Abstract. Altered sialylation, closely associated with tumor progression and metastasis, has been implicated in human thyroid carcinoma. The present study investigated the alteration in expression of ST6GalNAcII involved in invasion and to clarify the possible mechanism of ST6GalNAcII in the metastasis process in human follicular thyroid carcinoma cell lines. Using real-time PCR, western blot and IHC analysis, ST6GalNAcII differed in three follicular thyroid cancer cell lines (FTC133, primary and FTC238, lung metastasis). It also showed differential expression in follicular thyroid carcinoma and tissue specimens. In addition, we analyzed the PI3K/Akt signaling pathway. The altered expression of ST6GalNAcII corresponded to changed invasive phenotype of FTC-238 and FTC-133 cells in vitro and in vivo. Further studies showed that regulating ST6GalNAcII expression markedly modulated the activity of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. The altered expression of ST6GalNAcII corresponded to changed invasive phenotype of FTC-238 and FTC-133 cells in vitro and in vivo. Further studies showed that regulating ST6GalNAcII expression markedly modulated the activity of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. Targeting the PI3K/Akt pathway by its specific inhibitor LY294002, or by Akt small interfering RNA (siRNA) resulted in reduced capacity in invasion of FTC-238. In conclusion, our results imply that ST6GalNAcII activated the invasion in follicular thyroid cancer cells through regulating the activity of PI3K/Akt pathway.

Introduction

Thyroid cancers are the most common malignant tumors of the endocrine system. Metastasis of tumor cells frequently contributes to the failure of treatments in patients diagnosed with thyroid cancer. Substantial research has been devoted to elaborating the relationship between glycan alterations and invasive properties of malignant cells. The changes of glycans on cell glycoproteins play a physiological role in regulating metastatic efficiency of tumor cells (1). It is known that alteration in cell surface sialylated antigens affects many cellular properties (2-5). Sialyltransferases, a subset of glycosyltransferases, have been recognized to be involved in various diseases by catalyzing the biosynthesis of different glycoconjugates and saccharide structures (6). They use CMP-Neu5Ac as an activated sugar donor to catalyze the transfer of sialic acid residues to terminal positions of glycoprotein and glycolipid carbohydrate groups (7).

Sialyltransferases (ST) are a group of enzymes responsible for the transfer of sialic acid from cytidine 5-prime monophospho-N-acetylneuraminic acid (CMP-NeuAc) to terminal positions of glycoprotein and glycolipid carbohydrate groups. ST consisting of 20 members that are subjected into three subfamilies: α-2, 3-sialyltransferases, α-2, 6-sialyltransferases, α-2, 8-sialyltransferases (8). α-2, 6-sialyltransferases mediate the transfer of sialic acid with an α-2, 6-linkage to it with terminal Gal (ST6Gal 1-2) (9) or GalNAc residues (ST6GalNAc 1-6). Changes in specific ST6GalNAc family expression have been reported to be altered in several tumors. High level of ST6GalNAcI expression was associated with the tumorigenicity of MDA-MB-231 breast cancer cells (10). Suppression of ST6GalNAcII mRNA is not only associated with the pathological phenotype of IgA nephropathy but also with the poor prognosis in IgA nephropathy patients (11,12). ST6GalNAcIV promotes lung cancer metastasis through adhesion to galectins (13). ST6GalNAc V plays a positive role in mediating brain metastasis of breast cancer cells (14).

The phosphatidylinositol-3-kinase (PI3K)/Akt pathway is one of the core intracellular signaling pathways, it plays a crucial role in many cellular processes including proliferation, differentiation, apoptosis, cell cycle progression, cell motility and tumorigenesis, tumor growth, angiogenesis (15,16). Furthermore, aberrant activation of PI3K/Akt pathway has been reported to be a significant indicator of proliferation, invasion, metastasis in thyroid cancer. Activation of PI3K/ Akt/mTOR pathway sustains malignant features of MTC cell models (17). The proliferation and invasion of thyroid cancer cells are inhibited by curcumin via downregulation of PI3K/Akt signaling pathway (18). However, it remained largely unknown whether there is a certain correlation between

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Key words: ST6GalNAcII, invasive properties, follicular thyroid carcinoma, PI3K/Akt signaling
ST6GalNAcII and PI3K/Akt pathway in the progression of invasion, metastasis in thyroid cancer.

Therefore, we undertook to characterize the expression of ST6GalNAcII in FTC-238 and FTC-133 cell lines and thyroid cancer tissue samples. Besides, we investigated the correlation between ST6GalNAcII and PI3K/Akt pathway and their role in the thyroid cancer metastasis.

Materials and methods

Cell culture and tissues. Human follicular thyroid carcinoma cell lines FTC-133 and FTC-238 were purchased from Guangzhou Jennio Biotech Co. (China). The cell lines were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium containing 2 mM glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified CO₂ incubator at 37°C.

All specimens were obtained from the General Surgery Department of The Second Affiliated Hospital of Dalian Medical University (Liaoning, China) and included 101 samples of follicular thyroid cancer and the corresponding peritumoral tissues (3 cm from the tumor edge). For the use of these clinical materials for research purposes, prior consents from the patients and approval from the Ethics Committees of The Second Affiliated Hospital of Dalian Medical University were obtained, and all the procedures have been performed in compliance with the Helsinki Declaration. All specimens had confirmed pathological diagnosis and were staged according to the 2013 thyroid carcinomas staging system of the International Union against Cancer (UICC). These tissues were snap-frozen in liquid nitrogen and stored at -80°C until used.

RNA isolation and real-time PCR analysis. Real-time PCR was used to analyze gene expression. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. Real-time PCR was carried out on an ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using QuantiTect SYBR Green PCR kit (Qiagen). The primer sequences used for amplification were as follows: forward, 5'-CTCTGCTGCTCTTGCTGG-3' for GAPDH. The relative expression level of target gene was normalized to that of the respective GAPDH.

Western blot analysis. Extracted proteins were electrophoresed under reducing conditions in 10% sodium dodecyl sulfate-polyacrylamide gels, and then blotted onto a polyvinylidene difluoride membrane. After blocking with 5% skimmed milk in PBS containing 0.1% Tween-20 (PBST), the membrane was incubated with antibody (1/1,000 diluted; Abcam) and then with peroxidase-conjugated anti-rabbit IgG (1/10,000 diluted; GE Healthcare UK Ltd., Little Chalfont, UK). A GAPDH antibody (1/200 diluted; Santa Cruz Biotechnology) was used as a control. All bands were detected using ECL Western Blot kit (Amersham Biosciences, UK), and the bands were analyzed with LabWorks™ (ver 4.6, UVP, Bio-Imaging Systems).

Deregulation of ST6GalNAcII by RNAi. FTC-238 cells were incubated in appropriate antibiotic-free medium with 10% fetal bovine serum (Gibco), transferred to a 6-well tissue culture, and incubated at 37°C in a CO₂ incubator to obtain 60-80% confluence. The cell cultures were stably co-transfected with a plasmid vector containing the puromycin-resistance marker and the specific short hairpin RNA (shRNA) (ST6GalNAcII), respectively, which was prepared according to the protocol. Scrambled shRNA was used as the negative control. The transfection efficiency calculated by the percentage of fluorescent cells was about 82%, and cell viability was 89% by trypan blue dye exclusion assay. Four weeks later, we used puromycin to screen the cells stably expressing shRNA. Several colonies were picked and expanded for further study. The knockdown had no effects on the cell morphology.

Overexpression of ST6GalNAcII. The human ST6GalNAcII coding sequences were obtained from Takara Company (Dalian, China) and were inserted into the pEGFP-N2 vector (Invitrogen, Carlsbad, CA, USA) at the sites of EcoRI, Xhol. Cells were transfected with 5 µg of target gene expression vector or empty vector (EV) in 100-mm dishes using PolyFect transfection reagent (Qiagen) according to the manufacturer's instruction. After 4 weeks of screening, the cell lines stably expressing ST6GalNAcII (FTC-133/ST6GalNAcII) and empty vector (FTC-133/mock) were established. Then cells were collected for gene expression assay and for further explorations. The cell transfection efficiency was 79% and the survival rate was 87%.

In vitro extracellular matrix invasion assays. Invasiveness of tumor cells was examined using 24-well Transwell units (Corning, Corning, NY, USA) with 8-mm pore size polycarbonate filter coated with Matrigel (BD Biosciences) to form a continuous thin layer. Cells (3x10⁵) were harvested in serum-free medium containing 0.1% BSA and added to the upper chamber. The lower chamber contained 500 µl 90% RPMI-1640 and 10% FBS. At the end of incubation, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. The filters were fixed in methanol and stained with Wright-Giemsa. Cells invading the Matrigel that reached the lower surface of the filter were counted with light microscope at a magnification of x400. In migration assay, the upper chamber was not coated with Matrigel. Samples were acquired in triplicate and data expressed as the average cell number in five fields.

In vivo tumorigenicity assay. The tumorigenicity of ST6GalNAcII in vivo was investigated using a xenograft tumor model in the nude mice. Forty-eight 5-week-old male athymic nude mice were provided with sterilized food and water and equally divided into three groups. Approximately, 1x10⁶ cells (with or without ST6GalNAcII shRNA interference and control shRNA) were subcutaneously inoculated into the right flank of each nude mouse. Once bearing palpable tumors (about 4 weeks after tumor cell inoculation), mice...
were sacrificed and their tumors were isolated, weighed, and photographed. Experiments were repeated three times.

**Inhibition of the PI3K/Akt signaling.** LY294002 (Sigma) was used to suppress the activity of the PI3K/Akt signaling in FTC-238 cells. Briefly, the tumor cells (1x10^4 cells/well) were incubated with dimethyl sulfoxide (DMSO) or the PI3K inhibitor LY294002 (20 mmol/l) dissolved in DMSO, and collected after 24 h. Tumorigenicity was analyzed when PI3K/Akt signaling was blocked in xenograft tumor model. Sixty female athymic nude mice (5-week-old) were divided into 4 groups and 1x10^7 FTC-238 cells (with DMSO, LY294002, control shRNA, Akt shRNA, respectively) were injected subcutaneously into the axillary regions of each nude mouse, respectively. Once bearing palpable tumors (about 4 weeks after tumor cell inoculation), mice were sacrificed and their tumors were isolated and weighed. Changes in protein expression were measured by western blot analysis.

**Immunohistochemical (IHC) staining analysis.** Tumors were removed from the mice and immunohistochemical (IHC) staining was conducted using formalin-fixed paraffin-embedded sections of tissues by the avidin-biotin-peroxidase complex (ABC) method. Four-micron sections of formalin-fixed paraffin-embedded tissues were cut with a microtome and dried overnight at 37˚C on a silicanized slide (Dako, Carpinteria, CA, USA). Samples were deparaffinized in xylene at room temperature for 80 min and washed with a graded ethanol/water mixture and then with distilled water. The samples were soaked in a citrate buffer and then microwaved at 100˚C for 10 min. The following steps were used. Before addition of the primary antibodies, endogenous peroxidase activity was blocked by incubation in methanol containing 1% H2O2 for 20 min, followed by 60 min incubation with normal donkey serum to reduce background staining. The primary antibodies, goat anti-human, ST6GalNAcII antibodies (Abcam, Cambridge, UK), were incubated at 4˚C for 8 h, followed by incubation with the biotinylated secondary antibodies (donkey anti-goat IgG; Santa Cruz Biotechnology) for 30 min and ABC complex for 30 min. The primary and secondary antibodies were used at 1:80 and 1:100 dilutions, respectively. The peroxidase binding sites were demonstrated by the diaminobenzidine method. A phosphate-buffered solution instead of the primary antibody was used in the protocols for negative controls. The level of expression level is measured by Image-Pro Plus software 6.0.

**Statistical analysis.** Each experiment was performed at least in triplicate, and the measurements were performed in three independent experiments. The data were expressed as mean ± standard deviation (SD) from the triple tests of each group. SPSS 17.0 software was used for statistical analysis and Student's t-test was selected to determine the significance of differences among the examined groups. P<0.05 was considered to be statistically significant.

**Results**

**Differential expression of ST6GalNAcII in follicular thyroid carcinoma cell lines.** Real-time PCR and western blot analysis was used to evaluate the expression level of ST6GalNAcII in mRNA (Fig. 1a) and protein expression (Fig. 1b). These data indicated that ST6GalNAcII may be associated with metastasis of human follicular thyroid carcinoma cells (FTC-238).

**Silence of ST6GalNAcII inhibits the invasive ability of follicular thyroid cancer cells in vitro and in vivo.** Owing to the high expression of ST6GalNAcII in FTC-238 cells, we silenced ST6GalNAcII with shRNA, in order to elucidate
the effect of ST6GalNAcII on the invasion and metastasis of thyroid cancer cells. As shown in Fig. 2a and b, the expression level of ST6GalNAcII was significantly reduced in FTC-238 transfectants compared to the control transfectants (P<0.05).
After ST6GalNAcII shRNA transfection, Transwell invasion assay was performed to further evaluate the invasion capability of cells with ST6GalNAcII knockdown on tumor cells in vitro. The invasion capability of FTC-238 cells

Figure 3. Overexpression of ST6GalNAcII gene enhances the invasive ability of FTC-133 cells both in vitro and in vivo. (a and b) After full-length sequence transfection, ST6GalNAcII mRNA and protein were increased notably in FTC-133 cells by real-time PCR and western blotting. (c) The invasiveness of cells was analyzed. FTC-133/ST6GalNAcII cells were significantly more invasive (P<0.05) than the FTC-133 and FTC-133/mock cells. (d) Tumor volumes were increased obviously in nude mice bearing FTC-133/ST6GalNAcII, as compared to the control group (P<0.05). (e) Upregulation of ST6GalNAcII was also shown by IHC staining in xenograft tumors derived from FTC-133/ST6GalNAcII cells (x400) (P<0.05). Data are the means ± SD of triplicate determinants (P<0.05).
transfected with ST6GalNAcII shRNA showed a significant reduction, as compared with control shRNA group, suggesting that cell invasion capability was inhibited by treatment with ST6GalNAcII shRNA (Fig. 2c).

Nude mice bearing FTC-238, FTC-238-control shRNA and FTC-238-ST6GalNAcII shRNA1 xenografts were used to analyze the differences by measuring tumor volumes. Fig. 2d showed a significant reduction of mean tumor volume in nude mice bearing FTC-238-ST6GalNAcII shRNA, as compared with control shRNA group.

IHC staining analysis of the tumor section revealed that ST6GalNAcII was reduced in tumors derived from FTC-238-ST6GalNAcII shRNA cells compared to control group (Fig. 2e).

The above results indicated that knockdown of ST6GalNAcII could inhibit the invasion ability of follicular thyroid carcinoma cells.

Overexpression of ST6GalNAcII enhances the invasive ability of follicular thyroid cancer cells in vitro and in vivo. After elucidating whether the effect of ST6GalNAcII suppresses the invasion ability of FTC-238, we transfected FTC-133 cells with ST6GalNAcII expression vector to determine the effect of overexpression of ST6GalNAcII on tumor cell invasion ability of FTC-133. As shown in Fig. 3a and b, increased levels of mRNA and protein of were detected in FTC-133 transfectants.

Transwell invasion assay revealed that the invasion capability of FTC-133 cells transfected with ST6GalNAcII

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Transwell invasion assay revealed that the invasion capability of FTC-133 cells transfected with ST6GalNAcII

Figure 4. ST6GalNAcII gene mediates the activity of PI3K/Akt signaling pathway in FTC-238 and FTC-133 cell lines. (a) Expression of PI3K/Akt/NF-κB signaling molecules were downregulated at protein levels with ST6GalNAcII shRNA1 transfection in FTC-238-ST6GalNAcII shRNA1 cells (*P<0.05). (b) The increased protein levels of PI3K/Akt/NF-κB signaling molecules were determined by western blotting in FTC-133/ST6GalNAcII cells (*P<0.05). Data are the means ± SD of triplicates (*P<0.05).
expression vector was obviously increased compared with the FTC-133/mock cells (Fig. 3c).

Nude mice were inoculated with tumor cells FTC-133, FTC-133/mock, and FTC-133/ST6GalNAcII. Tumor volumes were increased obviously in nude mice bearing FTC-133/ST6GalNAcII, as compared to the FTC-133/mock group (Fig. 3d).

High expression levels of ST6GalNAcII in tumor cells of FTC-133/ST6GalNAcII, was also detected using IHC staining, as shown in Fig. 3e.

Therefore, the upregulation of the ST6GalNAcII gene was able to increase the invasion ability of follicular thyroid carcinoma cells.

ST6GalNAcII regulates the activity of PI3K/Akt signaling pathway in thyroid cells. Having established the pivotal role of PI3K/Akt pathway in tumor cells, we investigated whether ST6GalNAcII activated the PI3K/Akt pathway and whether this pathway played a central role in ST6GalNAcII-mediated cell invasion. Fig. 4a shows that following the decreased expression level of ST6GalNAcII, the expression and activity of the PI3K/Akt pathway was inhibited. PI3K expression decreased the protein and phosphorylation levels of Akt. The degree of phosphorylation of Akt at Ser473 and Thr308 and its downstream effector NF-κB was also downregulated after ST6GalNAcII silencing. No variation could be detected in the total amount of Akt protein, indicating a true decrease in the
phosphorylation status. In addition, as illustrated in Fig. 4b, over-expression of ST6GalNAcII in FTC-133 cells showed the reverse tendency. The above suggested that the variation of ST6GalNAcII expression levels alters the PI3K/Akt signaling pathway.

**PI3K/Akt inhibition mediates the invasion ability of FTC-238 cells both in vitro and in vivo.** After inhibition of PI3K and Akt by LY294002 and Akt siRNA, the invasion ability was significantly inhibited in FTC-238 cells. By western blotting, FTC-238 cells treated with LY294002, and Akt siRNA treatment exhibited apparently decreased expression levels of the main signal molecules of PI3K/Akt pathway (Fig. 5a). As shown in Fig. 5b, the inhibition of PI3K/Akt pathway made the FTC-238 cells less invasive. In Transwell invasion assay, the invasive capability of FTC-238 cells transfected with LY294002 and Akt siRNA was obviously decreased in the Transwell migration and invasion assay. Similar results were also observed in vivo analysis where reduced tumor weight was measured in the mouse group bearing FTC-238 tumors with impaired PI3K/Akt signaling (Fig. 5b). Altered expression levels of the main signal molecules of the PI3K/Akt pathway in the mouse group bearing FTC-238 tumors with LY294002 or Akt shRNA treatment were also validated using IHC staining. These data implicated a role of PI3K/Akt signaling in regulating the invasive properties of FTC-238 cells.

Clinical implications of ST6GalNAcII expression in thyroid carcinoma. The ST6GalNAcII expression status was detected in thyroid cancer with the corresponding pericarcinomatous tissue samples by immunohistochemistry staining (Table I). The data as shown in Table I are number of cases, and the expression of ST6GalNAcII was classified as high if >30% of tumor cells are stained and as low if <30% of cancer cells were stained. It is shown that follicular thyroid cancer tissues had a higher expression level of ST6GalNAcII compared with transitional tissues (P=0.004). There was no significant association between ST6GalNAcII expression and age, or distant metastasis in follicular thyroid carcinoma patients (P>0.05). Interestingly, the expression of ST6GalNAcII was also closely correlated with histological grade, lymph node metastasis, and clinical stage (P=0.004, 0.002, 0.006, respectively).

**Discussion**

Metastasis remains the major cause of mortality and relapse for most solid malignancies. Some research is currently underway to identify the signaling pathways and molecular mechanisms of metastasis in thyroid cancer (19-22). In this study, we investigated the expression of ST6GalNAcII, via PI3K/Akt signaling pathway, to assess whether it effectively regulated the invasiveness of follicular thyroid carcinoma cell lines FTC-238 (lung metastasis) and FTC236 (lymph node metastasis) and FTC-133 (primary tumor). We further analyzed the differential expression of ST6GalNAcII, which was reported to be related with clinicopathological characteristics of human follicular thyroid cancer.

Alteration in the expression pattern of glycogen synthase kinase 3 correlates with the invasive potential of various types of cancer (23,24). The biosynthetic pathway of sialylated glycans highlights the importance of sialyltransferases. Using real-time PCR analysis, we revealed that the expression profile

<table>
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<th>Characteristics</th>
<th>n</th>
<th>ST6GalNAcII(high) (%)</th>
<th>ST6GalNAcII(low) (%)</th>
<th>P-value</th>
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of ST6GalNAcII gene was remodeled between FTC-238 and FTC-133 and FTC-236. As depicted in Fig. 1a, FTC-238 cells showed a higher expression of ST6GalNAcII mRNA compared to FTC-133 and FTC-236 cells. This result suggested that ST6GalNAcII gene was active in the cells with high metastatic potential. Our previous report also indicated the crucial role of ST6GalNAcII gene in promoting high metastatic potential cell invasion in breast carcinoma (25). The altering expression of ST6GalNAcII gene in the two follicular thyroid cell lines may be more important as indicators and functional regulators of tumor metastasis.

Recent findings show that the overactivation of the PI3K/Akt signaling pathway plays a crucial role in regulating tumor invasive ability (26,27). We investigated the molecular mechanism by which ST6GalNAcII-mediated PI3K/Akt signaling pathway regulates follicular thyroid cancer cells invasiveness. Our study explored a novel mechanism that the invasion and chemosensitivity of human hepatocarcinoma cells can be regulated by the activation of ST6Gal1 or ST8Sia2-mediated PI3K/Akt (28). In this study, we indicated that ST6GalNAcII exerts the role of tumor metastasis of human follicular thyroid carcinoma through activation of the PI3K/Akt/NF-κB signal pathway. We demonstrated that FTC-238 cells exhibited higher PI3K/Akt activity than FTC-133. In addition, the invasive properties of FTC-238 cells were reversed by the inhibition of the PI3K/Akt pathway (Fig. 2e). These results indicated that ST6GalNAcII-mediated follicular thyroid carcinoma cell invasion was, at least in part, PI3K/Akt-dependent.

With altered mRNA expression in carcinoma tissues, sialyltransferases are regarded as prognostic factors and potential targets for therapeutic approaches (29,30). In this study, we utilized immunohistochemistry to evaluate protein expression of ST6GalNAcII in follicular thyroid cancer specimens of 101 cases. The result illustrated that follicular thyroid cancer tissues had a higher expression level of ST6GalNAcII than in the normal thyroid tissues (Table I). In addition, we found the expression of ST6GalNAcII was associated with histological grade, lymph node metastasis and clinical stage (Table I). The results from the clinical samples indicated that the altered level of ST6GalNAcII may play an important role in promoting invasion and metastasis of follicular thyroid cancer. Furthermore, it may be possible to utilize ST6GalNAcII as a useful biomarker for clinical diagnosis of thyroid cancer metastasis.

In conclusion, our work reveals differential expression of ST6GalNAcII gene in two human follicular thyroid carcinoma cell lines and follicular thyroid cancer specimens. ST6GalNAcII elucidated the unusual properties of invasion in thyroid cancer cells via modulating the PI3K/Akt signaling pathway. Besides, the elevated expression of ST6GalNAcII was associated with histological grade, lymph node metastasis and clinical stage of follicular thyroid cancer. Seeking for agents that simultaneously inhibit ST6GalNAcII gene may be a promising strategy for blocking follicular thyroid carcinoma metastasis in patients.


