The mucin-type glycosylating enzyme polypeptide N-acetylgalactosaminyltransferase 14 promotes the migration of ovarian cancer by modifying mucin 13

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Abstract. A high expression of O-glycosylated proteins is one of the prominent characteristics of ovarian carcinoma cells associated with cell migration, which would be attributed to the upregulated expression of glycosyltransferases. Therefore, elucidating glycosyltransferases and their substrates may improve our understanding of their roles in tumor metastasis. In the present study, we reported that knockdown of polypeptide N-acetylgalactosaminyltransferase 14 (GALNT14) by small interfering RNA significantly suppressed the cell migration and altered cellular morphology. Immunoprecipitation and western blot analyses indicated that GALNT14 contributed to the glycosylation of transmembrane mucin 13 (MUC13), which was significantly higher in ovarian cancer cells compared with the normal/benign ovary tissues. Furthermore, interleukin-8 (IL-8), which could regulate the migration ability of epithelial ovarian cancer (EOC) cells, had no remarkable effect on the expression of GALNT14 and the tumor-associated carbohydrate epitope Tn antigen. In addition, extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor modulated the expression levels of GALNT14. Our findings provide evidence that GALNT14 may contribute to ovarian carcinogenesis through aberrant glycosylation of MUC13, but not through the IL-8 pathway. These data provide novel insights into understanding the function of MUC13 on neoplasm metastasis and may aid in the development of new anticancer drugs for EOC.

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Abbreviations: GALNT14, polypeptide N-acetylgalactosaminyltransferase 14; MUC13, mucin 13; IL-8, interleukin-8; ERK1/2, extracellular signal-regulated kinase 1/2; EOC, epithelial ovarian cancer; EGFR, epidermal growth factor receptor; IGFBP-3, insulin-like growth factor binding protein-3; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

Key words: polypeptide N-acetylgalactosaminyltransferase 14, mucin 13, interleukin-8, migration, O-glycosylation, epithelial ovarian cancer cell

Introduction

Epithelial ovarian carcinoma (EOC) is the most aggressive tumor among gynecologic malignancies; it can be treated effectively with surgery and chemotherapy during the early stage (1,2). However, the difficulty of early diagnosis and the rapid metastasis represent outstanding clinical challenges associated with EOC, which lead to the frequent failure of current treatment strategies (3). To date, the mechanism of cancer metastasis has yet to be clarified. To improve the 5-year survival rate of EOC patients, further investigations into EOC pathogenesis and the development of novel treatment agents are required.

The mucin-type O-glycosylation is a common post-translational modification of proteins, which is initiated by the family members of polypeptide N-acetylgalactosaminyltransferases (GALNTs) that transfer UDP-N-acetylgalactosamine (UDP-GalNAc) to the hydroxyl group of serine (S) or threonine (T) residues on the target proteins forming Tn antigen (GalNAca-S/T) (4,5). The GALNTs family consists of at least 20 members in humans, i.e. GALNT1 to 14 and GALNTL1 to L6 (6). Aberrant glycosylation is a characteristic of most types of human cancer and effects several cellular properties, such as cell proliferation, apoptosis, differentiation, migration, invasion, transformation and immune responses (7). Studies have shown that O-glycans and GALNT genes play important roles in various tumors. For example, GALNT2 mediates the malignant character of hepatocellular carcinoma by modifying epidermal growth factor receptor (EGFR) (8). Upregulated GALNT3 promotes pancreatic cancer cell growth (9). The overexpression of GALNT6 in breast cancer disrupts mammary acinar morphogenesis through O-glycosylation of fibronectin (10). GALNT14 expression is a potential biomarker for breast cancer, and may be involved in modulating the apoptotic activity of insulin-like growth factor binding protein-3 (IGFBP-3) (11,12). Death-receptor O-glycosylation by

GALNT14 mediates tumor-cell sensitivity to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (13).

Mucin 13 (MUC13), a membrane-bound mucin, is normally expressed in the large intestine, kidney, trachea, small intestine and gastric epithelium (14). It has a large 151-amino acid tandem repeat domain, which is rich in serine and threonine residues that act as glycosylation sites, a sea urchin sperm protein enterokinase arginine domain, and three epidermal growth factor-like domains in the extracellular component, followed by a short transmembrane domain and a 69-amino acid cytoplasmic domain (15-17). In recent studies, aberrant expression of MUC13 has been shown in ovarian, gastric, pancreatic and colorectal cancer, and has been involved in carcinogenesis and tumor progression (17-20). However, the precise molecular mechanisms by which MUC13 regulates EOC properties remain largely unknown.

MUC13 is one of the substrates of GALNT14 (21). In EOC cells, the expression pattern and function of GALNT14 have not been reported, although O-glycosylation can mediate multiple cellular properties. In the present study, we reported that GALNT14 is frequently upregulated in ovarian cancer cells. Moreover, GALNT14 modifies MUC13 O-glycosylation, and plays critical roles in migration and cytoskeletal regulation of ovarian cancer cells.

Materials and methods

Cell lines and materials. Human ovarian carcinoma cell lines (SKOV-3, OVCAR-3 and HO8910PM) were kindly donated by the Institute of Ultrasound of the Chongqing Medical University. HO8910 cells were a gift from the Institute of the Pathology of Chongqing Medical University. RPMI-1640 medium was supplied by Gibco (Grand Island, NY, USA). Recombinant human IL-8 was purchased from Noroprotein (Shanghai, China), Takara Taq™ and PrimeScrip® RT Reagent kit were supplied by Takara Biotechnology (Dalian, China). 3-(4,5-dimethylthiazol-2-thiazyl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), ERK1/2 inhibitor (PD98059), p38 MAPK inhibitor (SB203580) and PI3K inhibitor (LY294002) were obtained from Sigma (St. Louis, MO, USA). Rabbit anti-β-actin and RIPA buffer were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-MUC13 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-GALNT14 was purchased from Sigma. Biotinylated *Vicia villosa* agglutinin (VVA) was supplied by Vector Laboratories, Inc. (Burlingame, CA, USA). Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L), goat anti-mouse IgG (H+L), horseradish peroxidase streptavidin were from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Effectene Transfection Reagent was purchased from Qiagen (Germantown, MD, USA).

Cell culture and siRNA transfection. All cell lines were maintained in RPMI-1640 medium containing 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin in a cell culture incubator at 37˚C, 5% CO_2. Cells were tested to have no mycoplasma contamination prior to the experiments. Cell transfections were performed with Effectene Transfection Reagent according to the manufacturer's instructions using 25 nM siRNA targeting GALNT14 in a 6-well plate or 50-ml flask. The three GALNT14 target sequences used were: siRNA1, 5'-GAU CCG GGA AAU CAU AUU ATT-3' (sense) and 5'-UAA UAU GAU UUC CCG GAU CTT-3' (antisense); siRNA2, 5'-GCC AAC ACG UAU AUA AAG ATT-3' (sense) and 5'-UCU UUA UAU ACG UGU UGG CTT-3' (antisense); siRNA3, 5'-CCA UCC AGA AGG GCA AUA UTT-3' (sense) and 5'-AUA UUG CCC UUC UGG AUG GTT-3' (antisense). Negative control (NC) siRNA and β-actin siRNA were included. Cells were incubated for 48 h after transfection and used for further experiments.

Cell viability assay. Cell viability was evaluated by the MTT reduction assay. SKOV-3 cells were seeded in 96-well plates with $7.5x10^3$ cells/well. After growing to confluence, the cells were treated with or without IL-8 (25, 50, 100 and 500 μg/l). Following incubation for 24 h, the medium was discarded, and cells were incubated with MTT (5 mg/ml) in culture medium at 37˚C for 4 h. Then, culture medium was removed, and 100 μl of DMSO per well was added for formazan dissolution. The absorbance was measured at a wavelength of 490 nm by a Sunrise Remote Microplate Reader (Grodig, Austria) and then normalized the value to the control group.

Wound healing assay. HO8910PM cells were counted and seeded at a density of $1x10^5$ cells/well in 24-well plates to reach 80-90% confluence. A sterile 10-μl plastic pipette tip was used to create three artificial wounds across the cell monolayer per well, and the debris was removed by washing the cells three times with PBS. Cells that migrated into the wounded area were visualized and photographed randomly in each well at 0 and 24 h with a Nikon TEU 2000 inverted microscope. The cell migration ability was estimated by the relative distance of wound closure.

In vitro migration assays. A total of $5x10^4$ cells were suspended in 100 μl RPMI-1640 medium without serum or growth factors, and plated in the upper chamber with the non-Matrigel-coated polycarbonate membrane (24-well insert; 8-μm pore size, Corning Life Sciences). RPMI-1640 medium (600 μl) containing 10% FBS was added to the lower chamber as a chemoattractant. Following incubation for 24 h at 37˚C under 5% CO_2, cells that had not migrated through the pores were removed with a cotton swab, whereas cells on the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with hematoxylin. The number of stained cells was counted by an inverted microscope (Nikon TEU 2000).

RNA extraction and reverse transcription-PCR (RT-PCR) analysis. Total RNA was isolated from the ovarian cancer cell lines using TRIzol (Takara, Dalian, China) according to the manufacturer's specifications. The quantity of RNA samples was measured by UV absorbance at 260-280 nm using a DNA/RNA GeneQuant Calculator (Amersham Biosciences, Piscataway, NJ, USA). Total cDNA was synthesized from 2 μg total RNA in 20 μl of reaction mixture containing 50 pmol of oligo-dT, 5.0 units of AMV reverse transcriptase, 40 units of RNase inhibitor, 40 nmol of dNTP, 4 μl of 5X RT buffer (Bioer, Hangzhou, China). One microliter of the resulting cDNA samples was taken for the amplification of the different transcripts by the different primers. The amplification conditions were: 94˚C for 4 min, followed by 30 cycles of 94˚C for

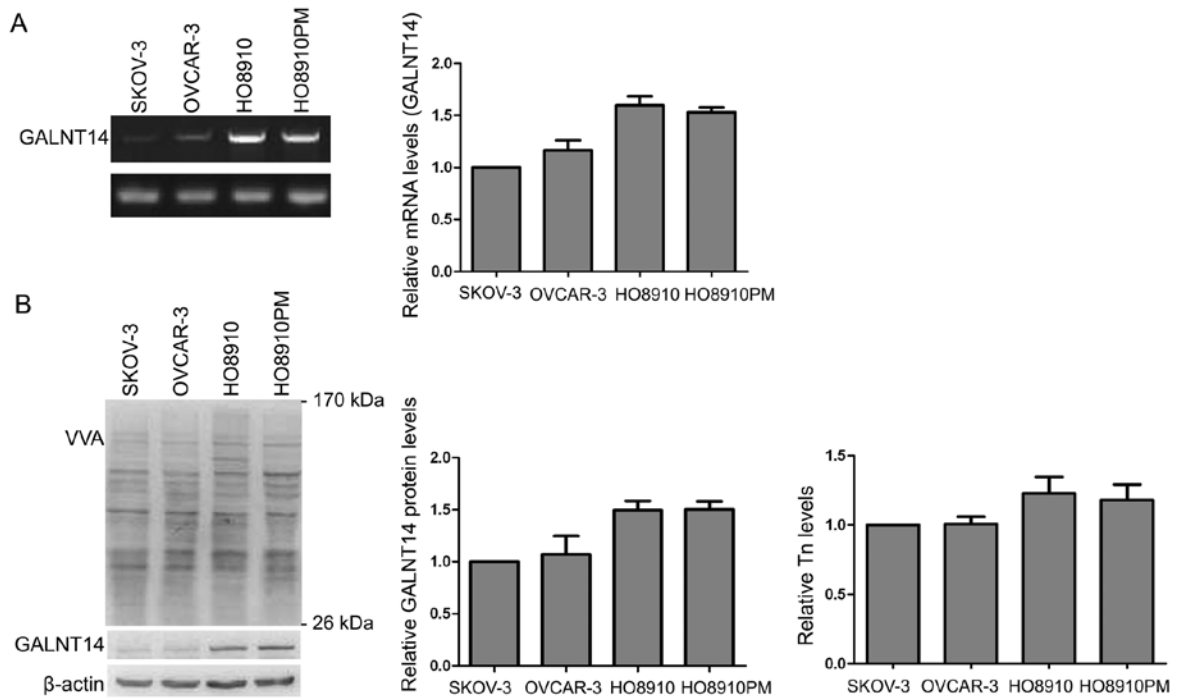

Figure 1. Expression of GALNT14 and Tn antigen in ovarian cancer cell lines. (A) Detection of GALNT14 mRNA expression in ovarian cancer cell lines. The GALNT14 mRNA expression was measured by RT-PCR analysis. The relative level of GALNT14 mRNA was normalized to GAPDH. n=3; error bars, means ± SD. (B) Expression of GALNT14 and Tn antigen were examined by western blotting, and β-actin was used as an internal control. The relative intensity of signals is presented as the means ± SD. n=3.

30 sec, 54˚C for 45 sec and 72˚C for 30 sec, and finally 5 min at 72˚C. The PCR reactions were performed in 20 μl volumes in the presence of 10 μM of each of the sense and the antisense primers using 2 units of BioReady rTaq Polymerase (Bioer). The following primers for RT-PCR were used: GALNT14 (474 bp): sense 5'-ACC TGG ACA CCT TCA CCT ACA T-3'; antisense 5'-CCA ATC TGC TCT CAA CAT TCC-3'. GAPDH (230 bp): sense 5'-CTC TCT GCT CCT CCT GTT CGA CAG-3'; antisense 5'-GTG GAA TCA TAT TGG AAC ATG T-3'. The PCR products were resolved by electrophoresis on 1.5% agarose gel containing 1% Goldview™.

Western blot, Vicia villosa agglutinin (VVA) lectin blot and immunoprecipitation. For extraction of total protein, ovarian cancer cell lines were harvested and lysed in 1X RIPA (CST) lysis buffer supplemented with 5 mM Na fluoride, 1 mM PMSF and 1 mM Na orthovanadate protease inhibitors. Extracted protein samples were measured by BCA protein assay (Pierce Chemical Co., Rockford, IL, USA). Total cell lysates containing 1 mg of protein were immunoprecipitated with primary antibodies and agarose beads at 4˚C. Then, the agarose beads were washed. Prior to western blotting, cell lysates or immunoprecipitated protein mixed with 5X SDS sample buffer were boiled for 10 min, electrophoresed on a 10% polyacrylamide minigel with equal amount (20 μg) and then transferred onto PVDF membranes. After blocking with 5% non-fat milk or 3% BSA in TBS for 1 h at room temperature, membranes blotted with proteins were incubated with the biotinylated VVA lectin or primary antibodies overnight at 4˚C. After washing three times (for 10 min) in TBST, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (dilution 1:4,000) or HRP-conjugated streptavidin (dilution 1:500) for 1 h at room temperature. After washing three times (for 10 min) in TBST, protein bands were visualized using ECL reagents and quantitated with a Fluor-S (Bio-Rad) instrument.

Statistical analysis. Statistical analysis was performed using SPSS version 10.0 package (SPSS Inc., Chicago, IL, USA). Student's t-test and one-way analysis of variance (ANOVA) were used for comparisons between groups. Data are presented as the means ± SD of 3 independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GALNT14 and Tn antigen expression in ovarian cancer cell lines. The mRNA expression profile of GALNT14 has been examined in primary normal and malignant tissue samples from ovary, skin, lung, pancreas, breast, endometrium, bladder and lymphoid cancer. Up to 30% of tissue samples from various types of human cancer including ovarian carcinomas showed GALNT14 mRNA overexpression (13). However, the GALNT14 functions in ovarian cancer remain largely unknown. Under these circumstances, we first investigated the GALNT14 expression in a panel of four EOC cell lines at the mRNA and protein levels by RT-PCR and western blot analysis to select suitable cell lines for the subsequent functional studies. Of these cell lines, SKOV-3 and OVCAR-3 cells showed a faint-GALNT14 expression, whereas HO8910 and HO8910PM showed a relatively high expression (Fig. 1A and B). Therefore, SKOV-3 and HO8910 were selected as representative of high- and low-GALNT14 expressing cell lines, respectively. HO8910 and HO8910PM were used to

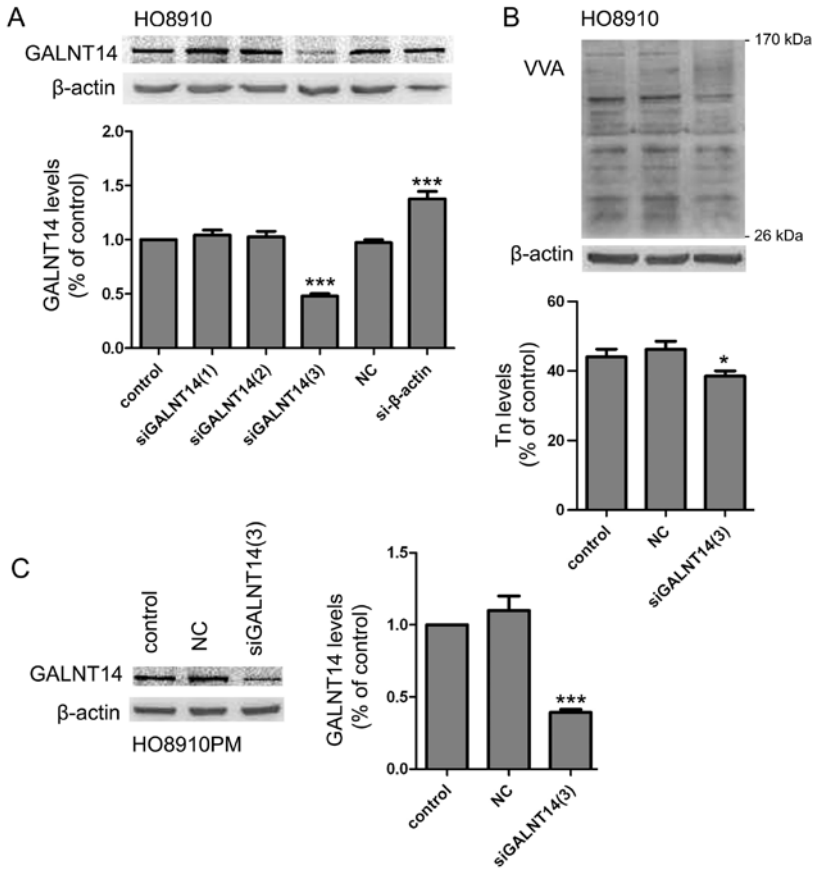

Figure 2. Knockdown of endogenous expression of GALNT14. (A) The expression of GALNT14 protein in HO8910 cells transfected with three different small interference RNAs (siRNAs) against GALNT14 or one negative control siRNA or one β-actin positive control siRNA by western blot analysis. β-actin served as an internal control. ***P<0.001; error bars, means ± SD; n=3. (B) The changes in carbohydrates on cellular proteins of HO8910 cells transfected with effective small interference RNA against GALNT14 were detected by *Vicia villosa* agglutinin (VVA), specific for GalNAc-O-Ser/Thr. *P<0.05; error bars, means ± SD; n=3. (C) Another type of EOC cell line, HO8910PM, was transfected with effective small interference RNA against GALNT14, and the effectiveness of interference was verified again by western blot analysis. ***P<0.001; error bars, means ± SD; n=3.

knock down the endogenous expression of GALNT14. *Vicia villosa* lectin (VVA) blot analysis, which preferentially recognizes terminal N-acetylgalactosamine residue linked to serine or threonine in a polypeptide (Tn antigen), was also performed to detect the activity of GALNTs (8,22). The expression profile of Tn antigen was simultaneously determined in these cell lines (Fig. 1B). We found that Tn antigen expression levels in each cell line were clearly different, which suggested the activities of GALNTs in different ovarian cancer cells were discrepant.

Knockdown of endogenous expression of GALNT14 reduces O-glycosylation protein expression. To assay the biological significance of GALNT14 in ovarian cancer cells, we adopted GALNT14 siRNA to transfect into HO8910 cells and to make cell mode which was knockdown endogenous expression of GALNT14. Results showed that the introduction of siGALNT14(3) resulted in a significant reduction of GALNT14 expression (Fig. 2A). The decreased glycoproteins were also observed by VVA lectin blot in cell lysates (Fig. 2B). To further confirm the efficiency of siGALNT14(3) interference, we measured another GALNT14-positive ovarian cancer cell line, HO8910PM, by western blot analysis (Fig. 2C). Therefore, siGALNT14(3) was selected for subsequent experiments.

GALNT14 knockdown reduces cellular migration and alters cellular morphologic characteristics. Increased cellular migration capability is required for a cancer cell to be competent for metastasis. Thus, we evaluated the effect of GALNT14 on the migration of HO8910 and HO8910PM cells by applying Transwell cell migration assay *in vitro*. As shown in Fig. 3A and C, knockdown of GALNT14 significantly suppressed cellular migration ability in HO8910 and HO8910PM cells (P<0.05). To further verify the role of GALNT14 in cellular migration, we performed wound healing assay in 24-well plates. Consistent with the above results, the number of cells migrating toward the center of the wound area was markedly depleted in the GALNT14 siRNA group compared with the control and NC siRNA groups (Fig. 3B). Notably, 2 days after transfection of siRNA, the clear, bipolar, elongated cell shape was converted to a relatively round morphology (Fig. 3D). These results suggest that GALNT14 is closely related to ovarian cancer metastasis.

MUC13 is a substrate of GALNT14 in ovarian cancer cell lines. MUC13, a transmembrane mucin, is highly expressed in ovarian cancer tissues compared with the normal/benign ovary samples. Moreover, the marked changes in cell-cell adhesion, cell motility, proliferation, and tumorigenesis are observed upon exogenous MUC13 expression (20), which are

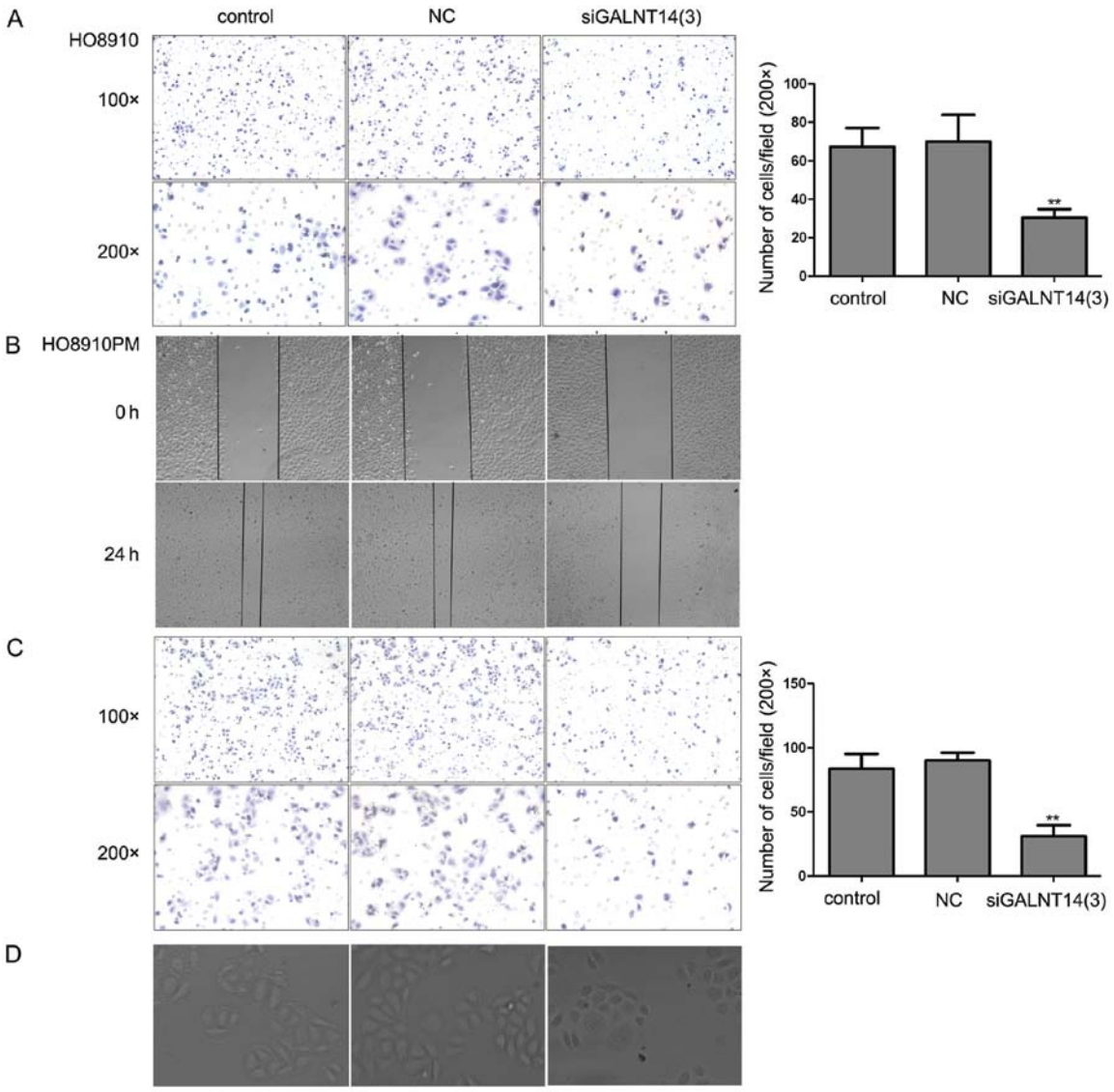

Figure 3. Effects of GALNT14 knockdown on cellular migration and cellular morphologic characteristics. (A) HO8910 cells were transfected with small interference RNA siGALNT14(3) for 48 h, and the changes of cell migration ability were analyzed by Transwell migration assays. Results are represented as means ± SD from 3 independent experiments. (Original magnification, x100 and x200). **P<0.01. (B) The HO8910PM cells treated with GALNT14 siRNA for 48 h were grown in a 24-well culture dish. Confluent monolayers were wounded with a 10-μl pipette tip and cultured for 24 h. Wound-healing migration is represented by the widths of injury lines. Representative images are shown (magnification, x100). (C) HO8910PM cells were transfected with siGALNT14(3) for 48 h, and cell migration ability was analyzed by Transwell migration assays. **P<0.01; error bars, means ± SD; n=3; (original magnification, x100 and x200). (D) Effect of GALNT14 knockdown on cellular morphologic characteristics in HO8910PM cells. The clear, bipolar, elongated cell shape was converted to a relatively round morphology. Representative images are shown.

partly similar to the function of GALNT14. In addition, a panel of mucin-derived peptide substrates such as MUC13, MUC2, MUC5AC and MUC7 could be glycosylated by glycosyltransferase GALNT14, which transfers GalNAc to the Ser/Thr residues on the target substrates (21). Moreover, according to previous research on the critical roles of MUC1 glycosylation by polypeptide N-acetylgalactosaminyltransferase 6 in mammary carcinogenesis (23), we proposed that MUC13 increased the migration of ovarian cancer cell lines by the glycosylating function of GALNT14. To verify this hypothesis, we first measured the expression levels of these two molecules in ovarian cancer cell lines by western blot analysis, and found that GALNT14 and MUC13 proteins were co-expressed in ovarian cancer cells (Fig. 4A). This finding indicated that GALNT14 might contribute to ovarian carcinogenesis through stabilization of the MUC13 protein. To investigate the interaction of GALNT14 and MUC13 in detail, we knocked down GALNT14 expression by siRNA and examined its effect on the MUC13 protein in HO8910PM cells. The results revealed that the knockdown of GALNT14 did not affect the expression of MUC13 at the protein level (Fig. 4B). However, compared to the control and NC groups, significantly lower levels of terminal N-acetylgalactosamine residue modified MUC13 were immunoprecipitated from HO8910PM cell lysates in the GALNT14 siRNA group, without any changes in the total expression of MUC13 protein (Fig. 4C). Collectively, these results suggested that GALNT14 may influence the post-translational modification and stabilization of MUC13 protein in ovarian cancer cells.

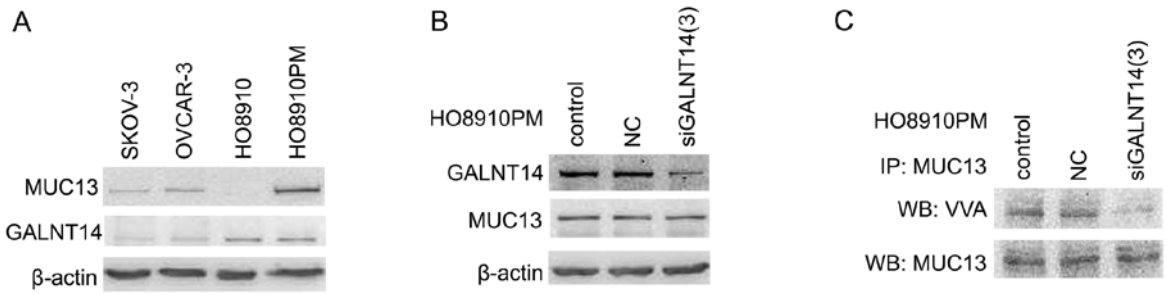

Figure 4. GALNT14 O-glycosylates MUC13 in ovarian cancer cell lines. (A) Western blot analysis revealed co-expression of GALNT14 and MUC13 in ovarian cancer cell lines. Representative data are shown. n=3. (B) Effect of GALNT14 knockdown on the expression of MUC13 protein in HO8910PM by western blot analysis. Data are expressed as means ± SD from 3 independent experiments. (C) GALNT14 knockdown decreased VVA binding to MUC13 protein. The HO8910PM cells treated with GALNT14 siRNA or blank control PBS or negative control siRNA (NC) were grown in a 6-well culture dish for 48 h, and cell lysates were immunoprecipitated with anti-MUC13 antibody, followed by immunoblotting analysis with VVA or anti-MUC13 antibody.

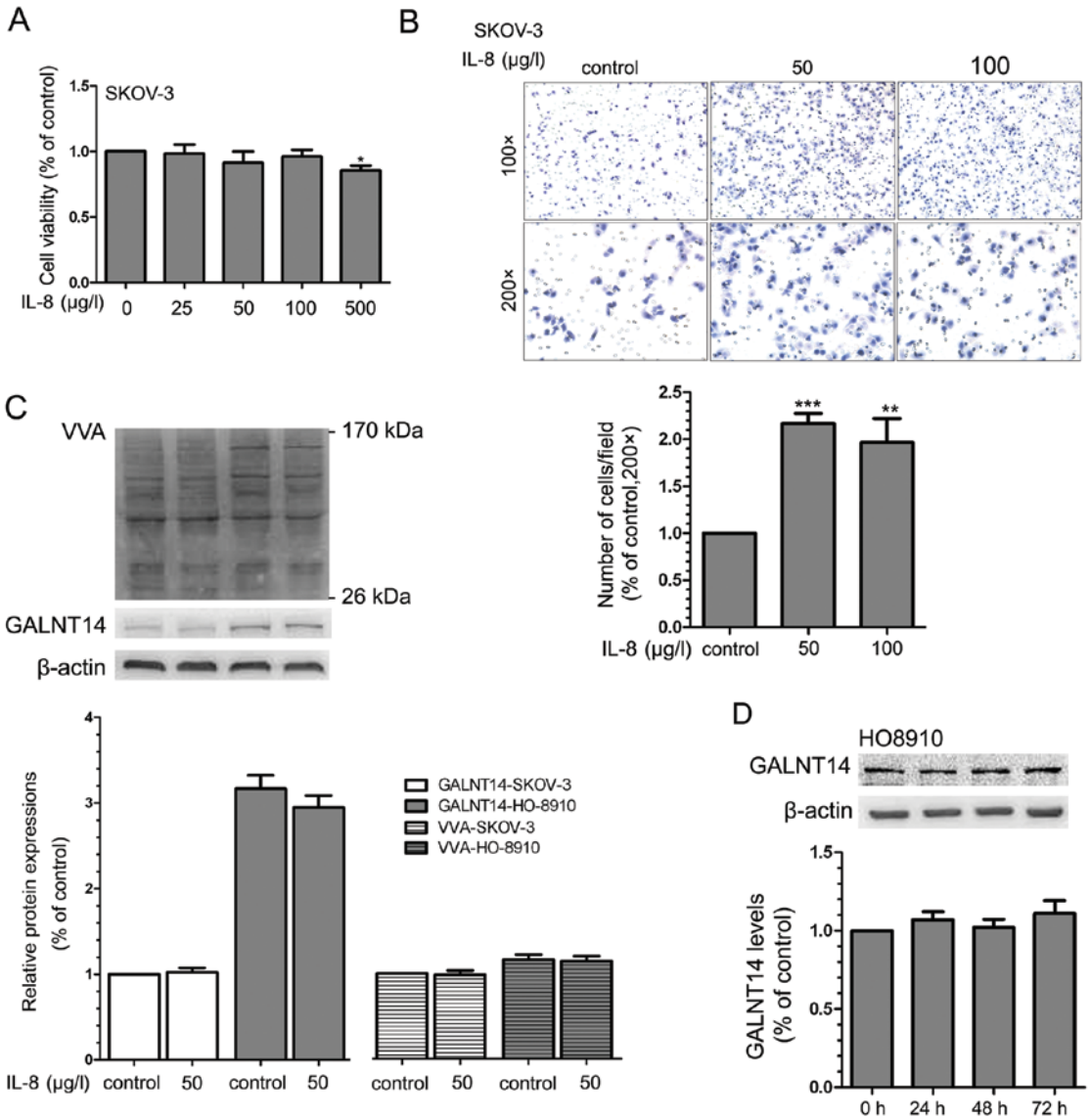

Figure 5. Effects of IL-8 on cell migration ability and the expression of GALNT14 and Tn antigen. (A) SKOV-3 cells were treated with various concentrations of IL-8 (0, 25, 50, 100 and 500 μg/l) for 24 h, and cell viability was analyzed by MTT analysis (n=5) as described in Materials and methods. *P<0.05. (B) Effects of various concentrations of IL-8 (0, 50 and 100 μg/l) on cell migration by Transwell migration assays. SKOV-3 and HO8910 (data not shown) cells were treated with various concentrations of IL-8 for 24 h, and cells migrating through the pores were counted in five random images and compared. Results are presented as means ± SD from 3 independent experiments. (Original magnification, x100 and x200). **P<0.01; ***P<0.001. (C) Effects of IL-8 (0 and 50 μg/l; 24 h) on the expression of GALNT14 and Tn antigen in SKOV-3 and HO8910 cell lines by western blotting. Representative results are shown. The protein bands were quantified from 3 separate experiments. Error bars, means ± SD. (D) Effects of IL-8 (50 μg/l; 0, 24, 48 and 72 h) on the expression of GALNT14 in HO8910 cell lines by western blot analysis. The relative level of GALNT14 was normalized to β-actin and obtained from 3 separate experiments. Error bars, means ± SD.

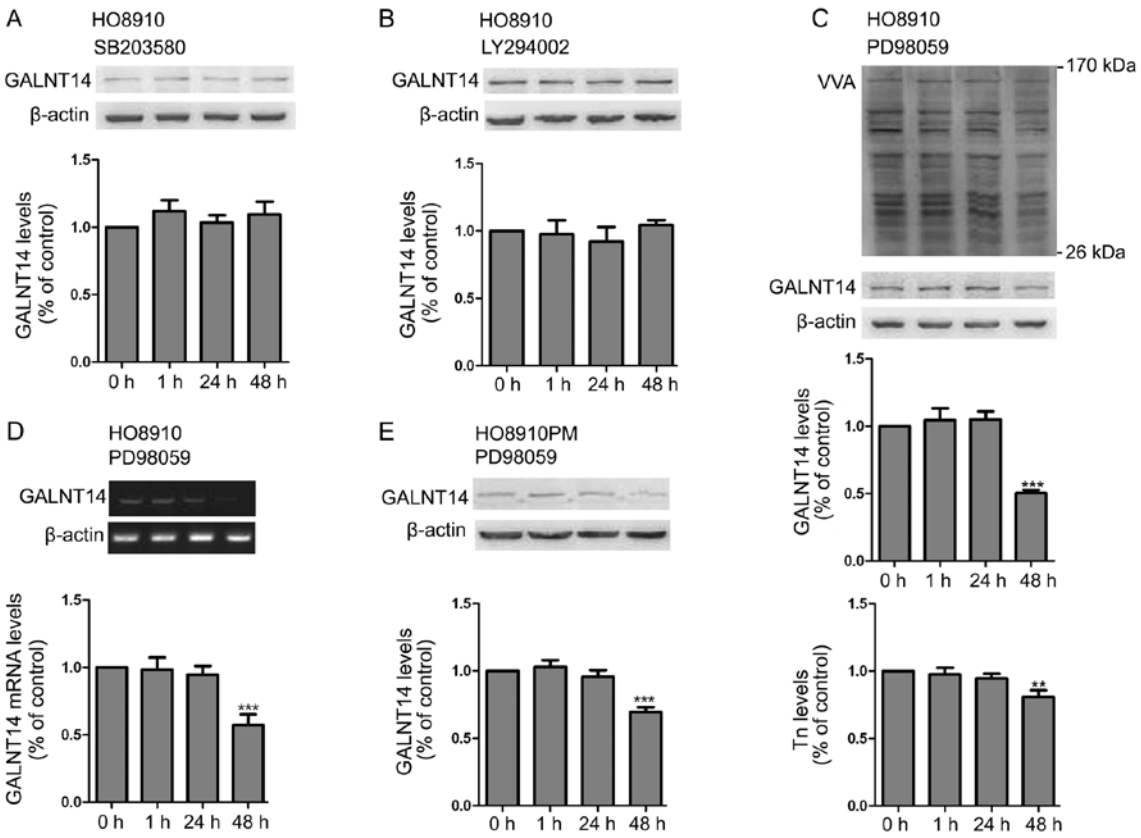

Figure 6. The ERK1/2 inhibitor PD98059 suppresses the expression of GALNT14 and Tn antigen. (A) HO8910 cells were treated with the p38 MAPK inhibitor SB203580 (10 μmol/l) for 0, 1, 24 and 48 h, and the expression profile of GALNT14 was detected by western blot analysis. (B) Effect of the PI3K inhibitor LY294002 (10 μmol/l; 0, 1, 24 and 48 h) on the expression of GALNT14 in HO8910 cell lines. (C) Effect of the ERK1/2 inhibitor PD98059 (10 μmol/l; 0, 1, 24 and 48 h) on the expression of GALNT14 protein and Tn antigen in HO8910 cell lines. The relative level of GALNT14 protein and Tn antigen were normalized to β-actin. n=3; error bars, means ± SD. **P<0.01; ***P<0.001. (D) Effect of the ERK1/2 inhibitor PD98059 (10 μmol/l; 0, 1, 24 and 48 h) on the expression of GALNT14 mRNA in HO8910 cell lines. The relative level of GALNT14 mRNA was normalized to GAPDH. n=3; error bars, means ± SD. (E) Another type of EOC cell line, HO8910PM, was treated with the ERK1/2 inhibitor PD98059 (10 μmol/l) for 0, 1, 24 and 48 h, and the expression of GALNT14 protein was detected by western blot analysis. Data are expressed as means ± SD. ***P<0.001; n=3.

GALNT14 regulation of the migration ability of tumor cells is not associated with the IL-8 pathway. It has been demonstrated that CXC-chemokine interleukin-8 (IL-8) mediates the metastasis of various tumor cells (24-26). Groux-Degroote *et al* (27) reported that IL-6 and IL-8 contribute to the increased expression of some glycosyltransferases and sulfotransferases participating in the biosynthesis of sialyl-Lewis^x and 6-sulfo-sialyl-Lewis^x epitopes in the human bronchial mucosa. However, the correlation between IL-8 and GALNT14 has not been reported. Thus, we analyzed the expression of GALNT14 in ovarian cancer cell lines which were stimulated by recombinant human IL-8. First, to select the suitable concentration of IL-8, its inhibitory efficiency on the proliferation of SKOV-3 cells was examined using MTT assay. Treatment with IL-8 did not suppress cell proliferation at a low dose (25-100 μg/l) for 24 h, but it induced marked cytotoxicity at a dose up to 500 μg/l compared with the control group (Fig. 5A). Furthermore, IL-8 (50-100 μg/l, 24 h) successfully increased the migration of SKOV-3 and HO8910 cell lines (Fig. 5B). However, the incubation of IL-8 for 24 h in two cell lines did not alter the expression of GALNT14 protein and Tn antigen (Fig. 5C). To further confirm this result, we extended the stimulation time of IL-8 to 72 h, but the expression levels of GALNT14 were not markedly different (Fig. 5D). These data suggest the GALNTs family including GALNT14 may not be involved in the role of IL-8 in cellular migration.

ERK1/2 regulates the expression of GALNT14 and Tn. Aberrant glycosylation modification affects the function of specific substrates targeted by these enzymes and the signaling pathways mediated by these substrates (8,23,28). However, the regulation of Golgi glycosyltransferases by signaling pathway mechanisms has not been studied extensively. Seales *et al* (29) recently found that the protein kinase C/Ras/ERK signaling pathway activates myeloid fibronectin receptors by altering β1 integrin sialylation. Thus, to find the relevant signaling pathways involved in regulating GALNT14 expression, we analyzed potential pathways using inhibitors of the p38 MAPK (SB203580), the ERK1/2 (PD98059), and the PI3K (LY294002) pathway in HO8910 cell lines. Among them, the ERK1/2 (PD98059) was the only compound to effectively attenuate the expression of GALNT14 at the mRNA and protein levels (Fig. 6A-D). Furthermore, the expression of Tn antigen, which existed in O-glycosylated substrates, was accordingly decreased (Fig. 6C). To further demonstrate these findings, we repeated the same experiments in another EOC cell line, HO8910PM (Fig. 6E). The result was consistent with the above findings.

Discussion

Mucin type O-glycosylation is initiated by members belonging to the GALNT family present in the Golgi apparatus, and is one of the common modifications that have various functions in the folding, stability and targeting of multiple glycoproteins (30). Accumulating evidence demonstrates that the GALNT family members are related to several cellular functions by catalyzing their specific substrates. For example, GALNT2 regulates the concentrations of plasma lipids by O-glycosylating angiopoietin-like protein 3 (31). The O-glycosyltransferase *pgant3* promotes cell adhesion during eukaryotic development by affecting the secretion and localization of the extracellular matrix integrin ligand, Tiggrin (32). Glycosyltransferase GALNT2 regulates the malignant character of hepatocellular carcinoma by modifying the epidermal growth factor receptor (8). Polypeptide N-acetylgalactosaminyltransferase 6 modulates mammary carcinogenesis through glycosylating MUC1 (23). It has been reported that the expression of GALNT14 mRNA is markedly higher in tumor tissues of the ovary, lung, breast, endometrium and bladder compared to these normal tissues (13). Accordingly, we investigated the GALNT14 expression in a panel of EOC cell lines by RT-PCR and western blot analysis. As expected, varying degrees of the expression of GALNT14 were detected in the four types of ovarian cancer cells. Subsequent functional analyses of GALNT14 revealed that GALNT14 could regulate cellular migration and cellular morphologic characteristics in ovarian cancer cells. In the present study, we showed for the first time that GALNT14 can mediate the malignant behavior of ovarian cancer cells.

Altered O-linked oligosaccharides expressed by cancer cells have several important functions in malignant transformation and tumor progression, including cell adhesion, invasion and metastasis (33). Short O-glycan Tn antigen is generally shielded by covalently bound terminal carbohydrate moieties in healthy and benign-diseased tissues, but it is unmasked in approximately 90% of human carcinomas, including ovarian cancer, due to defective O-glycosylation (34-36). Similarly, we showed different degrees of the expression of tumor-associated carbohydrate epitope, Tn antigen, in the four types of ovarian cancer cells. Tn antigen is rarely expressed in normal tissues, but it is widely expressed in human carcinomas (37). Thus, Tn antigen has attracted significant interest as a molecule target for tumor diagnosis and immunotherapy. A large number of anti-Tn IgG and IgM antibodies have been produced and analyzed for their potential feasibilities and antitumor activities (38-40). Although anti-Tn antibodies have been generated, some issues such as reduced effectiveness *in vivo*, immunogenicity and cross-reactivity against type-A blood antigen have to be resolved before applying to clinical therapy. Therefore, production of available anti-Tn antibodies requires further research and considerable clinical significance. It is also the focus and the follow-up research points of our laboratory.

Mucin glycosylating enzyme GALNT6 contributes to mammary carcinogenesis through abnormal glycosylation and stabilization of specific substrate MUC1 (23). GALNT2, sharing a high amino acid sequence homology with GALNT14, regulates the malignant character of hepatocellular carcinoma by modulating the structure of short O-glycan on substrate EGFR and the phosphorylation levels of EGFR and its downstream signaling molecules (8,21). Similar to other *N*-acetylgalactosaminyltransferases, GALNT14 has specific substrates including MUC13 (21,41). MUC13, a transmembrane mucin, is overexpressed in ovarian cancer tissues vs. the normal/benign ovary samples, and modulates cell-cell adhesion, cell motility, proliferation and tumorigenesis (20). We found that GALNT14 and MUC13 proteins were almost co-expressed in ovarian cancer cells. Accordingly, we hypothesized that GALNT14 contributes to ovarian carcinogenesis through aberrant glycosylation of MUC13. Consistent with our speculation, knockdown of GALNT14 had no marked influence on the expression profile of MUC13 protein, but the structure of short O-glycan on MUC13 was clearly attenuated. Moreover, the knockdown of GALNT14 was followed by the decrease of Tn antigen with different ranges of molecular weights. These suggest that GALNT14 may have additional functions through glycosylation of its other unidentified substrates in addition to MUC13 in EOC cells. Therefore, an in depth investigation of novel substrates of GALNT14 is warranted to elucidate unveiled pathophysiologic roles of GALNT14 in ovarian cancer.

It has been reported that CXC-chemokine interleukin-8 (IL-8), a pro-inflammatory cytokine initially described as a monocyte and neutrophil chemoattractant, mediates the metastasis of various tumor cells (24-26). Moreover, IL-6 and IL-8 promote the expression of glycosyltransferases and sulfotransferases involved in the biosynthesis of sialyl-Lewis^x and 6-sulfo-sialyl-Lewis^x epitopes in the human bronchial mucosa (27). Thus, to elucidate the relationship between IL-8 stimulation and the expression of N-acetylgalactosaminyltransferase GALNT14 and tumor-associated carbohydrate epitope Tn in ovarian cancer cells, we made the corresponding detections. Our findings indicated that, although IL-8 could regulate the migration ability of EOC cells, it had no marked effect on the expression of GALNT14 and Tn antigen. Thus, IL-8 modulates the migration ability of EOC cells but not by the GALNTs pathway. Considering the common mediating function of IL-8 and GALNTs in cellular biological behavior, we inferred that IL-8 may be the downstream signaling molecule of GALNTs pathway, or that IL-8 pathway and GALNTs pathway may be completely independent of one another. Therefore, further analysis is required.

To investigate the signaling pathways involved in GALNT14-induced alterations in cellular biological behavior, we determined enzyme activity of GALNT14 after using several common signaling pathway inhibitors. Seales *et al* (29) found that the protein kinase C/Ras/ERK signaling pathway activates myeloid fibronectin receptors by altering β1 integrin sialylation. Similarly, we found that ERK1/2 inhibitor modulated the expression level of GALNT14, which suggested that GALNT14 was the downstream signaling molecule of ERK1/2 in the regulation of cellular biological behavior. Based on the above results, we inferred that ERK1/2 inhibitor could also mediate the glycosylation of MUC13 and the cellular biological behavior of EOC cells. Additional studies are required to further verify our findings and our inferences. To our knowledge, the present study is the first to show that GALNT14 is modulated by the ERK pathway, which may provide novel insights into the pathophysiologic roles of GALNT14 in ovarian cancer progression.

In conclusion, the results of the present study suggest that GALNT14 could modulate MUC13 O-glycosylation and stabilization, and thereby mediate the malignant behavior of ovarian cancer cells. This study not only verifies a pathophysiologic role of GALNT14 in EOC cells but also provides insight into the significance of the regulation of the ERK pathway on GALNT14 expression in EOC tumor progression, although further verification tests are required to elucidate the exact mechanism of the ERK-GALNT14-MUC13 pathway in ovarian cancer cells. Understanding the correlative function and further mechanisms of O-glycosylation on the specific substrates by GALNT family genes may offer novel insights into the development of EOC anticancer drugs. These contain anti-microRNAs, carbohydrate mimetics, siRNAs, or small molecule compounds that can regulate GALNT gene expression or enzyme activity.

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References

1. Auersperg N, Edelson MI, Mok SC, Johnson SW and Hamilton TC: The biology of ovarian cancer. Semin Oncol 25: 281-304, 1998.
2. Auersperg N, Wong AS, Choi KC, Kang SK and Leung PC: Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr Rev 22: 255-288, 2001.
3. Lengyel E: Ovarian cancer development and metastasis. Am J Pathol 177: 1053-1064, 2010.
4. Tian E and Ten Hagen KG: Recent insights into the biological roles of mucin-type O-glycosylation. Glycoconj J 26: 325-334, 2009.
5. Ten Hagen KG, Fritz TA and Tabak LA: All in the family: the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases. Glycobiology 13: 1-16, 2003.
6. Tarp MA and Clausen H: Mucin-type O-glycosylation and its potential use in drug and vaccine development. Biochim Biophys Acta 1780: 546-563, 2008.
7. Hakomori S: Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci USA 99: 10231-10233, 2002.
8. Wu YM, Liu CH, Hu RH, Huang MJ, Lee JJ, Chen CH, Huang J, Lai HS, Lee PH, Hsu WM, Huang HC and Huang MC: Mucin glycosylating enzyme GALNT2 regulates the malignant character of hepatocellular carcinoma by modifying the EGF receptor. Cancer Res 71: 7270-7279, 2011.
9. Taniuchi K, Cerny RL, Tanouchi A, Kohno K, Kotani N, Honke K, Saibara T and Hollingsworth MA: Overexpression of GalNAc-transferase GalNAc-T3 promotes pancreatic cancer cell growth. Oncogene 30: 4843-4854, 2011.
10. Park JH, Katagiri T, Chung S, Kijima K and Nakamura Y: Polypeptide N-acetylgalactosaminyltransferase 6 disrupts mammary acinar morphogenesis through O-glycosylation of fibronectin. Neoplasia 13: 320-326, 2011.
11. Wu C, Shan Y, Liu X, Song W, Wang J, Zou M, Wang M and Xu D: GalNAc-T14 may be involved in regulating the apoptotic action of IGFBP-3. J Biosci 34: 389-395, 2009.
12. Wu C, Guo X, Wang W, Wang Y, Shan Y, Zhang B, Song W, Ma S, Ge J, Deng H and Zhu M: N-Acetylgalactosaminyltransferase-14 as a potential biomarker for breast cancer by immunohistochemistry. BMC Cancer 10: 123-130, 2010.
13. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, Lee D, von Goetz M, Yee SF, Totpal K, Huw L, Katta V, Cavet G, Hymowitz SG, Amler L and Ashkenazi A: Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat Med 13: 1070-1077, 2007.
14. Packer LM, Williams SJ, Callaghan S, Gotley DC and McGuckin MA: Expression of the cell surface mucin gene family in adenocarcinomas. Int J Oncol 25: 1119-1126, 2004.
15. Maher DM, Gupta BK, Nagata S, Jaggi M and Chauhan SC: Mucin 13: structure, function, and potential roles in cancer pathogenesis. Mol Cancer Res 9: 531-537, 2011.
16. Williams SJ, Wreschner DH, Tran M, Eyre HJ, Sutherland GR and McGuckin MA: Muc13, a novel human cell surface mucin expressed by epithelial and hemopoietic cells. J Biol Chem 276: 18327-18336, 2001.
17. Shimamura T, Ito H, Shibahara J, Watanabe A, Hippo Y, Taniguchi H, Chen Y, Kashima T, Ohtomo T, Tanioka F, Iwanari H, Kodama T, Kazui T, Sugimura H, Fukayama M and Aburatani H: Overexpression of MUC13 is associated with intestinal-type gastric cancer. Cancer Sci 96: 265-273, 2005.
18. Walsh MD, Young JP, Leggett BA, Williams SH, Jass JR and McGuckin MA: The MUC13 cell surface mucin is highly expressed by human colorectal carcinomas. Hum Pathol 38: 883-892, 2007.
19. Chauhan SC, Ebeling MC, Maher DM, Koch MD, Watanabe A, Aburatani H, Lio Y and Jaggi M: MUC13 mucin augments pancreatic tumorigenesis. Mol Cancer Ther 11: 24-33, 2012.
20. Chauhan SC, Vannatta K, Ebeling MC, Vinayek N, Watanabe A, Pandey KK, Bell MC, Koch MD, Aburatani H, Lio Y and Jaggi M: Expression and functions of transmembrane mucin MUC13 in ovarian cancer. Cancer Res 69: 765-774, 2009.
21. Wang H, Tachibana K, Zhang Y, Iwasaki H, Kameyama A, Cheng L, Guo J, Hiruma T, Togayachi A, Kudo T, Kikuchi N and Narimatsu H: Cloning and characterization of a novel UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase, pp-GalNAc-T14. Biochem Biophys Res Commun 300: 738-744, 2003.
22. Nagao M, Nakamura M, Oka N, Akiguchi I and Kimura J: Abnormal glycosylation of motor neurons with N-acetyl-D-galactosamine in a case of subacute motor neuronopathy associated with lymphoma. J Neurol 241: 372-375, 1994.
23. Park JH, Nishidate T, Kijima K, Ohashi T, Takegawa K, Fujikane T, Hirata K, Nakamura Y and Katagiri T: Critical roles of mucin 1 glycosylation by transactivated polypeptide N-acetylgalactosaminyltransferase 6 in mammary carcinogenesis. Cancer Res 70: 2759-2769, 2010.
24. Singh RK, Gutman M, Radinsky R, Bucana CD and Fidler IJ: Expression of interleukin 8 correlates with the metastatic potential of human melanoma cells in nude mice. Cancer Res 54: 3242-3247, 1994.
25. Luca M, Huang S, Gershenwald JE, Singh RK, Reich R and Bar-Eli M: Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. Am J Pathol 151: 1105-1113, 1997.
26. De Larco JE, Wuertz BR, Rosner KA, Erickson SA, Gamache DE, Manivel JC and Furcht LT: A potential role for interleukin-8 in the metastatic phenotype of breast carcinoma cells. Am J Pathol 158: 639-646, 2001.
27. Groux-Degroote S, Krzewinski-Recchi MA, Cazet A, Vincent A, Lehoux S, Lafitte JJ, Van Seuningen I and Delannoy P: IL-6 and IL-8 increase the expression of glycosyltransferases and sulfotransferases involved in the biosynthesis of sialylated and/or sulfated Lewis^x epitopes in the human bronchial mucosa. Biochem J 410: 213-223, 2008.
28. Freire-de-Lima L, Gelfenbeyn K, Ding Y, Mandel U, Clausen H, Handa K and Hakomori SI: Involvement of O-glycosylation defining oncofetal fibronectin in epithelial-mesenchymal transition process. Proc Natl Acad Sci USA 108: 17690-17695, 2011.
29. Seales EC, Shaikh FM, Woodard-Grice AV, Aggarwal P, McBrayer AC, Hennessy KM and Bellis SL: A protein kinase C/Ras/ERK signaling pathway activates myeloid fibronectin receptors by altering beta1 integrin sialylation. J Biol Chem 280: 37610-37615, 2005.
30. Carraway KL III, Funes M, Workman HC and Sweeney C: Contribution of membrane mucins to tumor progression through modulation of cellular growth signaling pathways. Curr Top Dev Biol 78: 1-22, 2007.
31. Schjoldager KT, Vester-Christensen MB, Bennett EP, Levery SB, Schwientek T, Yin W, Blixt O and Clausen H: O-glycosylation modulates proprotein convertase activation of angiopoietin-like protein 3: possible role of polypeptide GalNAc-transferase-2 in regulation of concentrations of plasma lipids. J Biol Chem 285: 36293-36303, 2010.
32. Zhang L, Tran DT and Ten Hagen KG: An O-glycosyltransferase promotes cell adhesion during development by influencing secretion of an extracellular matrix integrin ligand. J Biol Chem 285: 19491-19501, 2010.
33. Baldus SE, Engelmann K and Hanisch FG: MUC1 and the MUCs: a family of human mucins with impact in cancer biology. Crit Rev Clin Lab Sci 41: 189-231, 2004.

34. Springer GF, Desai PR, Ghazizadeh M and Tegtmeyer H: T/Tn pancarcinoma autoantigens: fundamental, diagnostic, and prognostic aspects. Cancer Detect Prev 19: 173-182, 1995.
35. Springer GF: Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy. J Mol Med 75: 594-602, 1997.
36. Li Q, Anver MR, Butcher DO and Gildersleeve JC: Resolving conflicting data on expression of the Tn antigen and implications for clinical trials with cancer vaccines. Mol Cancer Ther 8: 971-979, 2009.
37. Babino A, Oppezzo P, Bianco S, Barrios E, Berois N, Navarrete H and Osinaga E: Tn antigen is a pre-cancerous biomarker in breast tissue and serum in n-nitrosomethylurea-induced rat mammary carcinogenesis. Int J Cancer 86: 753-759, 2000.
38. Ando H, Matsushita T, Wakitani M, Sato T, Kodama-Nishida S, Shibata K, Shitara K and Ohta S: Mouse-human chimeric anti-Tn IgG1 induced anti-tumor activity against Jurkat cells in vitro and in vivo. Biol Pharm Bull 31: 1739-1744, 2008.
39. Takahashi HK, Metoki R and Hakomori S: Immunoglobulin G3 monoclonal antibody directed to Tn antigen (tumor-associated alpha-N-acetylgalactosaminyl epitope) that does not cross-react with blood group A antigen. Cancer Res 48: 4361-4367, 1988.
40. Zhang M, Yao Z, Saga T, Sakahara H, Nakamoto Y, Sato N, Nakada H, Yamashina I and Konishi J: Improved intratumoral penetration of radiolabeled streptavidin in intraperitoneal tumors pretargeted with biotinylated antibody. J Nucl Med 39: 30-33, 1998.
41. Elhammer AP, Poorman RA, Brown E, Maggiora LL, Hoogerheide JG and Kézdy FJ: The specificity of UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase as inferred from a database of in vivo substrates and from the in vitro glycosylation of proteins and peptides. J Biol Chem 268: 10029-10038, 1993.