to the core mannosyl residue of Asn (N)-linked glycan inside the Golgi body. An increase in the β1,6-GlcNAc branch on N-linked glycans is commonly observed upon neoplastic and malignant transformation

Abbreviations: ECM, extracellular matrix; GlcNAc, *N*-acetylglucosamine; GnT-V, *N*-acetylglucosaminyltransferase V; MT1-MMP, membrane-type 1 matrix metalloproteinase; TIMP-1, tissue inhibitor of metalloproteinase-1.

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http://dx.doi.org/10.1016/j.bbrc.2013.01.065

[16], and induction of mammary tumor growth and metastasis is significantly reduced in GnT-V knockout mice [17]. GnT-V directs the malignant transformation of cancer cells bilaterally; it produces an aberrant glycosylation of functional targets, thereby leading to perturbations in the molecular process in which the target proteins are involved, as illustrated by matriptase [18]. Previously, we demonstrated that a high invasive/metastatic potential is gained by the aberrant glycosylation of tissue inhibitor of metalloproteinase-1 (TIMP-1) by the catalysis of GnT-V [19,20]. Besides, overexpression of GnT-V appears to switch the expression of pro-invasive proteins by an indirect mechanism. Herein, we report that GnT-V is involved in cancer metastasis by regulating the expression of MT1-MMP and enhancing the proteolytic capacity in colon cancer.

2. Materials and methods

2.1. Stable transfectants

The WiDr cell line with stable overexpression was established as reported previously [19]. For knock-down experiments, the shRNA vector constructs for GnT-V and MT1-MMP were generated using pcDNA 3.1 vector (Invitrogen). The inserted DNA sequences for gene silencing are as follows: GnT-V, AGCTTCCCTCCTTTGACC CTAAGAATCTCGAGATTCTTAGGGTCAAAGGAGGGG and AGCTTG CTGGAGTCATGACAGCTTATCTCGAGATAAGCTGTCATGACTCCAGCG; MMP14, AGCTTCGGCCTTCTGTTCCTGATAAACTCGAGTTTATCAGG AACAGAAGGCCGGand AGCTTCGATGAAGTCTTCACTTACTTCTCGA GAAGTAAGTGAAGACTTCATCGG. The recombinant and control vectors were transfected using an electroporator according to the manufacturer's instructions (Neon™, Invitrogen).

2.2. Clinical samples

Tissue samples were obtained from colorectal cancer patients at Our Lady of Mercy Hospital at The Catholic University of Korea (Inchon, Korea) with agreement to participate obtained from all subjects. Tissues were ground with a mortar and pestle in the presence of sea sands, and clear protein preparations were obtained following centrifugation and filtration through a 0.45-μm syringe filter.

2.3. Immunoblot analyses

Proteins were resolved on 10–15% SDS–PAGE gels and transferred electrically onto PVDF membranes (Immobilon-P, Millipore). The membranes were blocked in 0.05% Tween 20-TBS plus 5% skim milk and then incubated with an anti-MT1-MMP (ab51074, Abcam) or anti-MMP-2 antibody (#4022, Cell Signaling). After incubation with HRP-labeled secondary antibodies (Cell Signaling), membranes were allowed to react with ECL™ Western blotting detection reagents (GE Healthcare) and exposed to X-ray film for 1–2 min. The band intensity was calculated from the digitalized, scanned files using ImageJ software (http://rsbweb.nih.gov/ij/).

2.4. Immunofluorescence

Mock and stably transfected cells were trypsinized and plated on cover slips in 6-well plates for 24 h. Cell mono-layers were washed with PBS, fixed with 4% paraformaldehyde, and completely washed with excess PBS-Tween 20 (0.02% v/v). After blocking with 1% (w/v) BSA, cells were treated with an anti-MT1-MMP antibody (MAB9181, R&D Systems) overnight. Cells were washed with PBS buffer three times for 5 min, and treated with goat anti-rabbit immunoglobulin G antibody conjugated with an FITC fluorescent dye (SantaCruz Biotechnology) in the blocking buffer at room temperature for 1 h. The cells were washed with PBS buffer and stained with DAPI (Sigma). Finally, each slide was examined by confocal fluorescence microscopy (LSM 510 Meta, Zeiss).

2.5. Cell invasion assay

Cell invasion assay was performed using the Matrigel-coated invasion chamber (Corning) with, as described elsewhere [19]. Briefly, 2×10^5 cells were placed inside the upper chamber and maintained in RPMI 1640 medium plus 1% BSA. Cells were incubated for 24 h and invading cells were counted with a microscope at a 400 magnification after fixation in methanol and staining with Toluidine blue.

2.6. In vitro MT1-MMP assay

Cells were incubated in dye-depleted RPMI1640 medium (Welgene) with the artificial substrate for MT1-MMP, MCA-PLA-C(OMe-Bz)-WAR(Dpa)-NH_2 (Calbiochem) at 10 μM. The MT1-MMP activity was calculated by measuring fluorescence in an LS 45 Luminescence Spectrometer (PerkinElmer) at an excitation and emission wavelengths of 328 and 396 nm, respectively.

2.7. Reverse-transcription PCR and quantitative real-time PCR

RNA was extracted from cells or tissues using RNeasy Miniprep kit (Qiagen), quantified and used for synthesis of cDNA, which was used as templates for RT-PCR and quantitative real-time PCR (qPCR). A gene-specific primer pair with sequences of TGTGTATGG CAAAGTGGATA (forward) and ACCATGGTTTTTCACGTAAC (reverse) was used to measure GnT-V expression level in these quantitative analyses. qRT-PCR was carried out using a QuantiTect SYBR Green RT-PCR kit (Qiagen) using an iCycler (Bio-Rad).

2.8. Statistics

The Pearson correlation coefficient (r) was used to evaluate the correlation between parameters. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. GnT-V stimulates MT1-MMP expression in cancer cells

Our previous study demonstrated the important role of GnT-V in the modulation of MMPs to create the high proteolytic burden in the tumor microenvironment [19,20]. Because membrane-type metalloproteinases (MT-MMPs) have recently been reported to be implicated in cancer progression, we attempted to test if GnT-V is involved in the regulation of MT-MMPs and the metastatic potential in cancer.

For this purpose, MT1-MMP is was chosen because it is a representative membrane-bound metalloproteinase that is best characterized among MT-MMPs and has been reported to be involved in a wide range of cancer progression [7]. And we investigated the relations between MT1-MMP and GnT-V expression in various cell lines, including both colon and non-colon cancer cells with varying levels of aggressiveness. WiDr, HepG2, and HeLa cells are among those that are known to show relatively less malignant phenotypes. For instance, HepG2 cells are epithelial in morphology and are not tumorigenic in nude mice [21]. These cells showed a low or basal level of GnT-V expression, and the MT1-MMP expression was also suppressed in those cell lines (Fig. 1A). However, a substantial expression of MT1-MMP was observed in the more aggressive or metastatic cell lines, such as LoVo, MDA-MB-231, and

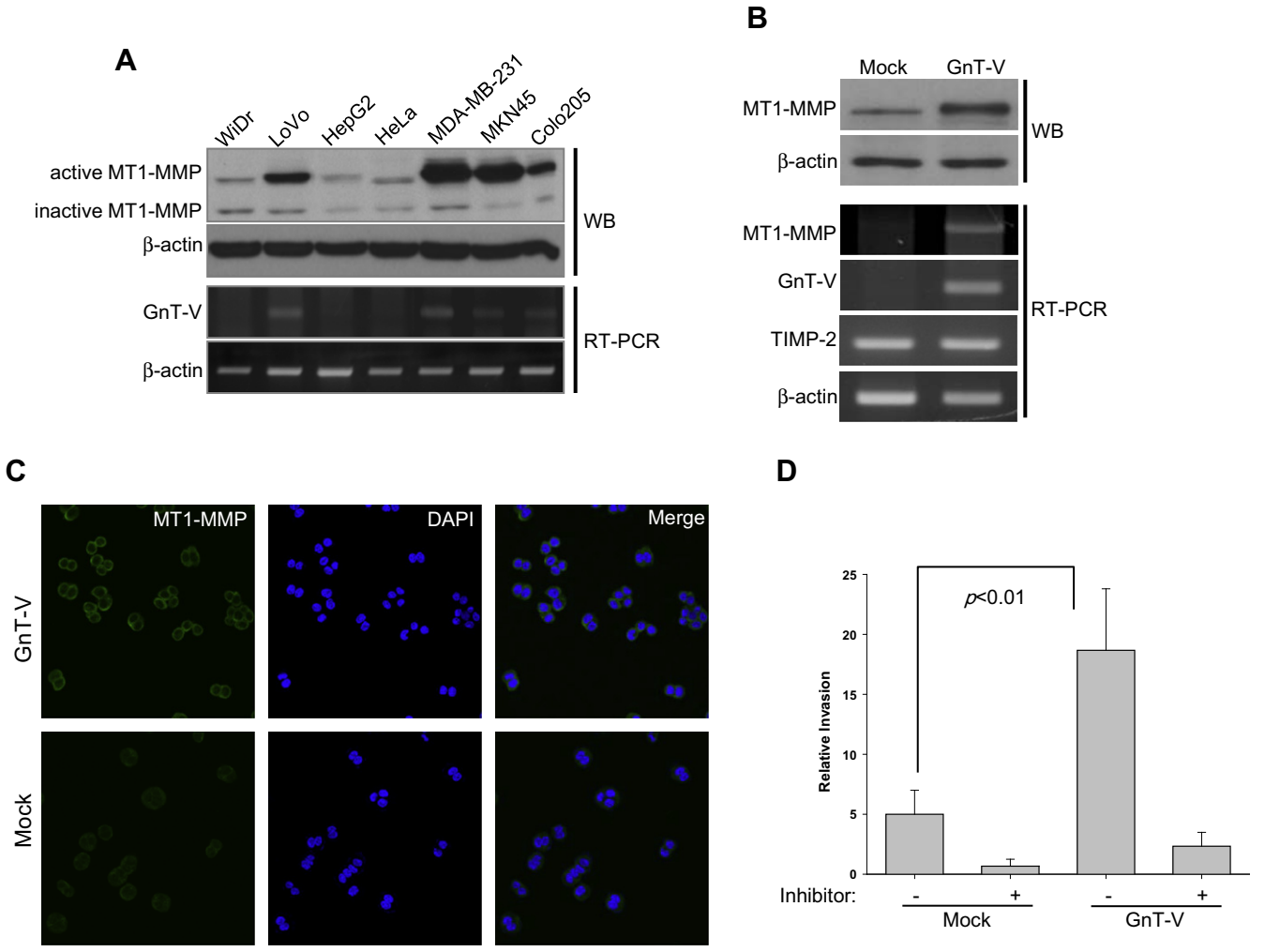

Fig. 1. Regulation of MT1-MMP expression by GnT-V in cancer cells. (A) Dependence of MT1-MMP expression on GnT-V expression level. Their expression levels were investigated in several cancer cell lines by Western blot analysis and RT-PCR. (B) MT1-MMP upregulation in GnT-V overexpressing WiDr cells. The transcription levels of GnT-V and TIMP-2 were also assessed by RT-PCR with β-actin as a loading control. (C) MT1-MMP expression levels on the cell surface as assessed by immunofluorescence. DNA was stained by DAPI. (D) Gain of high invasive potential by GnT-V overexpression. Invasion assay was performed in the Matrigel chambers in the presence or absence of a pan-MMP inhibitor.

Colo205. A relatively high level of active MT1-MMP was observed and found to be accompanied by a significant level of GnT-V expression. This result was confirmed by forced expression of GnT-V in WiDr, which is a colonic cancer cell line with marginal expression of GnT-V. Compared to mock cells, stable transfectant cells with an overexpression of GnT-V showed a dramatic increase in the expression of active MT1-MMP (Fig. 1B). The activity of MT1-MMP is reportedly regulated by several modulators with different molecular mechanisms [9–11]. To test if the increase in MT1-MMP expression is induced by transcriptional modulation or by other mechanisms, such as internalization and recycling pathway, the transcriptional level of MT1-MMP was also quantified by RT-PCR. The results show that the transcription level of MT1-MMP was marginal in mock cells but was dramatically increased by GnT-V expression. TIMP-2 is known to be co-expressed with MT1-MMP, and it counterbalances the proteolytic activity [22]. However, GnT-V did not affect the TIMP-2 transcription, thereby resulting in a net increase in MT1-MMP activity. The possibility that the GnT-V-induced increase in the MT1-MMP expression could be compromised by concomitant stimulation of the internalization of MT1-MMP by GnT-V was also investigated by immunofluorescence. As shown in Fig. 1C, a higher level of MT1-MMP was observed on the cell surface for GnT-V transfectant cells, compared to mock cells. Taken together, these results indicate the production of active MT1-MMP stimulated by GnT-V expression in various cancer cells.

The effect of GnT-V on cancer cell invasion was investigated *in vitro* using the Matrigel invasion chamber (Fig. 1D). WiDr cells did not show vigorous invasiveness, but GnT-V led to a substantial increase in invasion *in vitro*. Treatment with a pan-MMP inhibitor indicates involvement of the activities of MMPs during cancer cell invasion and that GnT-V stimulates cancer invasion in an MMP-dependent manner.

3.2. Suppressed GnT-V expression lowers the MT1-MMP level

To validate the effect of GnT-V on MT1-MMP expression, MT1-MMP expression was monitored in cancer cells after stable transfection of interference RNAs for GnT-V. For this purpose, the MDA-MB-231, MKN45, and Colo205 cell lines were selected because they show substantial levels of GnT-V expression. Vector constructs were made to produce small hairpin RNA (shRNA) and thus silence the gene expression via RNA interference. Two independent regions for the GnT-V gene were used to construct shRNA vectors as mentioned in Section 2, and either scrambled or specific shRNA vectors were stably transfected into the cells. Each stable

clone was selected and used for subsequent quantification as assessed by RT-PCR and immunoblot analysis.

An RT-PCR analysis revealed that we could generate clones in which GnT-V expression was significantly suppressed for all the three cell lines, although the extent of suppression differed between cell lines (Fig. 2A). As expected, MT1-MMP expression was concomitantly suppressed by the downregulation of GnT-V and the extent of suppression of MT1-MMP expression was proportional to that of GnT-V expression. Identical results were observed in the immunoblot analysis (Fig. 2B), which revealed the presence of an active form and a cleaved, inactive form of MT1-MMP in each cell line. RNA interference for the GnT-V gene suppressed both active and inactive forms of MT1-MMP. Taking the results together, we concluded that there appears to be equilibria in the levels of active and inactive forms of MT1-MMP, and the suppressed expression of GnT-V resulted in the down-regulation of the active MT1-MMP and simultaneously the cleaved, inactive MT1-MMP product. Quantitative analysis of the data obtained from RT-PCR and immunoblot analysis showed a strong correlation between the suppression of GnT-V and MT1-MMP expression (Fig. 2C).

3.3. Cancer invasion is stimulated by GnT-V-induced activation of both MT1-MMP and MMP-2

To assess the effect of GnT-V and MT1-MMP expression on the capacity of cancer cell invasion, an *in vitro* enzyme assay was performed using a fluorogenic peptide substrate for MT1-MMP. MT1-MMP activity produced a fluorescent cleavage product with a maximal fluorescent intensity at ~396 nm. The MDA-MB-231 cell line was selected as a model cell line because the cells show high expression levels of GnT-V and MT1-MMP as seen in Fig. 2A and B.

From the pre-experiment results (data not shown), an incubation time was fixed at 2 h and the cell number at 5×10^5 cells per ml of reaction mixture to secure a linearity in the measurement of MT1-MMP activity. Under these conditions, we examined how GnT-V affects the proteolytic activity of MDA-MB-231 cells. For this purpose, stable transfectant cells with RNA interference for GnT-V and MT1-MMP gene expression were established (Fig. 3A). As expected, two sets of shRNA for GnT-V were similarly effective to interfere with both MT1-MMP and GnT-V expression. MT1-MMP expression was effectively suppressed by RNA interference for MT1-MMP without affecting GnT-V expression itself. These manipulated cells were used to measure the *in vitro* proteolytic activity. Either each transfectant or the parental MDA-MB-231 cell was incubated, and the proteolytic activity of MT1-MMP was assessed by the increment in the fluorescence intensity. Fig. 3B shows the relative MT1-MMP activity for each cell. It was obvious that knock-down of MT1-MMP expression mitigated the proteolytic capacity, and the lowered MT1-MMP activity was also attained by RNA interference for GnT-V.

An *in vitro* invasion assay was performed, showing that MT1-MMP activity is, if not sufficient, necessary for cancer cells to invade the ECM components (Fig. 3C). More importantly, RNA interference for the GnT-V gene significantly inhibited invasion, and down-regulation of MT1-MMP caused by the suppression of GnT-V expression was largely responsible for the decreased invasiveness. Considering the effect of MT1-MMP on MMP-2 activation, the invasiveness can be partially attributed to the altered MMP-2 activity of MDA-MB-231 cells. Actually, MMP-2 activation was dependent on the MT1-MMP activity (Fig. 3D). Knock-down of MT1-MMP led to a decrease in the active MMP-2 in MDA-MB-231 and LoVo cells. This result was in line with the data obtained from the *in vitro* enzyme assay. These results suggest that stimulated GnT-V expression is accompanied by enhanced MT1-MMP activity and promotes invasion during metastasis.

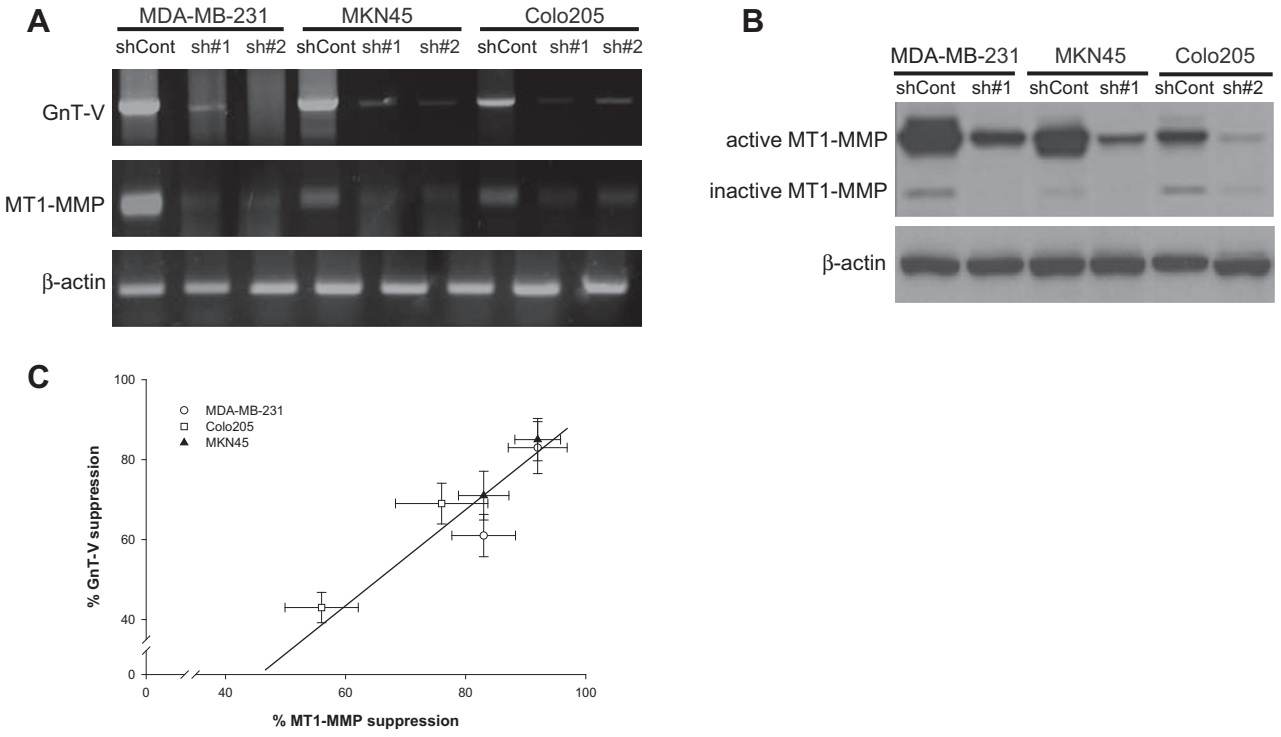

Fig. 2. Validation of the effect of GnT-V expression on the expression levels of MT1-MMP. (A) Suppression of GnT-V expression and the effect on MT1-MMP expression. Two independent regions for the GnT-V gene were targeted for the gene suppression by a small hairpin RNA in MDA-MB-231, MKN45, and Colo205 cells. (B) Immunoblot analysis for MT1-MMP expression in mock cells and cells with suppressed GnT-V expression. (C) Correlation of GnT-V and MT1-MMP expression levels. A plot for the expression levels of GnT-V in relation to MT1-MMP levels revealed a linear positive correlation of both enzymes.

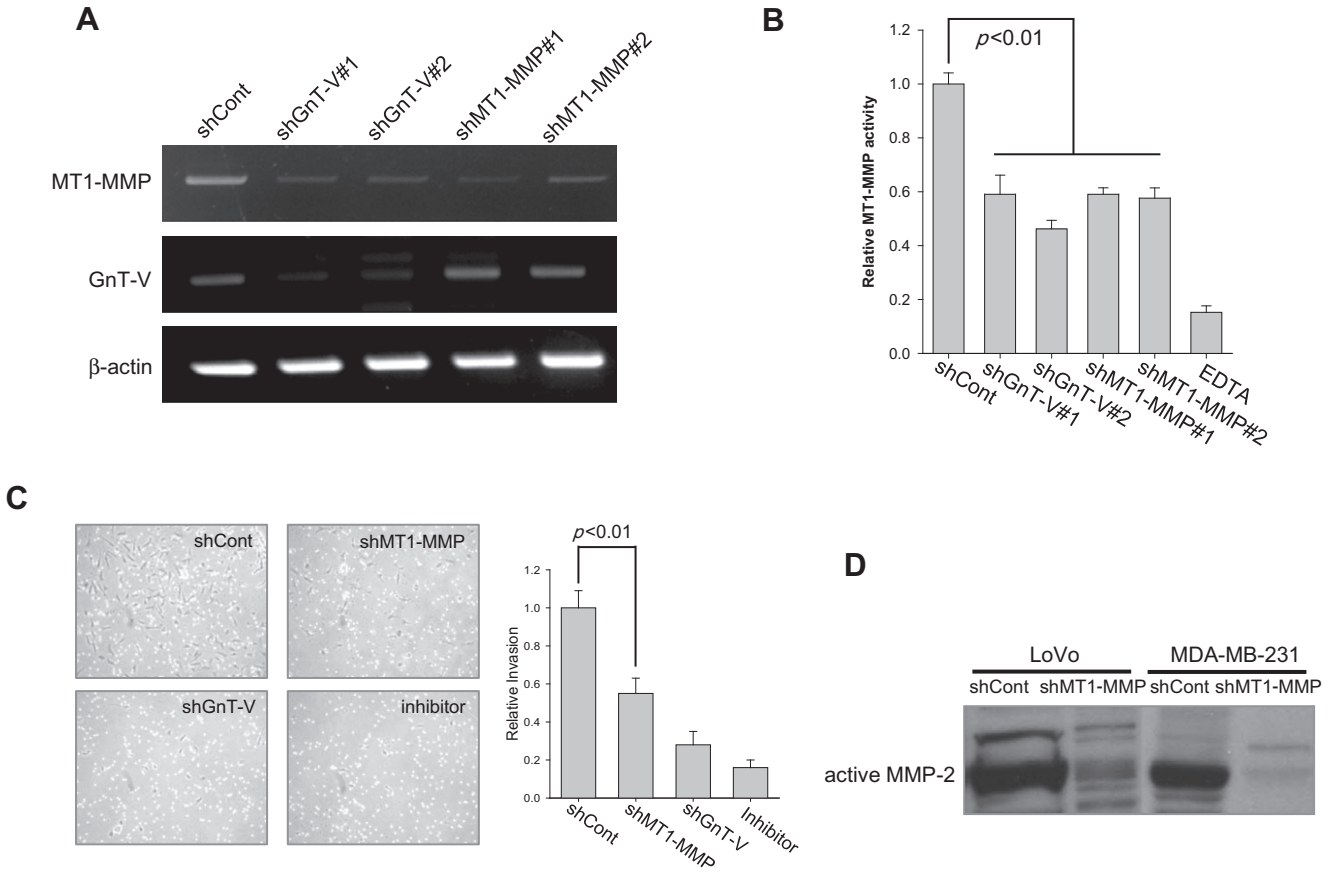

Fig. 3. Effect of GnT-V expression on the elevated invasiveness via MT1-MMP overexpression. (A) Establishment of stable transfectants of MDA-MB-231 cells with suppressed expression of GnT-V and MT1-MMP. (B) Downregulation of MT1-MMP activity in stable transfectant cells with suppressed expression of GnT-V and MT1-MMP. In this experiment, 5×10^5 cells were incubated for 2 h in reaction mixture, and the fluorescence intensity was measured at 395 nm. (C) Effect of downregulation of GnT-V and MT1-MMP on *in vitro* invasion. (D) Dependence of MMP-2 activation on MT1-MMP expression. MT1-MMP expression was transiently suppressed by expression of a short hairpin RNA for MT1-MMP, and MMP-2 expression was investigated by immunoblot analysis in LoVo and MDA-MB-231 cells.

3.4. GnT-V overexpression is synchronously observed with MT1-MMP upregulation in cancer tissues

There is mounting evidence that MT1-MMP is a multi-functional enzyme implicated in ECM degradation [7], angiogenesis [23], and MMP-2 activation [24]. For this reason, MT1-MMP is frequently overexpressed in a wide variety of human cancers [9–11]. To reveal the correlation of GnT-V and MT1-MMP expression, we investigated the expression patterns of the two enzymes in cancer and adjacent normal tissues. Fifty pairs of colon cancer and normal tissues that had been frozen in liquid nitrogen immediately after biopsy were divided into two pieces, with one half used for a Western-blot analysis and the other for quantitative real-time polymerase chain reaction (qRT-PCR).

Fig. 4A shows representative data from the Western-blot analysis of MT1-MMP and from the qRT-PCR of GnT-V. MT1-MMP was up-regulated in tumor tissues, compared to their normal counterparts. Importantly, the upregulation of MT1-MMP was correlated to the mRNA level of GnT-V, which was deduced from the cycle threshold (Ct) value for GnT-V subtracted from that for β-actin. The analysis was expanded to 50 tissue pairs, and a graph was drawn showing MT1-MMP level in relation to the relative mRNA level of GnT-V (Fig. 4B). In this graph, cancer tissues were classified into two groups; one group belongs to a lower Astler–Coller grade (A-B2) and the other to a higher grade (B3-D). Two important conclusions could be drawn from the result. First, MT1-MMP is upregulated in cancer tissues and the extent of upregulation increases as cancer progresses. Second, MT1-MMP upregulation shows a relatively strong correlation with GnT-V overexpression with the Pearson's correlation coefficient being 0.618. These data confirm that cancer cells reinforce the invasive/metastatic potential by the overexpression of GnT-V and the resultant up-regulation of MT1-MMP during cancer progression.

MT1-MMP has been reported to be involved in a variety of pathological processes during tumor progression. The membrane-bound metalloproteinase has been intensively characterized with respect to proteolytic activation, internalization/recycling pathway, molecular inhibition by TIMPs, MMP-2 activation, angiogenesis, etc. [23,24]. However, little has been known about upstream regulators associated with transcriptional activation or suppression. Herein, we report that GnT-V directs the transcriptional activation of MT1-MMP on the surface of cancer cells, which, in turn, leads to the activation of MMP-2. The resultant activation of the two MMPs by GnT-V leads to elevated proteolytic activity of cancer cells, thereby intensifying the invasive/metastatic potential. In fact, a relatively strong correlation was observed between GnT-V overexpression and MT1-MMP upregulation. To our best knowledge, this is the first observation that a certain enzyme is associated with transcriptional regulation of MT1-MMP in cancer.

GnT-V is a glycosyltransferase, and it has been known to promote cancer progression by inducing an aberrant glycosylation of several substrate glycoproteins. Previously, we reported that TIMP-1 is aberrantly glycosylated by GnT-V, and the aberrant TIMP-1 substantially loses the inhibitory capacity for gelatinases [19]. In addition to TIMP-1, PTPk [25], matriptase [18], and β_1 integrin [26] are also included in the list of substrates for GnT-V that is

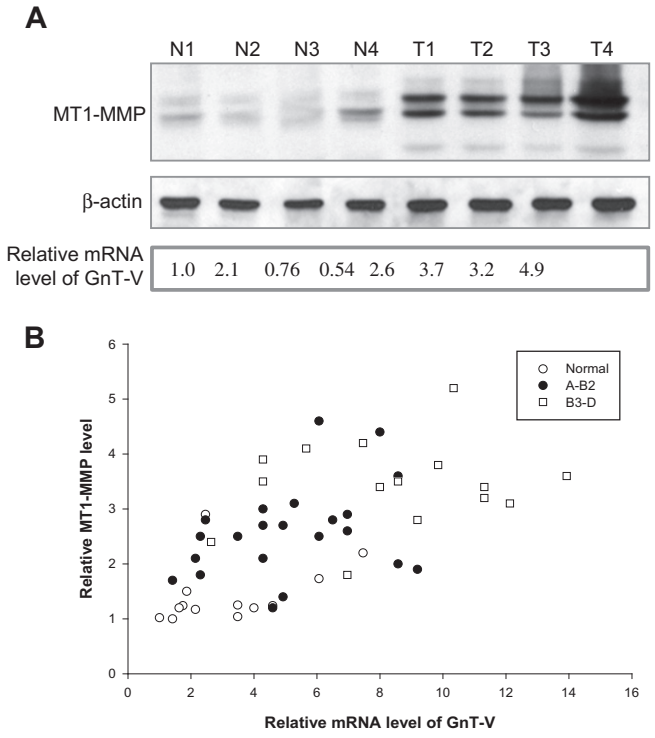

Fig. 4. Synchronous overexpressions of GnT-V and MT1-MMP in cancer tissues (A) Expression of MT1-MMP and mRNA levels of GnT-V in cancer tissues and their normal counterparts. Relative mRNA level of GnT-V was derived from the cycle threshold (Ct) value subtracted from that for β-actin in the quantitative RT-PCR data. (B) Correlation of the levels of GnT-V and MT1-MMP expression in cancer tissues. The expression levels were quantified from immunoblot analysis and qRT-PCR data of 50 tissue pairs, in which cancer tissues were classified into a lower Astler–Coller grade (A-B2) and a higher grade (B3-D). A relatively strong correlation between MT1-MMP upregulation and GnT-V overexpression was observed with the Pearson's correlation coefficient being 0.618.

implicated in tumor progression. Besides the substrate-targeted mechanism, GnT-V appears to direct tumor progression by a more indirect mechanism by which the glycosyltransferase may act as a switch for a certain molecular signaling. This notion is thought to be best exemplified by a previous report [27]; the galectin/glycoprotein lattice is maintained by GnT-V, and it acts dominantly to protect the epithelial growth factor receptor (EGFR) from negative regulation and immobilization through interaction with oligomerized Cav1. As a result, the GnT-V-involved lattice forms a favorable environment for EGFR signaling. Because a treatment with EGF to MDA-MB-231 cells did not affect the MT1-MMP expression (data not shown), GnT-V appears to utilize another signaling pathway to trigger the transcriptional control for MT1-MMP. Accordingly, future study should investigate which signaling pathway is involved and which is a key regulatory molecule in the GnT-V-triggered transcriptional regulation of MT1-MMP.

Acknowledgments

This work was supported by the 'Convergence Research Center Program (2011K000891)' of the Ministry of Education, Science and Technology and Grants from the KRIBB Research Initiative Program (KGM3231211).

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