Analysis of the correlation between alterations in N-glycans and invasiveness in liver cancer cell lines

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Abstract. The N-glycoforms of glycoproteins modify protein function and control a number of biological pathways. The aim of the present study was to investigate the correlation between alterations in N-glycans and cancer aggressiveness in terms of cancer cell invasion ability. The expression of urokinase-type plasminogen activator (uPA) and N-acetylglucosaminyltr ansferase V (GnT-V) in liver cancer cell lines was analyzed by western blotting. Cell invasiveness was analyzed by Matrigel invasion assays. uPA and GnT-V expression in liver cancer cell lines was knocked down by RNA interference. Furthermore, uPA was overexpressed in liver cancer cells using lentiviral vectors, and a mutant strain of HepG2 cells overexpressing uPA deficient in N-glycans was established. A glycoblotting-assisted matrix-assisted laser desorption/ionization-time-of-flight/mass spectrometry-based quantitative analysis of liver cancer cell lines was performed, in which invasiveness was altered by modifying the expression of uPA and GnT-V. N-glycan profiles were found to differ between the highly invasive liver cancer cell line HLE and the less invasive cell line HepG2. The expression of several N-glycans, including a form with m/z=1892, was changed according to invasiveness controlled by knockdown and overexpression of uPA. The invasiveness of HepG2 cells with mutant uPA did not increase regardless of the level of expression of uPA. Following GnT-V knockdown and N-glycan alteration, uPA expression did not change, whereas cell invasiveness decreased. One N-glycan (m/z=1892) was common among N-glycans in the comparative analysis between HLE and HepG2, HLE and uPA knockdown HLE, HepG2 and uPA-overexpressing HepG2, and HLE and GnT-V knockdown HLE cells and among N-glycan profiles in human uPA. Therefore, N-glycosylation is an important factor controlling invasiveness of liver cancer cells, and a specific N-glycan (m/z=1892) associated with the invasion of liver cancer cells via uPA was identified in the present study.

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Introduction

Glycosylation is one of the most common post-translational protein modifications. During the process of glycosylation, sugars are attached to proteins or lipids and cause immense structural and functional variations in the majority of eukaryotic cell proteins (1,2). N-glycans modify proteins and control several biological pathways (3). Alterations in N-glycan profiles have been suggested to play important roles in the proliferation, differentiation, invasion and metastasis of malignant cells (4). Glycan species may be analyzed and characterized using mass spectrometry (MS), and profiling of these molecules when they are secreted or shed from cancer cells is also performed (5,6). A novel glycomics method, SweetBlot, which facilitates high-throughput and large-scale glycome analysis using an automated glycan purification system, has been developed (7). This approach enables quantitative profiling of serum N-glycans. Using this quantitative N-glycomics procedure based on glycoblotting technology, which is both highly accurate and can be conducted on a large scale, specific N-glycan alterations were reported in the sera of 369 patients with liver cancer. These specific alterations were independent risk factors for shorter overall and disease-free survival (8). These alterations were also correlated with invasiveness based on analyses of clinicopathological factors. Specific glycosylation patterns related to the malignancy of human brain tumors at different stages were also reported (9). Altered N-glycan profiles may affect the malignancy of several types of cancer (10-14). Compared with normal cells, cancer cells frequently express N-glycans at different levels and with

fundamentally different structures; therefore, elucidation of these glycosylation patterns may provide a novel approach to cancer therapy.

The invasiveness of cancer cells is a major factor determining malignancy. Urokinase-type plasminogen activator (uPA) and N-acetylglucosaminyltransferase V (GnT-V) are important factors associated with cancer malignancy. uPA is a serine protease that converts plasminogen to plasmin. Through this enzymatic effect, uPA plays an important role in fibrinolysis, which mediates cancer cell invasiveness (15). uPA was found to be significantly upregulated in liver cancer tissues in comparison with normal liver tissues in patients with liver cancer (16). Another study suggested that uPA activity was the most important factor affecting liver cancer invasion in the tissue fibrinolysis system controlled by uPA and a strong predictor of liver cancer recurrence (17). It was also reported that the concentration of uPA is correlated with invasiveness in liver cancer cell lines, and that controlling uPA expression makes it possible to interfere with invasiveness (18). GnT-V is a glycosyltransferase that forms β1,6 branches. β1,6-branching also affects cellular pathways involved in adhesion, motility, angiogenesis and apoptosis. Thus, GnT-V expressed on cancer cells acts as a master regulator of metastasis (19,20) and affects malignancy in several types of cancer (21). GnT-V also affects the expression and activation of matriptase and uPA (22-24).

However, the association between alterations in N-glycans and cancer malignancy in terms of invasiveness is incompletely understood. The aim of the present study was to investigate the correlation between invasiveness and alterations in N-glycans using liver cancer cell lines with modified uPA and GnT-V expression.

Materials and methods

Cell culture. The human liver cancer cell lines HLE and HepG2 were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and they were authenticated by short tandem repeat profiling. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 100 U/ml penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS at 37˚C under 5% CO_2. The cells were harvested using trypsin and pelleted by centrifugation (1,500 x g, 3 min, 4˚C).

Establishment of uPA and GnT-V knockdown HLE cells. Stealth siRNA transduction particles targeting uPA (PLAUHSS108076, forward sequence, 5'-GCCCUCCUC UCCUCCAGAAGAAUUA-3' and reverse, 5'-UAAUUC UUCUGGAGGAGAGGAGGGC-3'; and PLAUHSS108078, forward sequence, 5'-CCAUCCCGGACUAUACAGACCAUC U-3' and reverse, 5'-AGAUGGUCUGUAUAGUCCGGGAUG G-3') and GnT-V (MGAT5HSS106510, forward sequence, 5'-GAAAGCGGAAGAAAGUCCUCGUUCA-3' and reverse, 5'-UGAACGAGGACUUUCUUCCGCUUUC-3'; and MGAT5HSS181096, forward sequence, 5'-CGCUGGAGU CAUGACAGCUUAUGAU-3' and reverse, 5'-AUCAUAAGC UGUCAUGACUCCAGCG-3') and negative control (Stealth™ RNAi Negative Control Duplex) were purchased from Thermo Fisher Scientific, Inc. siRNAs were transfected into HLE cells with Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Approximately $30x10^4$ cells in 10% FBS-supplemented medium were seeded in a 10-cm dish at 37˚C under 5% CO_2. Following incubation for 24 h, stealth siRNA (10 nM) was added and cells were transfected for 48 h.

Establishment of uPA-overexpressing cells. Whole uPA primer, a primer for amplifying human uPA (forward, 5'-TCG ACTCGAGATGAGAGCCCTGCTGGC-3', reverse, 5'-TCG ACTCGAGTCAGAGGGCCAGGCCATTC-3', Thermo Fisher Scientific, Inc.), was designed based on the uPA gene sequence (1,296 bp). The cDNA of uPA was amplified by reverse transcription-PCR (RT-PCR) with the cDNA of HLE cells as template, whole uPA primer and QIAquick Spin (Qiagen GmbH). The cDNA of uPA was cloned into the pLVSIN-IRES-ZsGreen1 vector (Takara Bio, Inc.) according to the manufacturer's instructions. Lentiviral supernatants were produced using the Lenti-X 293T Packaging System (Takara Bio, Inc.) and used for the transduction of HepG2 cells. HepG2 cells were transduced with supernatants containing the empty vector for the negative control. The insert was checked by sequencing with a Big Dye Termination Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Fluorescence-activated cell sorting was performed twice to select stable clones.

Establishment of uPA-mutant HepG2 cells. uPA has a glycosylation site including asparagine at the 302nd amino acid residue. This asparagine was replaced with glutamine by a substitution point mutation with uPA mutation primer (5'-CCGGATAGA GATAGTCGGTAGATTGCTCTTTTCC-3', Thermo Fisher Scientific, Inc.) and QIAprep Spin Mini Kit (Qiagen GmbH). The mutant plasmid was transfected into HepG2 cells, which resulted in the production of uPA without N-glycan modification, as the N-glycan is bound to the amide nitrogen atom on the side chain of asparagine.

Western blot analysis. Cellular proteins were extracted using the Subcellular Protein Fractionation Kit for Cultured Cells (Pierce; Thermo Fisher Scientific, Inc.). Samples were mixed with SDS sample buffer (Bio-Rad Laboratories, Inc.), boiled at 95˚C for 5 min, separated by SDS-PAGE, and transferred to PVDF membranes (GE Healthcare). The membranes were immunoblotted using primary antibodies against uPA (goat monoclonal anti-human, cat. no. sc-19088, diluted 1:750; Santa Cruz Biotechnology, Inc.), GnT-V [rabbit polyclonal anti-human, cat. no. EP6274 (2), diluted 1:200; Abcam] and GAPDH (rabbit monoclonal anti-human, cat. no. D16H11, diluted 1:2,000; Cell Signaling Technology, Inc.). The antibodies were diluted with 1X Tris-buffered saline with 0.1% Tween-20. The secondary antibodies were diluted 1:5,000 for uPA and GAPDH and 1:2,000 for GnT-V.

Cell invasion assay. Invasion assays were performed in 24-well Matrigel invasion chambers (Corning, Inc.) according to the manufacturer's instructions. Medium containing 10% FBS was added to the lower chambers. Approximately $3x10^4$ HLE cells or $15x10^4$ HepG2 cells in FBS-free medium were seeded in the upper chambers. After 48 h, cells on the upper chamber were removed with a cotton swab. The membranes

were fixed with 99% methanol for 2 min and stained with staining solutions I and II for 2 min each at room temperature using the Diff-Quik staining kit (Sysmex Corporation), and the cells were counted at a magnification of x20 using a fluorescence microscope (Keyence Corporation).

Extraction of glycoproteins. For N-glycan analysis, glycoproteins were extracted as described previously (25). Briefly, cell pellets consisting of $1x10^6$ cells were homogenized using an Ultrasonic Homogenizer (Taitec Corporation) in 100 mM Tris-acetate buffer (100 μl; pH 7.4) supplemented with 2% SDS as a surfactant for the complete dissolution of cell pellets. Reductive alkylation of the cellular proteins was performed by the addition of 500 mM tris(2-carboxyethyl)phosphine (Sigma-Aldrich; Merck KGaA) at room temperature for 60 min, followed by the addition of 200 mM iodoacetamide (Sigma-Aldrich; Merck KGaA) at room temperature for 30 min. After reductive alkylation, ethanol precipitation was carried out by adding a 4-fold volume of cold ethanol and incubating for 3 h at -30˚C. Supernatants and precipitated proteins were separated by centrifugation at 20,000 x g for 10 min at 4˚C, and the precipitates were again washed with cold ethanol. Collected precipitates containing glycoproteins/N-glycans were dried at 37˚C for 10 min.

For urokinase from human urine (P.N.: U0633-25UG, Lot: SLBN9831V, Sigma-Aldrich; Merck KGaA), the sample was dissolved in 100 mM solution of ammonium bicarbonate/0.1% Triton X-100/1% SDS containing 120 mM 1,4-dithiothreitol (10 μl). After incubation at 60˚C for 30 min, 123 mM iodoacetamide (10 μl) was added to the mixture, followed by incubation at room temperature to enable reductive alkylation. After 60 min, the mixture was treated with 10 μl trypsin (Sigma-Aldrich; Merck KGaA) at 37˚C overnight, followed by heat inactivation of the enzyme at 90˚C for 10 min. After cooling to room temperature, the mixture was treated with 10 μl trypsin (Sigma-Aldrich; Merck KGaA) at 37˚C overnight.

Pre-treatment of uPA. For urokinase from human urine (P.N.: U0633-25UG, Lot: SLBN9831V, Sigma-Aldrich; Merck KGaA), the sample was dissolved in 100 mM solution of ammonium bicarbonate/0.1% Triton X-100/1% SDS containing 120 mM 1,4-dithiothreitol (10 μl). After incubation at 60˚C for 30 min, 123 mM iodoacetamide (10 μl) was added to the mixture, followed by incubation at room temperature to enable reductive alkylation. After 60 min, the mixture was treated with 5 μG of trypsin (FUJIFILM Wako Pure Chemical Corporation) at 37˚C overnight, followed by heat inactivation of the enzyme at 90˚C for 10 min. After cooling to room temperature, the mixture was treated with 100 U of PNGase F from *Flavobacterium meningosepticum* (New England BioLabs Japan, Inc.) at 37˚C overnight. Then, the sample mixture was dried up by SpeedVac (Thermo Fisher Scientific, Inc.).

Glycoblotting analysis

Experimental procedures: Cell culture N-glycomics based on glycoblotting. N-glycans from samples of cell culture and urokinase from human urine were purified by glycoblotting using BlotGlyco H, which are commercially available synthetic polymer beads with high-density hydrazide groups (Sumitomo Bakelite Co., Ltd.). All procedures used the SweetBlot automated glycan purification system containing a 96-well plate platform (System Instruments Co., Ltd.).

N-glycan purification and modification by glycoblotting. Glycoblotting of sample mixtures containing urokinase from human urine was performed in accordance with previously described procedures. Commercially available BlotGlyco H beads (250 μl; 10 mg/ml suspension; Sumitomo Bakelite Co., Ltd.) were aliquoted into the wells of a MultiScreen Solvinert hydrophilic polytetrafluoroethylene 96-well filter plate (EMD Millipore). After removal of water using a vacuum pump, PNGase F-digested samples were applied to the wells, followed by the addition of 20 μl of internal standard (Sialylated glycan 2.5 μM) and 180 μl of 2% acetic acid in acetonitrile. The filter plate was then incubated at 80˚C for 45 min to capture the N-glycans onto the beads by chemically stable and reversible hydrazine bonds. The beads were then washed using 200 μl of 2M guanidine-HCl in 10 mM ammonium bicarbonate, followed by washing with the same volume of water and 1% triethylamine in methanol. Each washing step was performed twice. The N-glycan-linked beads were then incubated with 10% acetic anhydride in methanol for 30 min at room temperature so that unreacted hydrazide groups would become capped by acetylation. After capping, the reaction solution was removed under vacuum, and the beads were serially washed with 200 μl of 10 mM HCl, methanol and dioxane (this is a pretreatment for sialic acid modification). On-bead methyl esterification of carboxyl groups in sialic acid was carried out with 100 μl of 20 mM 3-methyl-1-p-tolyltriazene (Tokyo Chemical Industry Co., Ltd.) in dioxane at 60˚C for 90 min to dryness. After methyl esterification of the more stable glycans, the beads were serially washed in 200 μl of dioxane, water, methanol and water. The captured glycans were then subjected to a transiminization reaction with 20 μl of aminooxy-functionalized peptide reagent (aoWR; 20 mM in Milli Q; Sumitomo Bakelite Co., Ltd.) in 180 μl 2% AcOH/CAN solution for 45 min at 80˚C. After this reaction, WR-tagged glycan was eluted in 100 μl of water and purified by Mass PREPTM HILIC μEluent Plate (Waters Corporation); the recovered sample was dried up under vacuum.

Matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) MS analysis. The N-glycans purified by glycoblotting were diluted with 5 μl of 1% AcOH in 5% ACN/water. A total of 1 μl of sample was mixed with 1 μl of 2,5-dihydroxy benzoic acid (FUJIFILM Wako Pure Chemical Corporation) as ionic liquid matrices, and spotted onto a MALDI target plate. The analytes were then subjected to MALDI-TOF MS analysis using an Ultraflex time-of-flight mass spectrometer III (Bruker Daltonics, Inc.) in a reflector, using the positive-ion mode and typically accumulating 2,000 shots. The N-glycan peaks in the MALDI-TOF MS spectra were selected using FlexAnalysis v. 3 (Bruker Daltonics, Inc.). The glycan structures were estimated using the GlycoMod Tool (http://br.expasy.org/tools/glycomod/).

Statistical analysis. One-way ANOVA with Tukey's post hoc test was performed for experiments that involved morethan two groups, and Student's t-test was performed for comparisons

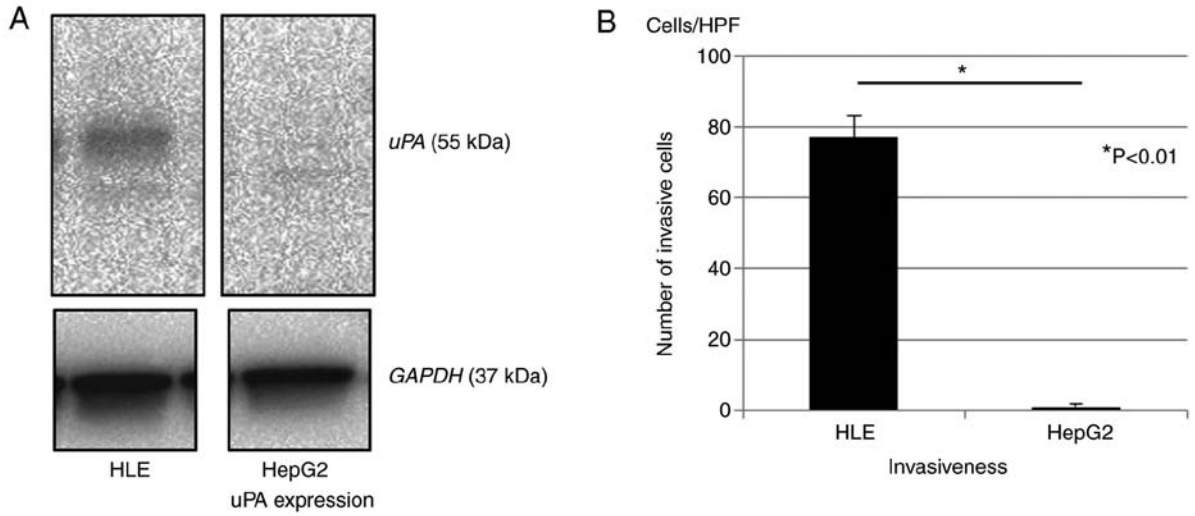


Figure 1. Expression of uPA and invasiveness. (A) uPA expression was detected in HLE cells, but not in HepG2 cells, by western blot analysis. uPA and GAPDH were exposed for 40 and 10 sec, respectively. (B) Cell invasion assay showed that HLE cells exhibited higher invasiveness than HepG2 cells. uPA, urokinase-type plasminogen activator. Experiment was repeated three times and the statistical significance is denoted by *P<0.05.

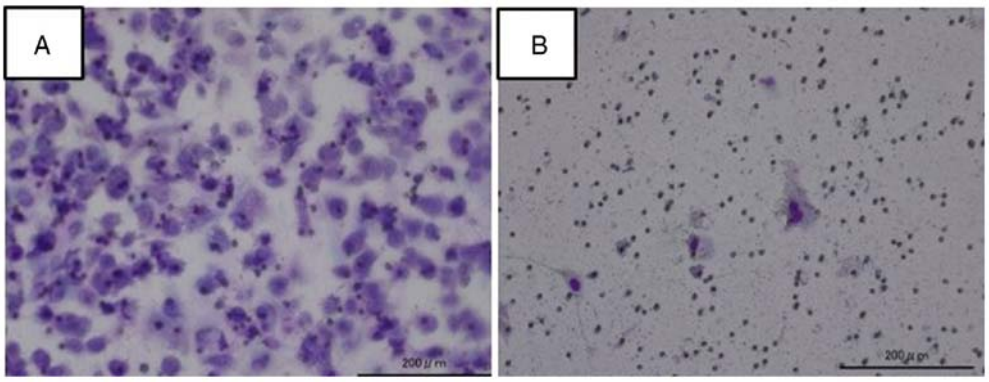


Figure 2. Invasion assay of (A) HLE and (B) HepG2 cells.

between two groups. Statistical analyses were performed using JMP Pro 12.0.1 for Windows (SAS Institute, Inc.). P<0.05 was considered to indicate statistically significant differences.

Results

uPA expression, invasiveness and glycoblotting analysis of HLE and HepG2 cells. Cell invasion assays revealed that HLE cells exhibited higher invasiveness compared with HepG2 cells (Figs. 1B and 2). Western blot analysis was performed to examine the expression of uPA in liver cancer cell lines. The expression of uPA in the highly invasive HLE cell line was higher compared with that in the less invasive HepG2 cell line (Fig. 1A). Glycoblotting analysis was performed in HLE and HepG2 cells to examine the correlation between invasiveness and N-glycan expression. In this assay, 75 N-glycans were identified, and the concentrations of 10 N-glycans (m/z=1851, 1892, 2029, 2054, 2070, 2216, 2521, 2887, 3192 and 3252) were higher in HLE compared with HepG2 cells (Fig. 3).

uPA expression, invasiveness and glycoblotting analysis of uPA knockdown HLE cells. The association between uPA expression and invasiveness was examined. uPA knockdown HLE cell lines were established using two specific siRNAs against uPA (HLE uPA siRNA1 and HLE uPA siRNA2) and a control HLE cell line (HLE control). Western blot analysis demonstrated that the expression of uPA was decreased in HLE uPA siRNA1 and HLE uPA siRNA2 cells compared with that in HLE control cells (Fig. 4A). Matrigel invasion assays demonstrated that the invasiveness of HLE uPA siRNA1 and HLE uPA siRNA2 cells was significantly lower compared with that of HLE control cells (P<0.01; Figs. 4B and 5). By glycoblotting analysis, 86 N-glycans were identified in the uPA knockdown HLE and HLE control cells. The concentrations of 14 N-glycans (m/z=1502, 1664, 1826, 1988, 2150, 2312, 2474, 1810, 1542, 1689, 1705, 1851, 1892 and 2095) decreased by >20% in HLE uPA siRNA1 and HLE uPA siRNA2 cells compared with those in HLE control cells (Fig. 6).

uPA expression, invasiveness and glycoblotting analysis of uPA-overexpressing HepG2 cells. A HepG2-uPA cell line (HepG2uPAWT) that overexpressed uPA was constructed using a lentivirus vector and a HepG2 control cell line (HepG2control) using an empty vector. A HepG2-uPA cell line (HepG2uPAMUT) that overexpressed a mutant uPA protein and had glutamine as the 302nd amino acid residue

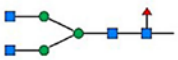
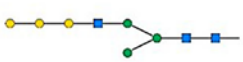
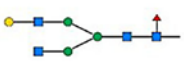
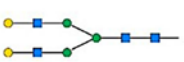
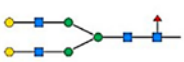
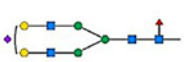
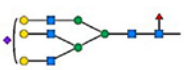
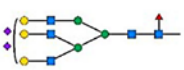
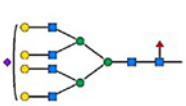
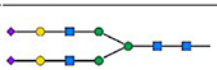
	m/z	Glycan structure	Presumptive composition	HepG2 (pmol)	HLE (pmol)
Hybrid /Complex	1851		(Hex)1 (HexNAc)1 (Deoxyhexose)1 +(Man)3 (GlcNAc)2	0.190	0.259
	1892		(HexNAc)2 (Deoxyhexose)1 +(Man)3 (GlcNAc)2	0.315	0.723
	2029		(Hex)3 (HexNAc)1+(Man)3 (GlcNAc)2	0.074	0.098
	2054		(Hex)1 (HexNAc)2 (Deoxyhexose)1 +(Man)3 (GlcNAc)2	0.019	0.028
	2070		(Hex)2 (HexNAc)2+(Man)3 (GlcNAc)2	0.021	0.540
	2216		(Hex)2 (HexNAc)2 (Deoxyhexose)1 +(Man)3 (GlcNAc)2	0.051	2.798
	2521		(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	1.815	3.276
	2887		(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	0.135	0.204
	3192		(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 +(Man)3 (GlcNAc)2	0.015	0.046
	3252		(Hex)4 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	0.018	0.023
Internal standard	2478		(Hex)2 (HexNAc)2 (NeuAcAm)2 +(Man)3 (GlcNAc)2	10.000	10.000

Figure 3. The concentration of 10 N-glycans was higher in HLE compared with HepG2 cells. Hex, hexose; HexNAc, N-acetylhexosamine; Man, mannose; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid.

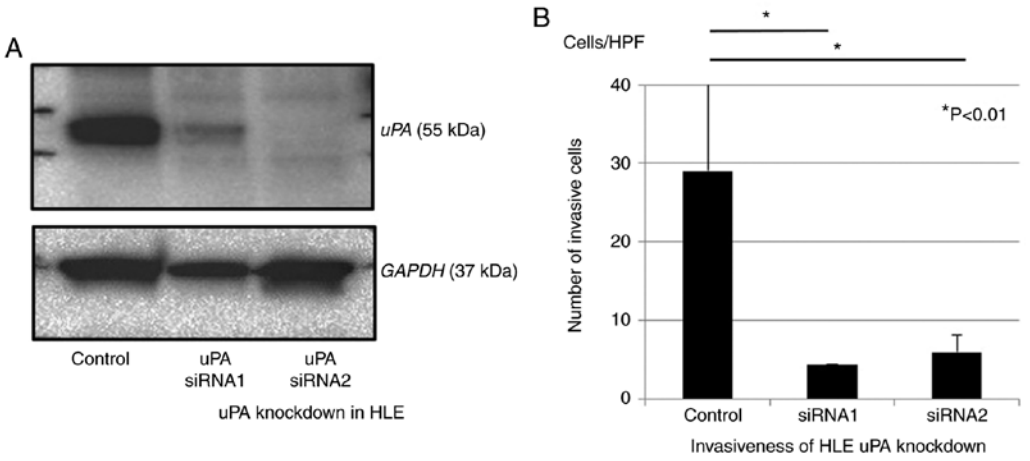


Figure 4. uPA knockdown and HLE cell invasiveness. (A) Two siRNAs against uPA effectively downregulated uPA protein levels in HLE cells as detected by western blot analysis. uPA and GAPDH were exposed for 60 and 6 sec, respectively. (B) Knockdown of uPA in HLE cells inhibited invasion. uPA, urokinase-type plasminogen activator; HPF, high-power field. Each experiment was repeated three times and the statistical significance is denoted by $^{*}P<0.05$.

instead of asparagine to prevent N-glycosylation was also constructed. Western blot analysis demonstrated that the expression of uPA was increased in HepG2uPAWT and HepG2uPAMUT cells compared to that in HepG2control cells (Fig. 7A). HepG2uPAWT cells expressed higher levels of uPA compared with HepG2uPAMUT cells. The invasiveness of HepG2uPAWT cells was significantly increased compared with that of HepG2control cells

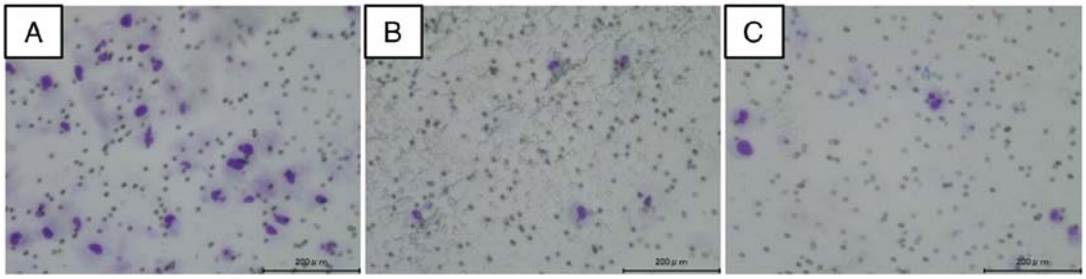


Figure 5. Invasion assay of HLE cells with uPA knockdown. (A) HLE control. (B) HLE uPA siRNA1. (C) HLE uPA siRNA2. uPA, urokinase-type plasminogen activator.

	m/z	Glycan structure	Presumptive composition	Control (pmol)	u-PA siRNA1 (pmol)	u-PA siRNAi2 (pmol)
High-Man	1502		(Hex)1+(Man)3 (GlcNAc)2	1.852	1.333	1.340
	1664		(Hex)2+(Man)3 (GlcNAc)2	19.517	11.834	8.318
	1826		(Hex)3+(Man)3 (GlcNAc)2	77.666	49.953	39.162
	1988		(Hex)4+(Man)3 (GlcNAc)2	86.449	69.903	49.005
	2150		(Hex)5+(Man)3 (GlcNAc)2	177.160	142.312	104.646
	2312		(Hex)6+(Man)3 (GlcNAc)2	106.915	67.117	67.992
	2474		(Hex)7+(Man)3 (GlcNAc)2	3.885	2.697	2.532
	1810		(Hex)2 (Deoxyhexose)1 +(Man)3 (GlcNAc)2	0.101	0.068	0.032
Hybrid /Compex	1543		(HexNAc)1+(Man)3 (GlcNAc)2	0.351	0.271	0.160
	1689		(HexNAc)1 (Deoxyhexose)1 +(Man)3 (GlcNAc)2	4.476	3.885	1.966
	1705		(Hex)1 (HexNAc)1+(Man)3 (GlcNAc)2	0.836	0.647	0.381
	1851		(Hex)1 (HexNAc)1 (Deoxyhexose)1 +(Man)3 (GlcNAc)2	5.584	4.116	2.590
	1892		(HexNAc)2 (Deoxyhexose)1 +(Man)3 (GlcNAc)2	4.445	2.467	0.706
	2095		(HexNAc)3 (Deoxyhexose)1 +(Man)3 (GlcNAc)2	0.586	0.298	0.118
Internal standard	2478		(Hex)2 (HexNAc)2 (NeuAcAm)2 +(Man)3 (GlcNAc)2	20.000	20.000	20.000

Figure 6. Decreased N-glycans in HLE cells were correlated with changes in invasiveness. Hex, hexose; HexNAc, N-acetylhexosamine; Man, mannose; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid.

(P<0.01), whereas the invasiveness of HepG2uPAMUT cells deficient in N-glycans did not increase (Figs. 7B and 8). By glycoblotting analysis, 72 N-glycans were identified in the uPA-overexpressing HepG2 and HepG2control cells. The concentrations of 22 N-glycans (m/z=1502, 2312, 2474, 1543, 1689, 1705, 1851, 1982, 2010, 2095, 2156, 2172, 2334, 2375, 2512, 2668, 2681, 2741, 2827, 2887, 3192 and 3497) increased by >20% in HepG2uPAWT cells compared with

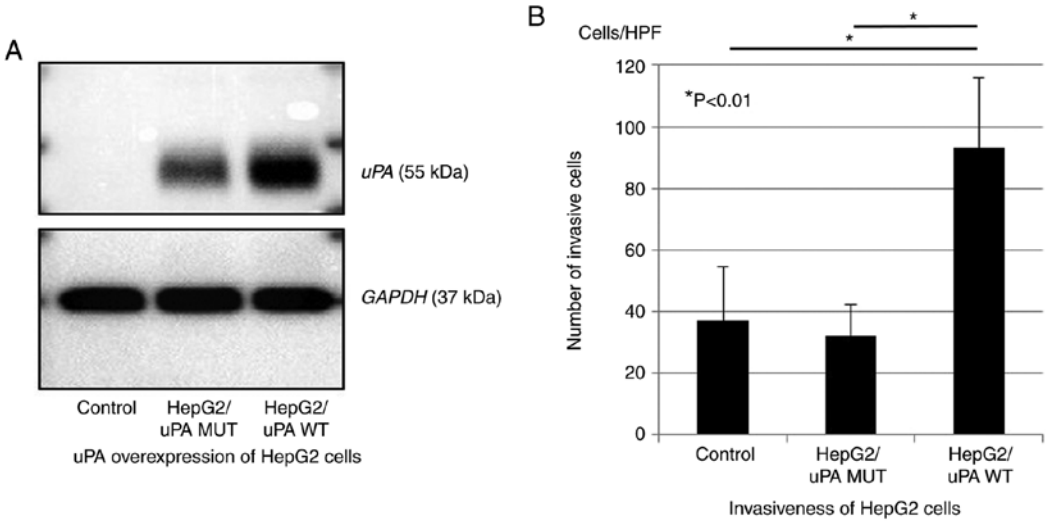


Figure 7. uPA overexpression and HepG2 cell invasiveness. (A) uPA expression increased in HepG2uPAWT cells, which overexpressed uPA using lentiviral vectors, and HepG2uPAMUT cells. uPA and GAPDH were exposed for 30 and 20 sec, respectively. (B) The invasion of HepG2uPAWT cells, which overexpressed mutant uPA proteins and had glutamine as the 322nd amino-acid residue instead of asparagine, was increased, while that of HepG2uPAMUT cells was not increased. uPA, urokinase-type plasminogen activator; WT, wild-type; MUT, mutant; HPF, high-power field. Each experiment was repeated three times and the statistical significance is denoted by $^{*}P<0.05$.

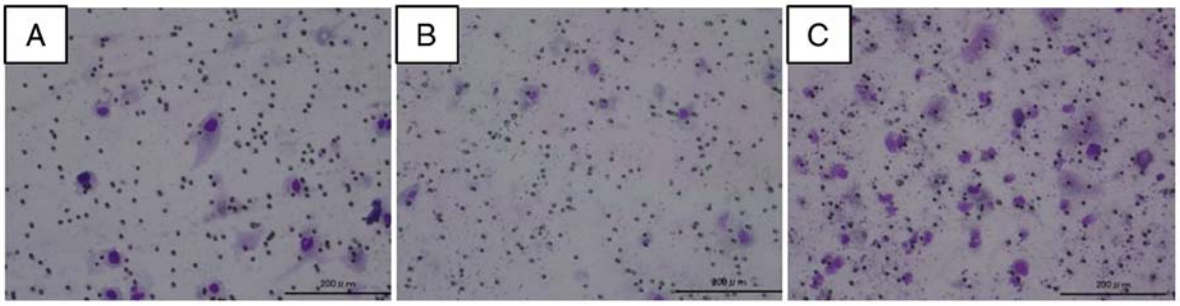


Figure 8. Invasion assay of HepG2 cells with uPA overexpression. (A) HepG2 control. (B) HepG2 uPA/MUT. (C) HepG2 uPA/WT. uPA, urokinase-type plasminogen activator; WT, wild-type; MUT, mutant.

those in HepG2control cells (Fig. 9). These 22 N-glycans were also increased in HepG2uPAMUT cells compared with those in HepG2control cells.

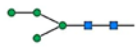
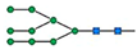
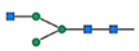
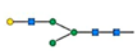
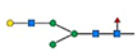
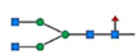
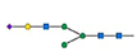
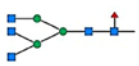
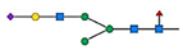
uPA expression, invasiveness and glycoblotting analysis of GnT-V knockdown HLE cells. The association between N-glycan alterations and invasiveness was next examined. GnT-V knockdown HLE cell lines were established using specific siRNAs against GnT-V (HLE GnT-V KD) and a control HLE cell line (HLE control). GnT-V knockdown was verified by western blot analysis and it was observed that uPA expression did not change in HLE GnT-V KD and HLE control cells (Fig. 10A). Matrigel invasion assays demonstrated that the knockdown of GnT-V significantly reduced invasiveness in HLE cells ($P<0.05$), despite the lack of change in uPA expression (Figs. 10B and 11).

By glycoblotting analysis, 97 N-glycans were identified in GnT-V knockdown HLE and HLE control cells. The concentrations of 18 N-glycans (m/z=1892, 2403, 2563, 2681, 2827, 2887, 2928, 3030, 3192, 3351, 3395, 3411, 3497, 3557, 3656, 3716, 3862 and 3922) decreased by >20% in HLE GnT-V KD cells compared with those in HLE control cells (Fig. 12).

N-glycan profiles in human uPA. A total of 16 N-glycans, 1746, 1892, 1908, 2038, 2054, 2070, 2095, 2152, 2172, 2299, 2445, 2459, 2501, 2563, 2604 and 2705, were identified by glycoblotting analysis (Figs. 13 and 14).

Comparing N-glycans in the analysis of HLE/HepG2, uPA knockdown HLE, uPA-overexpressing HepG2, GnT-V knockdown HLE cells and N-glycan profiles in human uPA. By comparing N-glycans in the analysis of HLE/HepG2 cells, 75 N-glycans were identified, and the concentrations of 10 N-glycans were higher in HLE cells compared with those in HepG2 cells. The concentrations of 13 N-glycans decreased by >20% in HLE uPA siRNA1 and HLE uPA siRNA2 cells compared with those in control HLE cells. The concentrations of 22 N-glycans increased by >20% in HepG2uPAWT cells compared with those in HepG2control cells. These 22 N-glycans were also increased in HepG2uPAMUT cells. The concentrations of 18 N-glycans decreased by >20% in HLE GnT-V KD cells compared with those in HLE control cells. A total of 16 N-glycans were identified by glycoblotting analysis of human uPA. One common N-glycan (m/z=1892) was correlated with invasiveness based on glycoblotting analysis of HLE/HepG2, uPA knockdown HLE, uPA-overexpressing HepG2 and GnT-V knockdown HLE cells.

A

	m/z	Glycan structure	Presumptive composition	Control (pmol)	HepG2 uPAMUT (pmol)	HepG2 uPAWT (pmol)
High-Man	1502		(Hex)1+(Man)3 (GlcNAc)2	11.518	15.581	13.951
	2312		(Hex)6+(Man)3 (GlcNAc)2	102.799	120.531	118.537
	2474		(Hex)7+(Man)3 (GlcNAc)2	5.957	12.364	10.266
Complex /Hybrid	1543		(HexNAc)1+(Man)3 (GlcNAc)2	0.843	1.223	1.217
	1689		(HexNAc)1 (Deoxyhexose)1+(Man)3 (GlcNAc)2	2.169	2.879	2.871
	1705		(Hex)1 (HexNAc)1+(Man)3 (GlcNAc)2	0.340	0.598	0.521
	1851		(Hex)1 (HexNAc)1(Deoxyhexose)1 +(Man)3 (GlcNAc)2	0.563	0.878	0.672
	1892		(HexNAc)2 (Deoxyhexose)1+(Man)3 (GlcNAc)2	1.857	2.834	2.447
	2010		(Hex)1 (HexNAc)1 (NeuAc)1+(Man)3 (GlcNAc)2	1.155	1.590	1.636
	2095		(HexNAc)3 (Deoxyhexose)1+(Man)3 (GlcNAc)2	0.566	0.902	0.668
	2156		(Hex)1 (HexNAc)1 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	1.492	2.598	1.910
Internal standard	2478		(Hex)2 (HexNAc)2 (NeuAcAm)2 +(Man)3 (GlcNAc)2	20.000	20.000	20.000

B

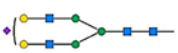
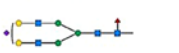
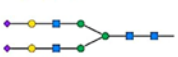
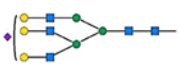
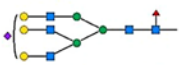
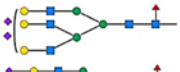
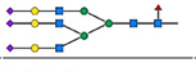
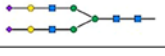
	m/z	Glycan structure	Presumptive composition	Control (pmol)	HepG2 uPAMUT (pmol)	HepG2 uPAWT (pmol)
	2172		(Hex)2 (HexNAc)1 (NeuAc)1+(Man)3 (GlcNAc)2	6.862	10.112	9.320
	2334		(Hex)3 (HexNAc)1 (NeuAc)1+(Man)3 (GlcNAc)2	7.156	10.703	10.358
	2375		(Hex)2 (HexNAc)2 (NeuAc)1+(Man)3 (GlcNAc)2	2.735	5.087	5.271
	2521		(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	2.905	4.702	5.064
	2668		(Hex)2 (HexNAc)2 (Deoxyhexose)2 (NeuAc)1 +(Man)3 (GlcNAc)2	0.255	0.781	0.463
	2681		(Hex)2 (HexNAc)2 (NeuAc)2+(Man)3 (GlcNAc)2	6.859	11.583	15.713
	2741		(Hex)3 (HexNAc)3 (NeuAc)1+(Man)3 (GlcNAc)2	0.105	0.201	0.216
	2827		(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)2 +(Man)3 (GlcNAc)2	12.814	18.095	25.088
	2887		(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	0.192	0.428	0.508
	3192		(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 +(Man)3 (GlcNAc)2	0.046	0.110	0.161
	3497		(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)3 +(Man)3 (GlcNAc)2	0.035	0.092	0.181
Internal standard	2478		(Hex)2 (HexNAc)2 (NeuAcAm)2 +(Man)3 (GlcNAc)2	20.000	20.000	20.000

Figure 9. Change of concentration of N-glycans in HepG2 cells with uPA overexpression. Hex, hexose; HexNAc, N-acetylhexosamine; Man, mannose; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid. uPA, urokinase-type plasminogen activator. (A) Concentrations of N-glycans (m/z=1502-2156). (B) Concentrations of N-glycans (m/z=2172-3497).

Discussion

To investigate the variations in the N-glycome profile according to the invasiveness of liver cancer cell lines, a glycoblotting-assisted MALDI-TOF/MS-based quantitative analysis was performed. N-glycan profiles were different between the highly invasive liver cancer cell line HLE and the less invasive HepG2 cell line. The expression of several N-glycans was changed according

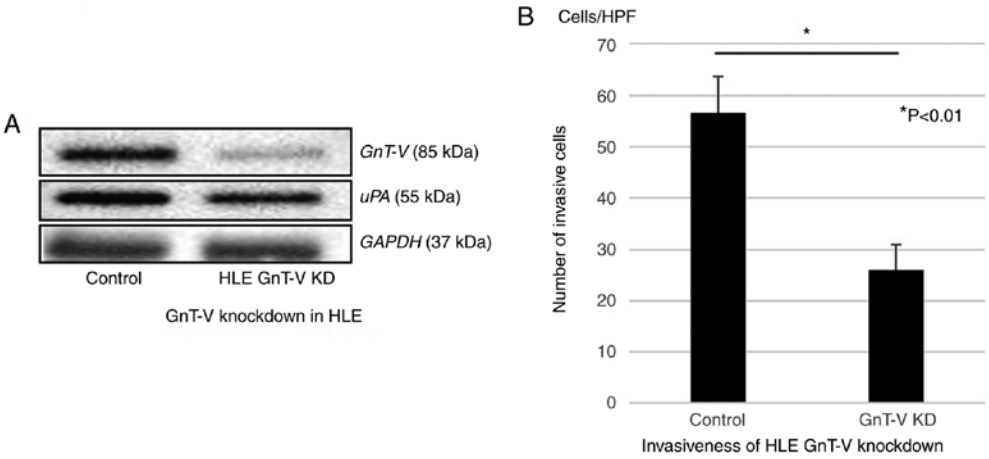


Figure 10. GnT-V knockdown and HLE cell invasiveness. (A) siRNAs against GnT-V effectively downregulated GnT-V protein levels in HLE cells as detected by western blot analysis. uPA expression did not decrease with GnT-V knockdown. GnT-V, uPA and GAPDH were exposed for 60, 60 and 20 sec, respectively. (B) Knockdown of GnT-V in HLE cells inhibited invasion without decreasing uPA expression. uPA, urokinase-type plasminogen activator; GnT-V, N-acetylglucosaminyltransferase V; HPF, high-power field. Experiment was repeated three times and the statistical significance is denoted by *P<0.05.

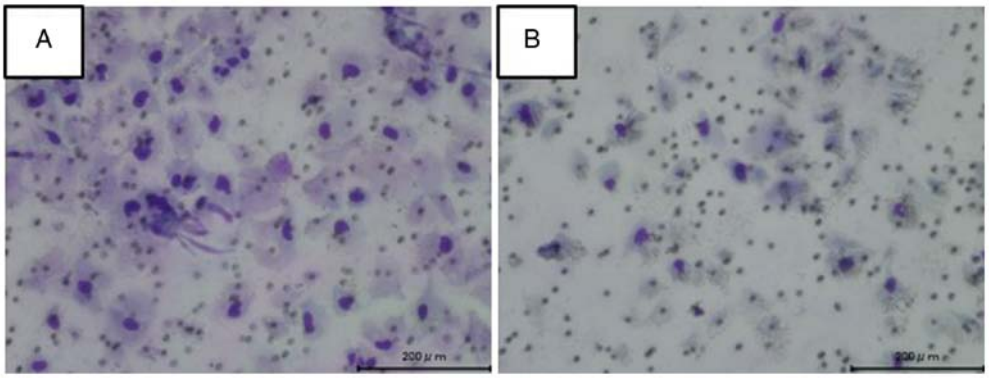


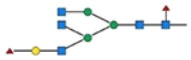
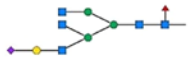
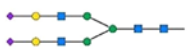
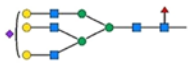
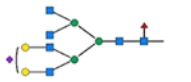
Figure 11. Invasion assay of HLE GnT-V knockdown. (A) HLE control. (B) HLE GnT-V knockdown. GnT-V, N-acetylglucosaminyltransferase V.

to cell invasiveness controlled by the knockdown and overexpression of uPA. The invasiveness of HepG2uPAMUT cells deficient in N-glycans did not increase regardless of the level of uPA expression. After GnT-V knockdown and N-glycan alterations, uPA expression did not change, whereas cell invasiveness decreased. Of note, one N-glycan (m/z=1892), was common in the comparative analysis among liver cancer cell lines with different invasiveness, and among N-glycan profiles in human uPA. In liver cancer cells, N-glycosylation was one of the important factors controlling invasiveness, and a specific N-glycan (m/z=1892) that was associated with the invasiveness of liver cancer cells via uPA was identified in the present study.

uPA is a mosaic glycoprotein composed of a COOH-terminal chymotrypsin-like serine protease domain and a modular non-catalytic NH_2-terminal region that mediates the specific cofactor activities of these proteins, exemplified by the cellular binding of uPA (26). The modular composition of the amino-terminal fragment of uPA includes an epidermal growth factor-like module and a kringle module, with the former being responsible for the specific cell binding of uPA (27). It has been reported that uPA was also correlated with invasion and growth in liver cancer cell lines (18). In the present study, the expression level of uPA in the highly invasive liver cancer cell line HLE was higher compared with that in the less invasive cell line HepG2, and the N-glycan profiles were different between these two cell lines. The invasiveness of uPA knockdown HLE cells decreased, and N-glycan profiles were different between HLE and uPA knockdown HLE cells. The invasiveness of uPA-overexpressing HepG2 cells increased, and the N-glycan profiles were also different between HepG2 and uPA-overexpressing HepG2 cells. These data demonstrated that the differences in invasiveness among different liver cancer cell lines may be attributed to the glycosylation changes. Moreover, a specific N-glycan (m/z=1892) was common among N-glycans in the comparative analysis between HLE and HepG2, HLE and uPA knockdown HLE, HepG2 and uPA-overexpressing HepG2, and HLE and GnT-V knockdown HLE cells. In addition, the N-glycan profiles of human uPA were evaluated, and they all shared one common N-glycan (m/z=1892). Therefore, N-glycan (m/z=1892) appears to play an important role in cell invasiveness controlled by uPA. These glycomic alterations may prove to be useful for the evaluation of tumor malignancy.

The glycosylation patterns of uPA have been previously analyzed (28,29). Pro-uPA is a glycoprotein containing 411 amino acids with 24 cysteine, 22 arginine and 27 lysine residues (28). N-glycosylation of human uPA occurs at Asn302 in the protease domain (30). The N-glycans of uPA contain Man, Gal, Fuc, GlcNAc and NeuSAc, as well as GalNAc residues (31).

A

	m/z	Glycan structure	Presumptive composition	Control (pmol)	GnTV KD (pmol)
Complex /Hybrid	1892		(HexNAc)2 (Deoxyhexose)1+(Man)3 (GlcNAc)2	14.382	11.697
	2403		(Hex)1 (HexNAc)3 (Deoxyhexose)2 +(Man)3 (GlcNAc)2	0.479	0.360
	2563		(Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	1.112	0.801
	2681		(Hex)2 (HexNAc)2 (NeuAc)2+(Man)3 (GlcNAc)2	13.017	9.958
	2827		(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)2 +(Man)3 (GlcNAc)2	19.330	12.676
	2887		(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	7.590	6.304
	2928		(Hex)2 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	0.338	0.254
	3030		(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 +(Man)3 (GlcNAc)2	0.724	0.521
	3192		(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 +(Man)3 (GlcNAc)2	10.032	6.292
Internal standard	2478		(Hex)2 (HexNAc)2 (NeuAcAm)2 +(Man)3 (GlcNAc)2	20.000	20.000

B

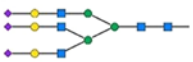
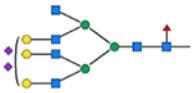
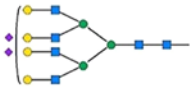
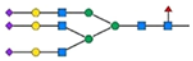
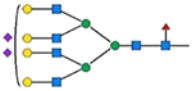
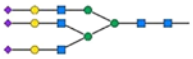
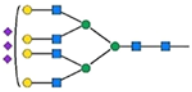
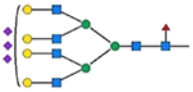
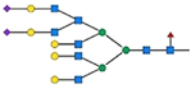
	m/z	Glycan structure	Presumptive composition	Control (pmol)	GnTV KD (pmol)
	3351		(Hex)3 (HexNAc)3 (NeuAc)3+(Man)3 (GlcNAc)2	2.537	2.145
	3395		(Hex)3 (HexNAc)4 (Deoxyhexose)1 (NeuAc)2 +(Man)3 (GlcNAc)2	0.333	0.229
	3411		(Hex)4 (HexNAc)4 (NeuAc)2+(Man)3 (GlcNAc)2	0.524	0.407
	3497		(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)3 +(Man)3 (GlcNAc)2	6.439	3.124
	3557		(Hex)4 (HexNAc)4 (Deoxyhexose)1 (NeuAc)2 +(Man)3 (GlcNAc)2	2.557	1.716
	3656		(Hex)3 (HexNAc)3 (NeuAc)4+(Man)3 (GlcNAc)2	0.434	0.255
	3716		(Hex)4 (HexNAc)4 (NeuAc)3+(Man)3 (GlcNAc)2	0.249	0.155
	3862		(Hex)4 (HexNAc)4 (Deoxyhexose)1 (NeuAc)3 +(Man)3 (GlcNAc)2	1.502	0.928
	3922		(Hex)5 (HexNAc)5 (Deoxyhexose)1 (NeuAc)2 +(Man)3 (GlcNAc)2	0.383	0.255
Internal standard	2478		(Hex)2 (HexNAc)2 (NeuAcAm)2 +(Man)3 (GlcNAc)2	20.000	20.000

Figure 12. Change of concentration of N-glycans in HLE cells with GnT-V knockdown. Hex, hexose; HexNAc, N-acetylhexosamine; Man, mannose; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid; GnT-V, N-acetylglucosaminyltransferase V. (A) Concentrations of N-glycans (m/z=1892-3192). (B) Concentrations of N-glycans (m/z=3351-3922).

In the present study, the overexpressed mutant uPA protein, which had glutamine as the 302nd amino acid residue instead of asparagine and was deficient in N-glycans, altered the invasiveness of liver cancer cells. Therefore, the glycosylation site at asparagine in the 302nd amino acid residue of uPA was found to be an important factor in controlling cancer cell invasiveness, although the glycosylation pattern of uPA does not affect its interaction with plasma proteins directly involved

	m/z	Glycan structure	Presumptive composition	(pmol)
High man	2459		(Hex)6 (Deoxyhexose)1+(Man)3 (GlcNAc)2	1.282
Complex /Hybrid	1746		(HexNAc)2+(Man)3 (GlcNAc)2	1.118
	1892		(HexNAc)2 (Deoxyhexose)1+(Man)3 (GlcNAc)2	8.118
	1908		(Hex)1 (HexNAc)2+(Man)3 (GlcNAc)2	0.696
	2038		(HexNAc)2 (Deoxyhexose)2+(Man)3 (GlcNAc)2	2.330
	2054		(Hex)1 (HexNAc)2 (Deoxyhexose)1 +(Man)3 (GlcNAc)2	2.274
	2070		(Hex)2 (HexNAc)2+(Man)3 (GlcNAc)2	0.682
	2095		(HexNAc)3 (Deoxyhexose)1+(Man)3 (GlcNAc)2	1.815
	2152		(HexNAc)4+(Man)3 (GlcNAc)2	0.702
	2172		(Hex)2 (HexNAc)1 (NeuAc)1+(Man)3 (GlcNAc)2	0.700
	2299		(HexNAc)4 (Deoxyhexose)1+(Man)3 (GlcNAc)2	55.235
	2445		(HexNAc)4 (Deoxyhexose)2+(Man)3 (GlcNAc)2	57.014
	2501		(HexNAc)4 (Deoxyhexose)1 (Sulph)2 +(Man)3 (GlcNAc)2	0.635
	2563		(Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	2.150
	2604		(HexNAc)4 (Deoxyhexose)1 (NeuAc)1 +(Man)3 (GlcNAc)2	15.870
	2705		(HexNAc)6 (Deoxyhexose)1+(Man)3 (GlcNAc)2	3.109
Internal standard	2478		(Hex)2 (HexNAc)2 (NeuAc)2+(Man)3(GlcNAc)1	50.000

Figure 13. N-glycans in uPA from human urine. Hex, hexose; HexNAc, N-acetylhexosamine; Man, mannose; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid. uPA, urokinase-type plasminogen activator.

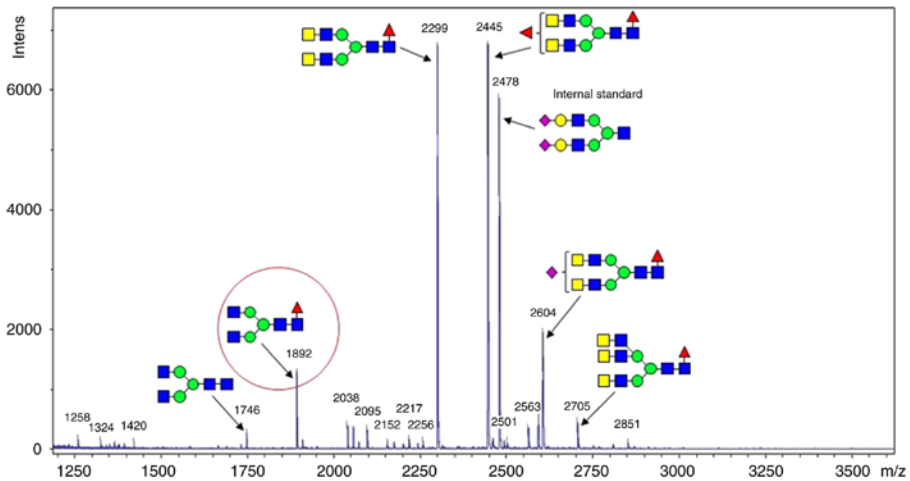


Figure 14. N-glycoforms of urokinase from human urine. Tag: aoWR, Matrix: 20 mg/mi DHB in 70% EtOH, Internal Standard: 50 pmol. There was one common N-glycan (m/z=1892) correlated with invasiveness based on glycoblotting analysis of HLE/HepG2, uPA knockdown HLE, uPA-overexpressing HepG2 and GnT-V knockdown HLE cells. uPA, urokinase-type plasminogen activator.

in its fibrinolytic function (32); additionally, the fibrinolytic activity of recombinant non-glycosylated single-chain uPA has been demonstrated to be similar to that of the glycosylated urinary and recombinant proteins (33).

GnT-V, encoded by the MGAT5 gene (34), is involved in the formation of a β1,6-branched antenna in complex-type glycans. GnT-V is the strongest enzyme associated with cancer progression and metastasis among all glycosyltransferases involved in the formation of multiantennary N-glycans (35-37). The expression of GnT-V was found to be correlated with metastasis in resected colorectal cancer specimens by immunohistochemical analysis (38). Formation of multiantennary N-glycans by GnT-V has been observed in excised liver cancer tissues (39). In addition, increased levels of GnT-V have been observed on immunohistochemical analysis of liver cancer tissues (40). The GnT-V levels were also found to be correlated with the metastatic potential of gastric (41), endometrial (42) and mucinous ovarian cancer (43). Matriptase is a serine protease that plays important roles in cell migration, extracellular matrix degradation, and the activation of uPA and hepatocyte growth factor (22,23). It was also reported that GnT-V mediated metastasis via the upregulation and increased stabilization of matriptase by adding β1-6GluNAc branching (44,45). However, in the present study, knockdown of GnT-V altered N-glycans, including specific N-glycans (m/z=1892) bound to uPA, and reduced the invasiveness of HLE cells without altering uPA expression. These data demonstrated that GnT-V modified N-glycan-binding to uPA, and N-glycosylation is necessary for uPA-mediated cancer cell invasiveness. Therefore, knocking down GnT-V may reduce the invasiveness of HLE cells through N-glycan alterations, in addition to the activation of uPA by matriptase, which was also upregulated by GnT-V. Moreover, N-glycan (m/z=1892) was revealed to be specific among N-glycan alterations when comparing HLE and GnT-V knockdown HLE cells, and was common among N-glycan alterations modulating uPA expression. It was recently reported that the spatial accessibility of N-glycans of substrate glycoproteins to the GnT-V active site likely defines the potential for site-specific modification (46).

There was a limitation to the present study: The expression of the GnT-V protein in HepG2 cells and overexpression of GnT-V in HepG2 cells was not evaluated. If the expression of GnT-V in HepG2 cells is low, overexpression of GnT-V in HepG2 cells and detection of the corresponding indicators may further support the conclusions of the present study. This point will be the focus of a future study.

In conclusion, N-glycan alterations occurred in response to changes in the cancer cell invasiveness triggered by uPA and GnT-V, and a specific N-glycan (m/z=1892) was found to be associated with this change in invasiveness. The function of uPA was controlled by N-glycan alterations mediated by GnT-V, even if not directly through the effect of uPA activation by matriptase. Therefore, glycomic alterations may be useful for the diagnosis of malignancy, and a specific N-glycan (m/z=1892) may be a candidate as a novel biomarker and target for molecular therapy.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ToK made substantial contributions to conception and design. HT, NK and TA analyzed and interpreted the data regarding the alterations in N-glycans and invasiveness in liver cancer cell lines. NH performed the histological examination of the kidney. AN, SS, TO, TaK, MF, HY, HK, SN and AT helped supervise the findings of this study. HT and ToK were major contributors to writing the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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