Effect of *N*-acetylglucosaminyltransferase V on the expressions of other glycosyltransferases

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Received 6 December 2003; revised 4 January 2004; accepted 11 February 2004

First published online 28 February 2004

Edited by Veli-Pekka Lehto

Abstract Transfection of sense cDNA of *N*-acetylglucosaminyltransferase V (GnTV) into H7721 human hepatocellular carcinoma cells resulted in the decreased expression of surface sialyl Lewis X (SLe^x), a sialylated fucose-containing antigen. The enzymatic mechanisms were speculated to be the concomitantly decreased expression of α1,3-fucosyltransferase (FucT)-III, -VI, -VII and the branching enzyme of *O*-glycans, core 2-β1,6-*N*-acetylglucosaminyltransferase (C2GnT)-I, -II. These two glycosyltransferase families were suggested to be the key enzymes in the synthesis of SLe^x. The expression of α2,3-sialyltransferase (ST3)-IV, but not ST3-I, -II and -III was elevated by sense GnTV. However, it did not cause the increase of SLe^x synthesis. Transfection of antisense GnTV into H7721 cells showed entirely opposite effects on the expression of above-mentioned SLe^x and glycosyltransferases as the sense GnTV.

Key words: *N*-acetylglucosaminyltransferase V; Sialyl Lewis X; α1,3-Fucosyltransferase; Sialyltransferase; Core 2-β1,6-*N*-acetylglucosaminyltransferase

1. Introduction

Alteration in the glycan structure on cancer cell surface is closely related to malignant behaviors such as invasion and metastasis. At least two surface glycan structures are frequently reported to closely associate with cancer metastasis. One is the *N*-acetylglucosamine (GlcNAc) β1,6-mannose-α1,6 branching structure on *N*-glycan of glycoproteins [1,2], which is synthesized by *N*-acetylglucosaminyltransferase V (GnTV), a well-known cancer-associated enzyme which is upregulated in many cancers [1–6]. We found that the in vitro metastatic potential (cell adhesion to laminin, cell migration and invasion through artificial membrane) of a human hepatocarcinoma cell line, H7721, was promoted and attenuated upon transfection of the sense and antisense cDNAs of GnTV respectively [7]. Moreover, transfection of oncogene (*H-ras* or *v-sis*) or anti-metastasis gene (*nm23*-H1) into H7721 cells resulted in the concomitant increase or decrease, respectively, in both GnTV expression and the metastatic potential [8,9]. The upregulation of GnTV expression is always accompanied by the increase in the amount of its product, β1,6-GlcNAc branch of *N*-glycans on the cell surface, indicating that the effect of GnTV is very likely mediated by this structure [7–9]. A second type of glycan structure related to metastasis is sialyl Lewis sugar antigens, including sialyl Lewis X (SLe^x) [sialic acid (SA)-α2,3-Gal-β1,4 (fucose-α1,3) GlcNAc-]; sialyl dimeric (difucosyl) Lewis X (SDLe^x) [SA-α2,3-Gal-β1,4 (fucose-α1,3) GlcNAc-β1,3-Gal-β1,4 (fucose-α1,3) GlcNAc-β1,3-] and sialyl Lewis A (SLe^a) [SA-α2,3-Gal-β1,3 (fucose-α1,4) GlcNAc-]. They are mainly located in the termini of glycans in glycolipids and *O*-glycans of glycoproteins, and also in the outer chains of *N*-glycans. Their sialylation and fucosylation steps are catalyzed by α2,3-sialyltransferase (α2,3-ST) and α1,3-fucosyltransferase (α1,3-FucT) respectively. Sialyl Lewis antigens are supposedly involved in the process of cancer metastasis, since these compounds serve as ligands of E- or P-selectin expressed on the surface of vascular endothelial cells. Thus, they could mediate the adhesion of malignant cells to vascular endothelium [10,11]. Patients with cancer cells highly expressing SLe^x or SLe^a have a significantly higher risk to develop hematogenous metastasis [12–14]. Our group demonstrated that surface SLe^x was upregulated on H7721 cells upon transfection of the metastasis-promoting gene *c-erb*B2/*neu* [15], or treatment of epidermal growth factor (EGF) and phorbol ester [16]. It was downregulated by metastasis-suppressive gene *nm23*H1 [17], *all-trans* retinoic acid and cyclic adenosine monophosphate (AMP) [16]. In addition, transfection of α1,3-FucT-VII into H7721 cells to increase the synthesis of SLe^x antigen led to the enhancement of the metastatic potential [15].

However, the relation between GnTV and α1,3-FucT or their products, β1,6-GlcNAc branch and sialyl Lewis antigens is still unresolved. Are these two enzymes or two products independent of each other in the development of cancer metastasis? Theoretically, the increased antennary (outer chain) number of *N*-glycans by GnTV should provide more substrates for the synthesis of sialyl Lewis antigens at the chain termini, resulting in the increase of these antigens on the cell surface. In the present investigation, the expression of surface SLe^x was determined after H7721 cells were transfected with sense or antisense cDNA of GnTV, since SLe^x is the predom-

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Abbreviations: Gal, galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; SA, sialic acid; GnTV, *N*-acetylglucosaminyltransferase V; FucT, fucosyltransferase; ST, sialyltransferase; ST3Gal, cytidine monophosphate-*N*-acetylneuraminate:β-galactoside α2,3-sialyltransferase; ST6Gal, cytidine monophosphate-*N*-acetylneuraminate:β-galactoside α2,6-sialyltransferase; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell spectra; RT-PCR, reverse transcriptase-polymerase chain reaction; MAA, Maackia amurensis agglutinin; SNA, Sambucus nigra agglutinin; HRP, horseradish peroxidase; PBS, phosphate-buffered saline

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doi:10.1016/S0014-5793(04)00188-7

inant Lewis antigen on H7721 cell surface [15,17]. To our surprise, it was found that sense GnTV reduced, while antisense GnTV enhanced the expression of SLe^x. Then the effect of GnTV on the expressions of α1,3-FucT and cytidine monophosphate-*N*-acetylneuraminate:β-galactoside α2,3-sialyltransferase (ST3Gal), two glycosyltransferase families directly involved in SLe^x synthesis, was studied. In addition, core 2-β1,6-*N*-acetylglucosaminyltransferase (C2GnT), a glycosyltransferase needed for the branch formation of *O*-glycans in glycoproteins was also investigated. It has been reported to be a critical enzyme for the synthesis of SLe^x precursor [18], and SLe^x located predominantly at the branching termini of *O*-glycans.

2. Materials and methods

2.1. Materials

H7721 cell line was obtained from the Institute of Cell Biology, Academia Sinica. RPMI-1640 medium and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco/BRL. KM93 (anti-SLe^x monoclonal antibody) was a product of Seikagaku Company in Japan. Lectin Maackia amurensis agglutinin (MAA) and Sambucus nigra agglutinin (SNA), fluorescein isothiocyanate (FITC)-conjugated second antibodies (goat anti-mouse IgM) and horseradish peroxidase (HRP) were purchased from Sigma. Trizol and AMV reverse transcriptase were from Promega. Other reagents, including Taq enzyme, RNase inhibitor, deoxyribonucleoside triphosphate (dNTP), oligo(dT)-18 were commercially available in China.

The plasmid containing sense cDNA of GnTV (pcDNA3FluHuTV, 7.75 kb) was a kind gift from Prof. Pierce at the University of Georgia, USA. The characterization of this plasmid and the construction of antisense cDNA of GnTV were described in our previous papers [19,20]. The constructs containing sense and antisense cDNA of GnTV, named GnTV-S and GnTV-AS respectively, and the vector pcDNA3 were transfected into H7721 cells using the electroporation method as described previously [19,20]. The GnTV-S-transfected cells (GnTV-S/H7721) were characterized by the appearance of the transcript of exogenous GnTV-S cDNA and the increase of GnTV activity. Similarly, the cells transfected with GnTV-AS (GnTV-AS/H7721) were characterized by the appearance of the transcript of GnTV-AS cDNA, the decrease of endogenous GnTV mRNA and the GnTV activity [19,20].

2.2. Cell culture and treatment

Cells were cultured at 37°C, 5% CO_2 in RPMI-1640 medium containing 10% fetal calf serum (FCS), penicillin and streptomycin as previously described [19,20].

2.3. Detection of Lewis antigen SLe^x with flow cytometry

The cells (1×10^6) were detached with 2 mM ethylenediamine tetraacetic acid (EDTA), washed and resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), then incubated with 1:50 diluted monoclonal antibody to SLe^x (KM93) for 30 min at 4°C. After being washed twice, the cells were incubated for 45 min at 4°C with 1:200 diluted FITC-conjugated secondary antibody, then the cells were washed again and 1×10^4 cells/sample were subjected to flow cytometry for fluorescence analysis [15–17]. A '(−) Control' sample without KM93 was set up in each run to normalize the assay condition. Fluorescence-activated cell spectra (FACS) were drawn automatically, and the left or right shift of the curve indicated the decrease or increase of the mean fluorescence intensity (MFI) respectively, as indicated by the 'M1' bar in the figures.

2.4. Determination of the expression of glycosyltransferase mRNAs with reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cell RNA was extracted with Trizol and the complementary DNAs (cDNAs) were synthesized with oligo(dT)-18 primer and AMV reverse transcriptase from 3 μg total RNA. The RT-PCR was performed in 50 μl of reaction mixture containing 5 μl cDNA, 0.2 μM of the primer pair of glycosyltransferases or β-actin (internal standard) according to the manual. The cDNA was subjected to denaturation at 95°C for 5 min, followed by 28 cycles (94°C, 61.5°C and 72°C, 1 min for each) of PCR, and incubated at 72°C for 10 min and 4°C for 5 min. Then 10 μl products were applied to agarose gel electrophoresis. The amplified DNA bands were scanned and the photographs were analyzed with NIH Image software. The semiquantitative data were obtained by the intensity ratios of the glycosyltransferase bands to the β-actin band. For each glycosyltransferase, three experiments were performed and the results were reproducible. The primers were as follows: FucT-III F: 5′-CCTCCCGACAGGACACCACTCC-3′, R: 5′-GCGTCCGTACACGTCCACCTGG-3′ [21]; FucT-VI F: 5′-AATGGGTCCCGCTTCCCAGACAG-3′, R: 5′-GCGTCCGTACACGTCCACCTTG-3′ [21]; FucT-VII F: 5′-CACCTCCGAGGCATCTTCAACTG-3′, R: 5′-CGTTGGTATCGGCTCTCATTCATG-3′ [21]; ST3Gal-I F: 5′-TATGGGCCTGAGATAGACAGTCAC-3′, R: 5′-GATCCGGATTTTATTGATGGAGGC-3′ [21]; ST3Gal-II F: 5′-GAGAACATGGATCTTCCACCGG-3′, R: 5′-GGCTGGGTTGTAGATCTGGACC-3′ [21]; ST3Gal-III F: 5′-ATGGAGGCGTTCTTGCCAACAAG-3′, R: 5′-ATGCGAACGGTCTCATAGTAGTG-3′ [21]; ST3Gal-IV F: 5′-TTGAACAATGCCCCAGTGG- CTGG-3′, R: 5′-TCTTGGGAGACATTATGGCCTGAC-3′ [21]; cytidine monophosphate-*N*-acetylneuraminate:β-galactoside α2,6-sialyltransferase (ST6Gal)-I F: 5′-GAGAGCATTAGGACCAAGGCTGG-3′, R: 5′-ACGTCAGTCTTGCGCTTGGATGG-3′ [21]; C2GnT-I F: 5′-GCAATGAGTGCAAACTGGAAGT-3′, R: 5′-AATTGCCCGTAATGGTCAGTGTT-3′ [22]; C2GnT-II F: 5′-TCAGGGGTCACCCGAGGGGACCAAG-3′, R: 5′-CTCCACCTCTTCTTTGCTCAGTGG-3′ [23]; β-actin F: 5′-GATATCGCCGCGCTCGTCGTCGAC-3′, R: 5′-CAGGAAGGAAGGCTGGAAGA- GTGC-3′ [21].

2.5. Analysis of α2,3-sialyl and α2,6-sialyl residues at the glycan terminal of cell protein

Cells were homogenized in HEPES buffer (pH 7.2) containing 2% Triton X-100 with a brief sonification, and centrifuged at $1000\times g$ at 4°C for 15 min. Protein concentration was determined by Lowry's method [24]. Western blot was performed with a modified method of Marone et al. [25] as described in our previous papers [9,15]. 60 μg of the protein samples was separated by 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% fat-free dried milk in PBS/0.05% Tween-20 at 4°C overnight and then incubated for 3 h with 1:100 dilution of MAA-HRP or SNA-HRP in 5% fat-free dried milk/0.05% Tween-20 in PBS. The lectin-HRP conjugates were prepared by the method of Wilson and Nakane [26]. Finally, the membrane was stained with the enhanced chemiluminescence (ECL) reagent and put under X-ray film for exposure. Three experiments were performed and the results were reproducible.

3. Results

3.1. Expression of surface SLe^x on GnTV-S- and GnTV-AS-transfected H7721 cells

Fig. 1 shows the expression of surface SLe^x as determined by flow cytometry. The mock (vector)-transfected H7721 cells expressed a moderate amount of SLe^x. On the surface of GnTV-S-transfected cells, the amount of SLe^x was decreased to 45.4% of the 'Mock' value ($P<0.01$). In antisense transfected cells, on the other hand, it was elevated to 177.3% compared to the 'Mock' cells ($P<0.01$).

3.2. Effect of GnTV on the expressions of α1,3-FucT subtypes

Up to date, six subtypes of human α1,3-FucTs have been cloned [27–32]. Four of them, α1,3-FucT-III, -V, -VI, -VII efficiently fucosylate sialylated acceptors and produce sialyl Lewis antigens, while α1,3-FucT-V and -IX prefer neutral acceptors and form non-sialyl Lewis antigens as their products. α1,3-FucT-III has both α1,3 and α1,4 fucosylation activities, leading to synthesis of both α1,3-fucosyl-containing Le^x or SLe^x, and α1,4-fucosyl-containing SLe^a, while α1,3-FucT-VII catalyzes the synthesis of SLe^x only. The gene for α1,3-FucT-V was reported to be a silent gene and rarely expressed

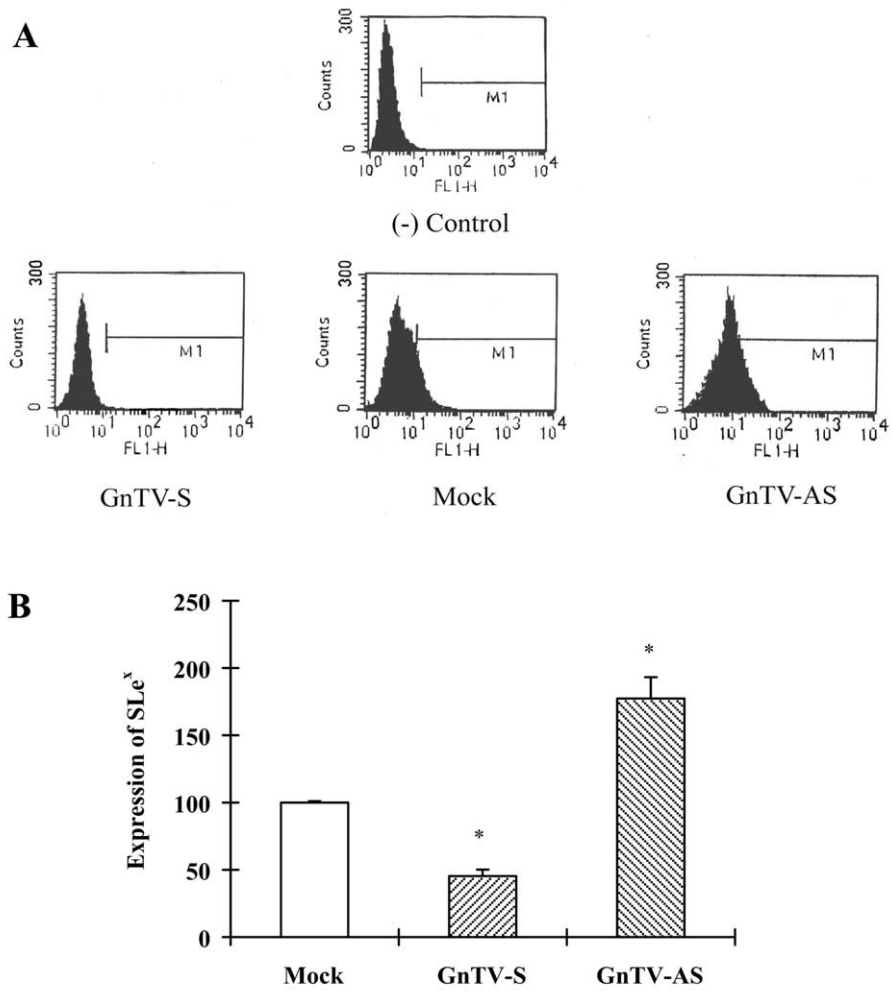

Fig. 1. Alteration of surface SLe^x expression on GnTV-S/H7721 and GnTV-AS/H7721 cells. A: FACS of SLe^x antigen. B: Relative expressions of SLe^x antigen in differently transfected cells (set the MFI value of 'Mock' as 100%). *: $P < 0.01$; $n = 3$. (−) Control: Sample without the addition of KM93; Mock: H7721 cells transfected with pcDNA3 vector; GnTV-S: H7721 cells transfected with pcDNA3/GnTV-S plasmid; GnTV-AS: H7721 cells transfected with pcDNA3/GnTV-AS plasmid. SLe^x: sialyl Lewis X. The experimental procedure was described in Section 2.

in tissue [33]. In untreated parent H7721 cells, α1,3-FucTs mRNA appear in order IV > III > VI > VII > IX, but the main enzymes responsible for SLe^x synthesis are considered to be α1,3-FucT-VI and -VII [34]. Therefore, the effects of GnTV on the expressions of α1,3-FucT-III, -VI and -VII were studied.

In Fig. 2, the expressions of α1,3-FucT-III, -VI and -VII in the cells are shown as determined by using RT-PCR. The products of α1,3-FucT-III, -VI, -VII and β-actin were 521, 534, 497 and 789 bp, respectively, in consistency with the reported lengths [21]. In mock-transfected cells, the expressions of α1,3-FucT were III > VI > VII, a result compatible with that using real-time PCR [34]. Interestingly, the expressions of all the three assayed α1,3-FucTs were significantly decreased in GnTV-S/H7721 cells ($P < 0.01$), representing only 37.6, 45.0 and 52.5% of the 'Mock' levels respectively. On the other hand, expressions of α1,3-FucT-III, -VI and -VII were remarkably elevated in GnTV-AS/H7721, up to 185.2, 223.0 and 187.5% of the 'Mock' value, respectively ($P < 0.01$).

3.3. Effect of GnTV on the expressions of STs

STs are divided in four specific subfamilies based on their substrate specificities and the types of the formed sialyl linkages in the products (such as α2,3, α2,6 and α2,8) [35,36]. In this investigation we concentrated to study the expression of ST3Gal because of its critical role in the synthesis of SLe^x. The ST3Gal subfamily transfers the sialyl group from cytidine monophosphate-*N*-acetylneuraminate to the galactose residue of glycans, forming a α2,3 linkage. ST3Gal can be further divided into five subtypes. The substrates of types I and II are the Gal-β1,3-*N*-acetylgalactosamine (GalNAc) sequence on *O*-glycans and glycolipids respectively, while the preferred substrates of ST3Gal-III and -IV are Gal-β1,3-GlcNAc- (precursor of SLe^a) and Gal-β1,4-GlcNAc- (precursor of SLe^x), respectively. Type V was evidenced as a ganglioside GM3 synthase. The expressions of types I–IV were studied in GnTV-S/H7721 and GnTV-AS/H7721 cells.

In Fig. 3 the expression levels of ST3Gal-I, -II, -III, -IV in the cells are shown as determined by using RT-PCR. The products of ST3Gal-I, -II, -III, and -IV were 543, 483, 529,

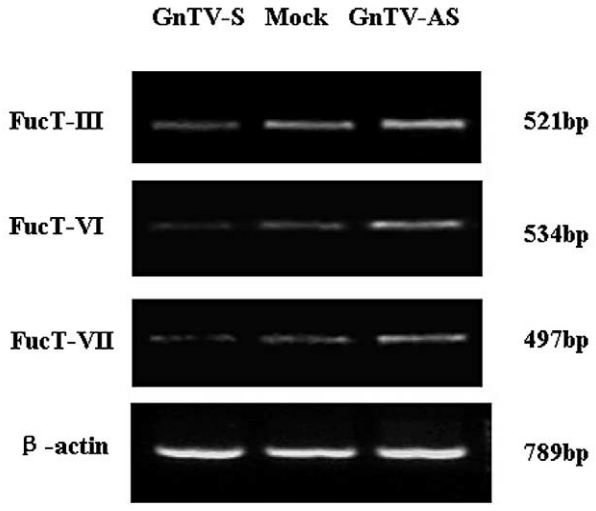

Fig. 2. Analysis of the expressions of α1,3-FucT-III, -VI and -VII in GnTV-S/H7721 and GnTV-AS/H7721 cells using RT-PCR.

and 503 bp, also in agreement with the reported lengths [21]. Only ST3Gal-IV was expressed in an apparent amount. Its level of expression varied so that it was increased to 216.7% in GnTV-S/H7721 and decreased to 25.3% in GnTV-AS/H7721 cells when compared with the controls.

In order to study whether the change of ST expression in GnTV-S- and GnTV-AS-transfected cells was specific for the ST3Gal subfamily, the expression of ST6Gal as a control glycosyltransferase for ST3Gal was also observed after the transfection of GnTV-S and GnTV-AS. To date, two subtypes, ST6Gal-I [35,36] and ST6Gal-II [37] were reported. The ubiquitously expressed ST6Gal-I was selected for study. The level of ST6Gal-I expression was the same in GnTV-S-, GnTV-AS- and mock-transfected cells (data not shown).

3.4. Effect of GnTV on the amount of α2,3- and α2,6-sialyl residues at the glycan termini of cell proteins

We next studied whether the alteration of ST3Gal-IV expression brings about an expected change in the amount of sialyl residues on surface glycans (Fig. 4). Results by using

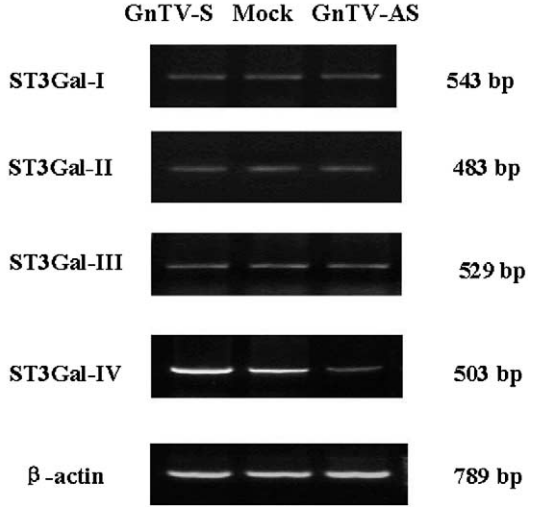

Fig. 3. Analysis of the expressions of four ST3Gals in GnTV-S/H7721 and GnTV-AS/H7721 cells using RT-PCR.

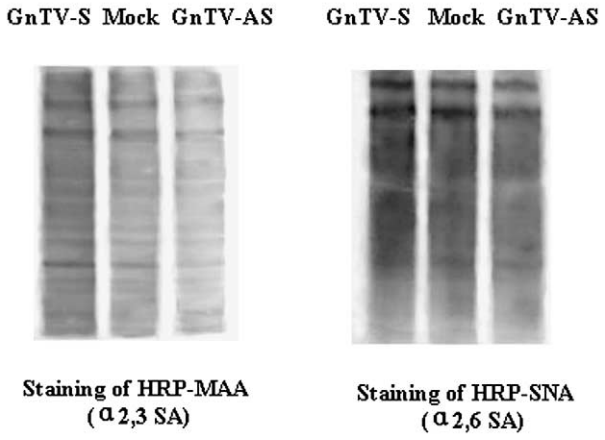

Fig. 4. Western blot analysis and lectin staining of α2,3- and α2,6-linked SA at the glycan termini of glycoproteins in GnTV-S/H7721 and GnTV-AS/H7721 cells.

HRP labeled MAA and SNA as specific probes for α2,3- and α2,6-linked sialyl residues [38,39] showed that the staining intensity of HRP-SNA (specific for α2,6-SA) was higher than that of HRP-MAA (specific for α2,3-SA) in all three types of the transfected cells. The intensity of α2,3-SA was approximately doubled on GnTV-S-transfected cells ($P < 0.01$), but was decreased to about the half level on GnTV-AS-transfected cells ($P < 0.01$) as compared to the 'Mock' cells. These findings were consistent with the results concerning the expression of ST3Gal-IV in the same cells. The results suggest that the increased and decreased terminal α2,3-SA residues on glycoproteins on GnTV-S/H7721 and GnTV-AS/H7721 cells, respectively, were the consequence of the altered expression of ST3Gal-IV. On the other hand, the intensity of α2,6-SA showed no apparent alteration among differently transfected cells.

3.5. Effect of GnTV on the expressions of C2GnT subtypes

C2GnT is a family of glycosyltransferases that catalyze the synthesis of the branching core 2 and core 4 of *O*-glycans [40,41]. These enzymes transfer the GlcNAc group from UDP-GlcNAc to the innermost GalNAc residue in the sequence of Gal-β1,3-GalNAc-Ser/Thr (precursor of core 2) or GlcNAc-β1,3-GalNAc-Ser/Thr (precursor of core 4) and form a branching GlcNAc-β1,6-GalNAc linkage. In this study we

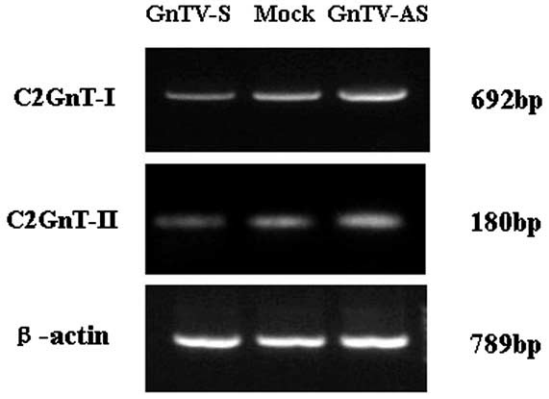

Fig. 5. Analysis of C2GnT-I and C2GnT-II expressions in GnTV-S/H7721 and GnTV-AS/H7721 cells using RT-PCR.

explored the expressions of C2GnT-I and -II as two representatives of the C2GnT family with different substrate specificities.

In Fig. 5 the expressions of C2GnT-I and -II are shown as determined by using RT-PCR. The products of C2GnT-I and C2GnT-II were 692 and 180 bp, respectively, compatible with earlier reports [22,23]. The expression of the former in H7721 cells was higher than the latter. The levels of C2GnT-I and -II reduced to 45.5 and 54.0% of the 'Mock' value ($P < 0.01$) in GnTV-S/H7721, and elevated to 190 and 160% of the 'Mock' level ($P < 0.01$) in GnTV-AS/H772 cells. Thus, the changes in the expression levels closely correlated with those of α1,3-FucTs.

4. Discussion

The findings in this study demonstrated that transfection of sense and antisense cDNA of GnTV was accompanied by changes in the expression levels of other glycosyltransferases related to SLe^x synthesis. On the other hand, we have previously shown that there is an elevated metastatic potential in the GnTV-transfected H7721 cells [7,8]. In the present study, these cells showed a reduced expression of SLe^x on cell surface, suggesting that SLe^x may not mediate the enhancement of metastatic potential induced by GnTV-S. On the other hand, GnTV transfection was associated with an elevated expression of ST3Gal-IV and an increased amount of terminal α2,3-SA on the glycans of glycoproteins (Figs. 3 and 4), suggesting that they could be important in mediating the enhanced metastatic capacity.

The decreased expression of SLe^x in GnTV-S-transfected cells was accompanied by a reduction of α1,3-FucT-III, -VI, -VII and C2GnT-I, -II, indicating a correlated role of these two glycosyltransferase families in SLe^x synthesis. α1,3-FucT-III, -VI, and -VII are directly involved in the last fucosylation step in SLe^x synthesis. C2GnTs could increase the branching of *O*-glycans, and in this way provide more substrate for α1,3-FucT and ST3Gal to synthesize SLe^x. Our findings support the results reported by Nakamura et al. [18] that SLe^x synthesis in B lymphocyte leukemia cell line KM3 was critically regulated by C2GnT-I. The present results showing that surface SLe^x correlated with the expression of α1,3-FucT-III/-VI/-VII in GnTV-S-, GnTV-AS- and mock-transfected cells also implicate α1,3-FucT as another key enzyme for SLe^x synthesis, such correlation is also seen in various cell lines transfected with *c-erb*B2/*neu* or *nm23*H1 [15,17]. Collectively, the downregulation of α1,3-FucT-III/-VI/-VII and C2GnT-I/-II seems to underlie the reduced SLe^x expression on the GnTV-S/H7721 cell surface. Conversely, the increased expressions of α1,3-FucT-III/-VI/-VII and C2GnT-I/-II could provide an explanation for the elevation of SLe^x on GnTV-AS/H7721 cells. Surprisingly, the change of ST3Gal-IV expression in transfected cells was just the opposite to the alteration of surface SLe^x on GnTV-S- and GnTV-AS-transfected cells. This could suggest that ST3Gal-IV is not a rate-limiting enzyme in the synthesis of SLe^x. In GnTV-S/H7721 cells, increased ST3Gal-IV could in principle provide more precursors for fucosylation to be converted by α1,3-FucTs to SLe^x. The fucosylation step, however, would be inefficient due to reduction of α1,3-FucT-III/-VI/-VII in these cells. Moreover, the downregulated C2GnTs would lead to a decrease in the C2 or C4 branching, thus reducing the substrates for terminal sialylation and fucosylation. Collectively, this could provide an explanation for the decrease of surface SLe^x on GnTV-S/H7721 cells. The elevation of β1,6-GlcNAc branch in *N*-glycans on GnTV-S/H7721 cell as shown previously in our lab [7] may be not important in the change of total surface SLe^x, since SLe^x mainly resides at the termini of *O*-glycans and glycolipids. Similarly, a reduced number of antennary of *N*-glycans and low level of ST3Gal-IV in GnTV-AS/H7721 cells will not lead to reduction in the synthesis of SLe^x, since the former is not important and the latter is not rate-limiting. Furthermore, the increase of α1,3-FucT-III/-VI/-VII in GnTV-AS/H7721 cells can promote the synthesis of SLe^x on *O*-glycans and glycolipids. In addition, the upregulation of C2GnTs further provides more branches on *O*-glycan and increases the possibility for SLe^x synthesis. As a consequence, SLe^x expression was increased on GnTV-AS/H7721 cells.

An interesting finding was that there was an increase of β1,6-GlcNAc branches in *N*-glycan in GnTV-S-transfected cells and a concomitant decrease in β1,6-GlcNAc in *O*-glycans. This occurred at the circumstance of a reduced C2GnT expression. Conversely, a decrease of this branch in *N*-glycan was seen in GnTV-AS-transfected cells accompanied by an increase of β1,6-GlcNAc branch in *O*-glycans. The biological significance of the phenomenon is unknown and will be further investigated.

In mock-transfected H7721 cells, the total expression of ST3Gal-I–IV was far higher than the expression of ST6Gal, but the content of α2,3-linked SA was lower than that of α2,6-linked SA (Fig. 4). This may be partially explained by that α2,6-SA is synthesized not only by ST6Gal, but also by the ST6GalNAc family. It consists of I–VI subtypes, which are the major enzymes for the α2,6-sialylation of *O*-glycans [35,36]. The total activity of ST6GalNAc-I–VI plus ST6Gal may exceed the total activity of ST3Gal-I–IV, resulting in the higher content of α2,6-SA than of α2,3-SA.

The mechanism of the effect of expressing sense or antisense GnTV on the expression of other glycosyltransferases is unresolved and currently being investigated in our lab. Most probably, however, the effect is indirect. Concerning the putative pathways, we found that the GlcNAc-β1,6-mannose-α1,6 branch of *N*-glycans on the receptor of epidermal growth factor (EGF-R) was increased on GnTV-S/H7721 cells. This altered glycosylation led to altered affinity of EGF-R to EGF and changes in transmembrane signal transduction, e.g. changes in the activation of MAPK (mitogen-activated protein kinase) and protein kinase B signaling pathways. In GnTV-AS/H7721 cells, the alterations were just opposite to those in GnTV-S/H7721 (to be published). From these results, we assume that the aberrantly glycosylated surface receptor and the subsequent alterations in signal transduction from some extracellular regulators to the genes of α1,3-FucTs, ST3Gal-IV and C2GnTs could provide one possible mechanism to explain the altered expression of these glycosyltransferases after GnTV-S or GnTV-AS transfection. In supporting that, we discovered that transfection of GnTV-S and GnTV-AS resulted in the alteration of the expression of integrin genes [20], revealing that GnTV might indirectly regulate some gene expressions. However, other mechanisms cannot be ruled out, such as the multi-antennary glycan products of GnTV protecting some regulatory factor(s) from degradation by intracellular proteases, and the active factor(s) may affect the gene transcription of the glycosyltransferases.

The expressions of other glycosyltransferases involved in the synthesis of *N*-, *O*-glycans and glycolipids unrelated to SLe^x synthesis may be also changed after transfection of GnTV-S and GnTV-AS. It will be very interesting to further study on this problem by using the gene chip of glycosyltransferases.

Acknowledgements: This work was supported by a grant from the National Natural Science Foundation of China (No. 30370343).

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