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Research Article

N-acetylglucosaminyltransferase V modifies the signaling pathway of epidermal growth factor receptor

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Abstract. Transfection of sense cDNA of N-acetylglucosamyltransferase V (GnTV-S) into human H7721 hepatocarcinoma cells resulted in an increase in the N-acetylglucosamineβ1,6mannoseα1,3- branch (GnT-V product) on the N-glycans of epidermal growth factor (EGF) receptor (EGFR), and promotion of its EGF binding and tyrosine autophosphorylation, but showed little effect on the expression of EGFR protein. The phosphorylation at T308, S473 and tyrosine residue(s) and the activity of protein kinase B (Akt/PKB) as well as the phosphorylation of p42/44 mitogen-activated protein kinase (MAPK) and MAPK kinase (MEK) before and after EGF stimulation were concomitantly increased. Conversely, in the antisense GnT-V (GnTV-AS)-transfected H7721 cells, all the results were the reverse of those with GnTV-S-transfected cells. After the cells were treated with 1-deoxymannojirimycin, an inhibitor of N-glycan processing at high mannose, or antibody against the extracellular glycan domain of EGFR, the differences in PKB activity, p42/44 MAPK and MEK phosphorylation among GnTV-S-, GnTV-AS- and mock-transfected cells were significantly attenuated. These findings indicate that the altered expression of GnT-V will change the glycan structure and function of EGFR, which may modify downstream signal transduction.

Key words. Receptor of epidermal growth factor; N-acetylglucosaminyltransferase V; phosphorylation; Akt/PKB; MEK; p44/42 MAPK.

Epidermal growth factor (EGF) receptor (EGFR) is an important surface receptor with N-glycans in its extracellular domain, and the glycosylation of EGFR is essential for its function [1, 2]. Rebbaa et al. [3] reported that changes in EGFR glycosylation on U373 MG glioma cells after transfection of N-acetylglucosaminyltransferase III (GnT-III), a glycosyltransferase responsible for the synthesis of bisecting β1,4 N-acetylglucosamine (GlcNAc) in N-glycans, resulted in the decrease of its binding to the ligand and autophosphorylation, and consequently altered the behavior of cell proliferation after EGF stimulation. Ihara et al. [4] also reported that overexpression of GnT-III in pheochromocytoma PC12 cells inhibited neurite outgrowth during differentiation, as well as the transient tyrosine phosphorylation and dimerization of nerve growth factor (NGF) receptor (Trk) after stimulation with NGF. These findings suggest that the modification of glycan structure on cell surface receptors may alter their function, and bisecting β1,4 GlcNAc may be a glycan structure, which blocks the binding of the receptors to their corresponding ligands. However, whether other alterations of N-glycan structure on surface receptors in addition to the increase of bisecting β1,4 GlcNAc affect the receptor functions remains unclear.

N-acetylglucosaminyltransferase V (GnT-V) is a Golgi enzyme involved in the processing of N-linked glycans (N-glycans). It catalyzes the transfer of the GlcNAc

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group from UDP-GlcNAc to the α1,6 mannoside of C_2C_2 biantennary or $C_{2,4}C_2$ triantennary N-glycans, and produces a β1,6 branching GlcNAc structure (GlcNAcβ1,6Manα1,6-) in $C_2C_{2,6}$ tri- or $C_{2,4}C_{2,6}$ tetra-antennary N-glycan products (C_2C_2, $C_{2,4}C_2$, $C_2C_{2,6}$ and $C_{2,4}C_{2,6}$ express the linkages of the bi-, tri- and tetra-antennae to the carbon atom positions of α1,3 and α1,6 mannosides in the N-glycan core). There is good evidence for GnT-V involvement in cell proliferation and cancer metastasis [5, 6]. Our laboratory has reported that transfection of some oncogenes or metastasis-suppressive genes into a human H7721 hepatocarcinoma cell line up- or downregulated GnT-V, respectively [7, 8]. We also found that the metastatic potential of the cells was enhanced after the transfection of the sense cDNA of GnT-V (GnTV-S) into H7721 cells [9]. Conversely, the metastatic potential was reduced and the cell susceptibility to all-trans retinoic acid-induced apoptosis was increased after the antisense cDNA of GnT-V (GnTV-AS) had been transfected [9, 10]. Recently, we discovered that the expressions of integrin α5 and α6 subunits were upregulated after the transfection of GnTV-S into H7721 cells, while the β1 subunit was downregulated by the transfection of GnTV-AS [11]. In addition, transfection of Gn-V-S or GnTV-AS led to an alteration in the expression of other glycosyltransferases [12]. In the light of these results, one can speculate that the transfection of sense or antisense GnT-V may change the expression of some genes. GnT-V is regulated by the mitogen-activated protein kinase (MAPK) and protein kinase B (PKB or Akt) signaling pathway of some growth factors, such as EGF [13, 14] and insulin [15]. Of interest is to study whether GnT-V transfection can influence the function of some surface receptors to ultimately alter the receptor signaling.

In the present investigation, EGFR was selected as the surface receptor to study its structural and functional changes, including the N-glycan structure, autophosphorylation and the EGF binding affinity of EGFR, after the transfection of GnTV-S or GnTV-AS. PKB, p42/44 MAPK and MAPK kinase (MEK) were chosen as the signaling molecules involved in EGFR signaling. The phosphorylation of PKB, MAPK and MEK as well as the activity of PKB were determined in the GnTV-S- and GnTV-AS-transfected H7721 cells and compared with the mock cells transfected with the vector.

Materials and methods

Materials

The 7721 human hepatocarcinoma cell line was obtained from the Institute of Cell Biology. Plasmid pcDNA3FluHuTV (7.75 kb) containing full-length GnT-V cDNA (2.3 kb) was generously provided by Dr. Pierce at Georgia University. RPMI 1640 medium was purchased from GIBCO/BRL. Rabbit polyclonal antibodies against human PKB (Akt), phospho-Akt (Thr308 and Ser473), MEK1/2, phospho-MEK1/2 (Ser217/221) and p42/44 MAPK, and monoclonal antibody against phospho-p42/44 MAPK (Thr202/Tyr204) were from Cell Signaling Technology. The Akt/PKB assay kit was from New England Biolabs. Horseradish peroxidase (HRP)-labeled secondary antibodies (goat anti-rabbit IgG and anti-mouse IgG) were obtained from DAKO. Polyvinylidene difluoride (PVDF) membrane was purchased from Bio-Rad. ^{125}I-labeled EGF and an enhanced chemiluminescence (ECL) assay kit were from Amersham. Phosphotyrosine antibody (PT66), neuraminidase (from *Clostridium perfringens*), HRP, lectins, concanavalin A (Con A) and *Datura stramonium* agglutinin (DSA), MES, HEPES, leupeptin, pepstatin and human EGFR monoclonal antibodies (CF4 and 29.1.1) and FITC-conjugated secondary antibody (goat anti-mouse IgG) were from Sigma. Human EGFR monoclonal antibody 528, protein G plus-agarose and monoclonal antibody for β-actin were from Santa Cruz Technology. 1-Deoxymannojirimycin (DMJ) was from Toronto Research Chemicals. Other reagents were commercially available in China.

Construction of the plasmids containing sense and antisense cDNA of GnT-V and characterization of the transfected cells

The characterization of plasmids containing sense GnT-V cDNA and the construction of antisense cDNA of GnT-V were described in our previous papers [10, 11]. The constructs containing sense or antisense cDNA of GnT-V, or the vector pcDNA3, were transfected into H7721 cells using the electroporation method described previously [10, 11]. The GnTV-S-transfected cells (GnTV-S/H7721) were characterized by the appearance of the transcript of exogenous GnTV-S cDNA and an increase in GnT-V activity. Similarly, the cells transfected with GnTV-AS (GnTV-AS/H7721) were characterized by the appearance of the GnTV-AS mRNA, the decrease of endogenous GnT-V mRNA and the GnT-V activity [10, 11].

Cell culture and treatment

Cells mock-transfected with the vector or the constructed plasmid were cultured at 37°C, 5% CO_2 in RPMI-1640 medium containing 10% fetal calf serum (FCS), penicillin and streptomycin as previously described by our laboratory [9–12]. In the experiments using EGF, the cells were precultured in FCS-free medium (serum starvation) for 24 h, and then 100 ng/ml EGF was added for 5-min incubation. When DMJ was used, its concentration was 5.0 μM and the incubation time was 48 h. In the experiment for blocking the EGFR, 50 μg/ml EGFR monoclonal antibody (29.1.1) against the extracellular glycan-rich domain of EGFR was added and incubated with the cells for 2 h.

Detection of EGFR on the cell surface with flow cytometry

Detection of cell surface EGFR subunits was performed according to the method reported previously by us [11]. Cells were detached with 2 mM EDTA, washed and blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 4 °C for 10 min, then 1×10^6 cells were incubated with the monoclonal antibody 528 against EGFR (final concentration: 5 µg/ml according to previous experiments) for 45 min at 4 °C. In the '(–) control' sample, the first antibody was omitted. After two washings with PBS, the cells were reincubated for 30 min at 4 °C with 1:128 FITC-conjugated secondary antibody (goat anti-mouse IgG), then the cells were washed again, suspended in 0.5 ml PBS and subjected to flow cytometry for fluorescence analysis. Fluorescence-activated cell spectra (FACS) were drawn automatically, and the left or right shift of the curve or its peak indicated the decrease or increase in the mean fluorescence intensity (MFI) respectively, as shown by the 'M1' bar in the figures.

Immuno precipitation of EGFR and PKB

The cultured cells were washed with cold PBS, and the monolayer cell was lysed by the addition of 200 µl of lysis buffer (50 mM pH 7.4 HEPES/150 mM NaCl/100 mM NaF/1 mM $MgCl_2$/1.5 mM EGTA/1% Nonidet P-40). The protein concentration of the supernatants was determined by the Lowry method. The cell lysate containing 500 µg protein was incubated with 5 µg of monoclonal EGFR antibody against the intracellular domain (CF4) and incubated at 4 °C for 1 h, then protein G plus-agarose was added and the sample was incubated at 4 °C for 3 h for immunoprecipitation of EGFR. PKB was immunoprecipitated according to the manual of the Akt/PKB assay kit.

Analysis of the glycan structure on EGFR with HRP-labeled lectins

In brief, the immunoprecipitated EGFR from differently transfected cells was treated with neuraminidase to remove the terminal sialic acids of the N-glycans on EGFR with a routine method in our laboratory [11]. After washing, the same amount of protein from each sample was equally divided into three parts, separately subjected to 8% SDS-PAGE and Western blotted to three different PVDF membranes according to the modified method of Marone et al. [16]. The membranes were then blocked with 5% fat-free dry milk in 0.1 M Tris buffer (pH 7.4)/0.15 M NaCl/0.05% Tween 20, and treated with 1:300 HRP-DSA, 1:300 HRP-Con A or 1:500-diluted EGFR monoclonal antibody (CF4)/1:500 diluted HRP-labeled anti-mouse IgG. The HRP-DSA and HRP-Con A complexes were prepared by the method of Wilson and Nakano [17]. Finally, the color of the EGFR bands was developed with ECL reagents. Densitometric analysis was used for quantification of the band intensities.

Determination of EGFR autophosphorylation and PKB tyrosine phosphorylation

The cells were incubated in FCS-free medium for 24 h, and treated with 100 ng/ml EGF for 5 min at 37 °C. After being washed twice with ice-cold PBS, the monolayer cells were lysed with 200 µl lysis buffer. EGFR was immunoprecipitated, divided into two parts and subjected to 8% SDS-PAGE and Western blot as described above, then the membranes were probed with 1:1000 mouse monoclonal phosphotyrosine antibody (PT66), or 1:500 EGFR antibody (CF4), followed by incubation with 1:500-diluted HRP-labeled secondary antibody. The color was also developed with ECL reagent. The tyrosine phosphorylation (Tyr p) of EGFR was calculated from the ratio of staining intensity of Tyr p to that of EGFR.

In the determination of tyrosine phosphorylation on PKB, the immunoprecipitated PKB was also equally divided into two parts, separately subjected to 10% SDS-PAGE, Western blotted to two membranes and detected by 1:500-diluted PKB antibody or 1:1000-diluted PT66, followed by 1:500-diluted secondary antibody and ECL reagent. Finally, the tyrosine phosphorylation (Tyr p) of PKB was calculated from the ratio of the staining intensity of Tyr p to that of PKB.

^{125}I-EGF binding assay

The competitive method reported by Rebbaa et al. [3] was adopted. H7721 cells were seeded into 24-well plates at a density of 5×10^4/ml, and incubated overnight in RPMI 1640 medium containing 10% FCS. Then the medium was changed to FCS-free RPMI 1640, and further incubated at 37 °C for 15 h. After the cells had been washed twice with binding buffer (PBS containing 0.1% BSA), ^{125}I-labeled EGF (1.5×10^4 cpm) was added in the presence of 0–5.0 nM unlabeled EGF. Nonspecific binding was determined by the addition of 1 µM cold EGF. The cells were incubated for 1 h at room temperature, and the unbound EGF was aspirated, then the cells were washed three times with binding buffer, and lysed with 200 µl 0.2 M NaOH. The cell-bound radioactivity of ^{125}I-EGF was counted and expressed as cpm/10^4 cells.

Analysis of the expression and phosphorylation of PKB, MEK and p42/44 MAPK using Western immunoblot

Briefly, the cells were homogenized in 0.1 M MES buffer (pH 6.5)/150 mM NaCl/2% TritonX-100/25% glycerol/0.1 mg% leupeptin and pepstatin, then centrifuged at 1000 g at 4 °C for 15 min. Aliquots of 50 µg of protein samples were subjected to 10% SDS-PAGE and Western blot. The membranes were treated with 1:500-diluted antibody of Akt/PKB, 1:300-diluted antibody of phospho-

Akt (Thr308) or phospho-Akt (Ser473), 1:1000 antibody of MEK1/2 or phospho-MEK1/2 (Ser 217/221), 1:1000 antibody of p42/44 MAPK or phospho-p42/44 MAPK (Thr202/Tyr204) in 5% fat-free dry milk in Tris-buffered saline, followed by incubation with 1:500-diluted HRP-labeled secondary antibody, and stained with ECL reagent. Beta-actin was used as loading control and stained with 1:800-diluted monoclonal antibody and 1:500 HRP-labeled secondary antibody. The bands were also quantified with densitometric analysis. The phosphorylation of PKB, MEK and p42/44 MAPK was calculated from the ratio of staining intensity of phosphorylated protein to total protein after normalizing with β-actin. The procedure for detecting tyrosine phosphoryalation on PKB in serum-starved cells was described above.

Assay of PKB activity

A PKB assay was performed with an Akt/PKB assay kit according to the instruction manual. Briefly, after protein determination, 500 µg of cell lysate was mixed with immobilized PKB antibody and incubated at 4 °C for 2–3 h to immunoprecipitate PKB. The pellet was suspended in 40 µl kinase buffer (25 mM Tris/HCl, pH 7.5/1 mM β-glycerolphosphate/2 mM dithiothreitol/0.1 mM Na_3VO_4/10 mM $MgCl_2$) and used as enzyme preparation, which was supplemented with substrates ATP (200 µM) and 1 µg GSK-3α/β fusion protein (paramyosin fused to GSK-3α/β crosstide corresponding to residues surrounding Ser21/9 of GSK-3α/β, CGPKGPGRRGRRRTSSFAEG). After incubation at 30 °C for 60 min, the phosphorylated GSK-3α/β fusion protein was detected by Western blot using phospho-GSK-3α/β (Ser21/9) antibody, followed by treatment with secondary antibody and ECL reagents. The intensity of the phospho-GSK-3α/β band was also subjected to densitometric analysis.

Statistical analysis

Values are expressed as mean ± SD. Statistical significance was determined with SPSS 10.0. Results were evaluated by Student's t tests. $p < 0.05$ and $p < 0.01$ were considered statistically significant and very significant, respectively.

Results

Alterations in the amount, N-glycan structure and autophosphorylation of surface EGFR on GnTV-S/H7721 and GnT-AS/H7721 cells

Figure 1 A, B shows that the expression of surface EGFR detected with flow cytometry was not significantly changed in GnTV-S- or GnT-AS-transfected cells compared with the mock cells transfected with the vector. Figure 1 C indicates that the protein amount of immunoprecipitated EGFR was also unchanged. However, in GnTV-S/H7721 cells, the HRP-DSA staining of EGFR was increased to 181.9%, while HRP-Con A staining was decreased to 43.1% of the 'mock' values ($p < 0.01$; fig. 1 C, D. The opposite results were found in GnTV-AS/H7721 cells: the HRP-DSA and HRP-Con A staining were decreased to 49.2% and increased to 181.0% of the 'mock' values, respectively ($p < 0.01$). DSA specifically and strongly binds to tri- and tetra-antennary complex-type N-glycans with the GlcNAcβ1,6Manα1,6- branch, the product of GnT-V [18], while Con A specifically binds to C_2C_2 biantennary complex-type N-glycans with no bisecting GlcNAc structure, the substrate of GnT-V [19]. Therefore, the above results provided the evidence that total GlcNAcβ1,6Manα1,6- branch was increased in EGFR from GnTV-S/H7721 cells but decreased in that from GnTV-AS/H7721 cells. EGFR has multiple potential N-glycosylation sites [1]. It has been speculated that not all of the N-glycans on EGFR were modified by GnT-V to increase a β1,6 GlcNAc branch.

Autophosphorylation at tyrosine residue(s) is an important event in the signal transduction of many growth factor receptors, including EGFR. The tyrosine autophosphorylation of EGFR in GnTV-S/H7721 and GnT-AS/H7721 cells is shown in figure 1 E, F. It was increased 71.0% ($p < 0.05$) and decreased 58.6% ($p < 0.01$) of the 'mock' levels on GnTV-S- and GnTV-AS-transfected cells, respectively.

Alteration in EGF binding to surface EGFR on GnTV-S/H7721 and GnT-AS/H7721 cells

As shown in figure 2, the competitive binding assay showed that the binding affinity of EGFR to EGF was strengthened in GnTV-S but weakened in GnTV-AS cells.

PKB phosphorylation and activity in GnTV-S/H7721 and GnT-AS/H7721 cells

The expression of PKB protein was not obviously altered in GnTV-S/H7721 or GnTV-AS/H7721 cells; however, the phosphorylation at T308 and S473 residues of the PKB protein was apparently elevated in GnTV-S/H7721, but obviously reduced in GnTV-AS/H7721 cells when compared with the 'mock' cells (fig. 3 A, B). After densitometric quantification, the phosphorylation at T308 and S473 was 296.1% and 185.6% of the 'mock' level, respectively, in GnTV-S transfected cells ($p < 0.01$). In contrast, it was 42.3% and 25.4%, respectively, in GnTV-AS-transfected cells ($p < 0.01$). Full activation of PKB depends on phosphorylation at both T308 and S473 [20, 21]. Recently, tyrosine phosphorylation of PKB (maybe at Y315 and Y326) was also reported to participate in the full activation of PKB [21, 22]. Therefore, the tyrosine phosphorylation of PKB was determined in the differently transfected cells (fig. 3 A, B). It was 190.0% and 38.6% of the 'mock' value in GnTV-S/H7721 and GnTV-AS/H7721 cells, respectively ($p < 0.01$).

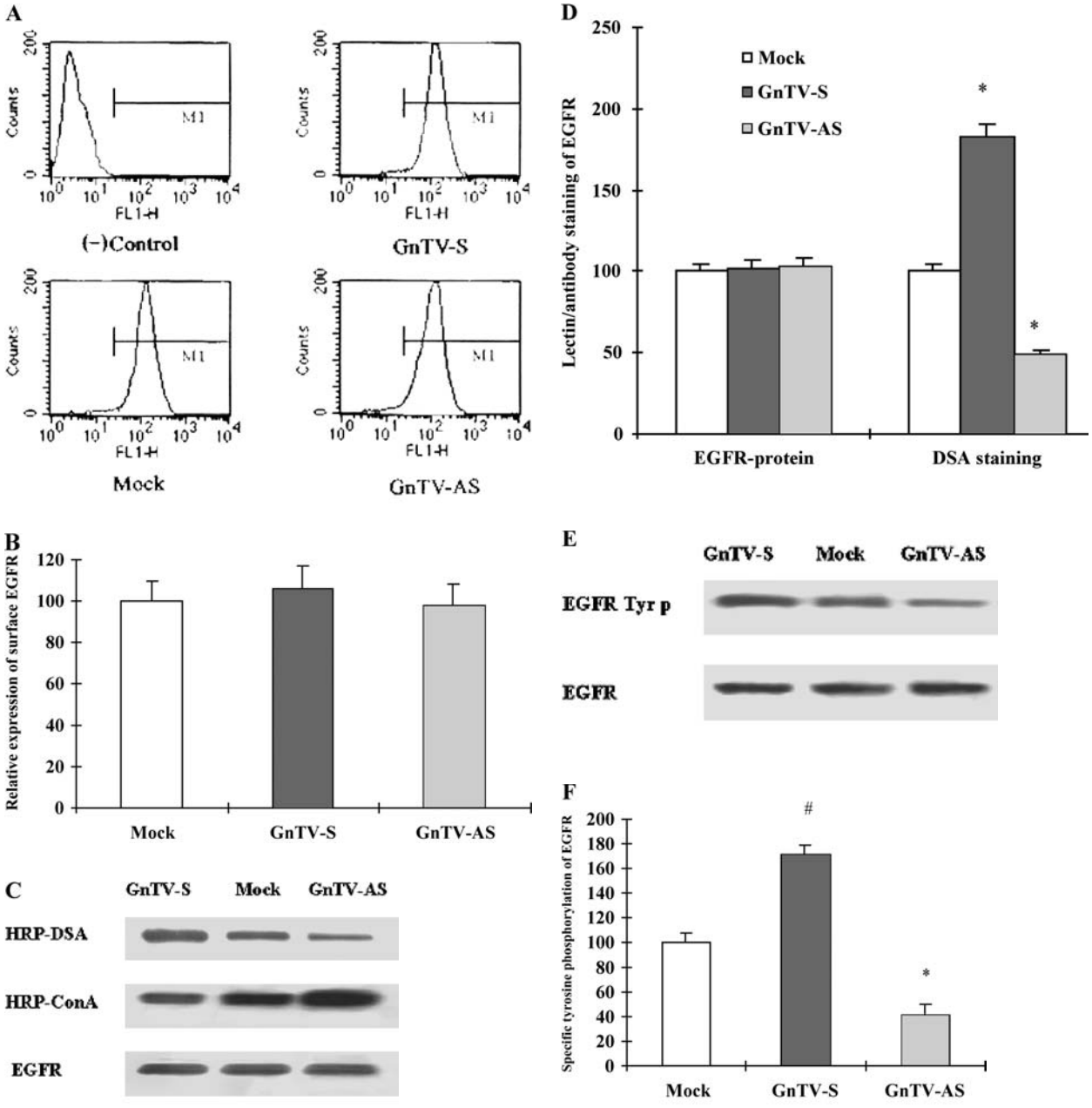

Figure 1. Expression, N-glycosylation and autophosphorylation of EGFR on GnTV-S- and GnTV-AS-transfected H7721 cells. Mock, cells mock-transfected with pcDNA3 vector; GnTV-S, cells transfected with sense cDNA of GnT-V; GnTV-AS, cells transfected with antisense cDNA of GnT-V. Three reproducible experiments were performed. *$p < 0.01$; #$p < 0.05$ compared to Mock. The experimental procedure is described in Materials and methods. (*A*) Flow-cytometric analysis of EGFR on the cell surface. (–)Control; sample without addition of first antibody. (*B*) Densitometric analysis of *A* for the quantification of the flow-cytometric analysis of EGFR on the cell surface. (*C*) Western blot profiles of immunoprecipitated EGFR after staining with HRP-labeled lectins, or EGFR antibody/secondary antibody. (*D*) Densitometric analysis of *C* for the quantification of lectin staining. (*E*) Western blot profiles of immunoprecipitated EGFR after staining with phosphotyrosine antibody (PT66) or EGFR antibody/secondary antibody (Cells were cultured in FCS-free medium for 24 h. then treated with 100 ng/ml EGF for 5 min). (*F*) Densitometric analysis of *E* for the quantification of EGFR phosphotyrosine.

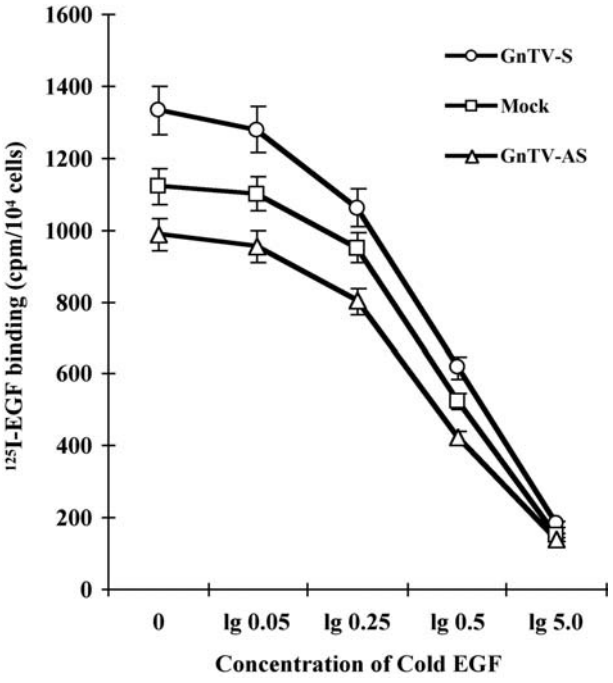

Figure 2. Binding of ^{125}I-EGF to EGFR on the cell surface of GnTV-S- and GnTV-AS-transfected H7721 cells. Mock, GnTV-S, GnTV-AS, same as in figure 1. Data are expressed as the mean ± SD from three independent experiments. The experiment procedure is described in Materials and methods.

The altered phosphorylation of PKB resulted in a change in PKB activity, which was indicated as the amount of phosphorylated GSK3α/β substrate. As shown in figure 3C, D, the PKB activity was elevated to 180.2% of the 'mock' value in GnTV-S-transfected cells ($p < 0.01$) and reduced to 47.1% in GnTV-AS-transfected ones ($p < 0.01$).

Phosphorylation of MEK and p42/44 MAPK in GnTV-S/H7721 and GnT-AS/H7721 cells before and after EGF stimulation

As indicated in figure 4, there was only one protein band of MEK, but two protein bands of p42/44 MAPK appeared after Western blot and staining. The lower band of MAPK was p42 while the upper one was p44. The expression of both MEK and p42/44 MAPK were not apparently altered in GnTV-S/H7721 and GnT-AS/H7721 cells, and were not different before and after EGF stimulation (to avoid the interference of the endogenous growth factors in FCS on the phosphorylation activity, the cells were starved in FCS-free medium for 24 h before the addition of EGF). In the absence of EGF, p42/44 MAPK was not phosphorylated, but the phosphorylated MEK (p-MEK) could be observed. The relative phosphorylation of MEK, as expressed by the ratio of p-MEK to MEK protein, was upregulated to 141.3% ($p < 0.01$) and downregulated to 67.7% ($p < 0.05$) in GnTV-S/H7721 and

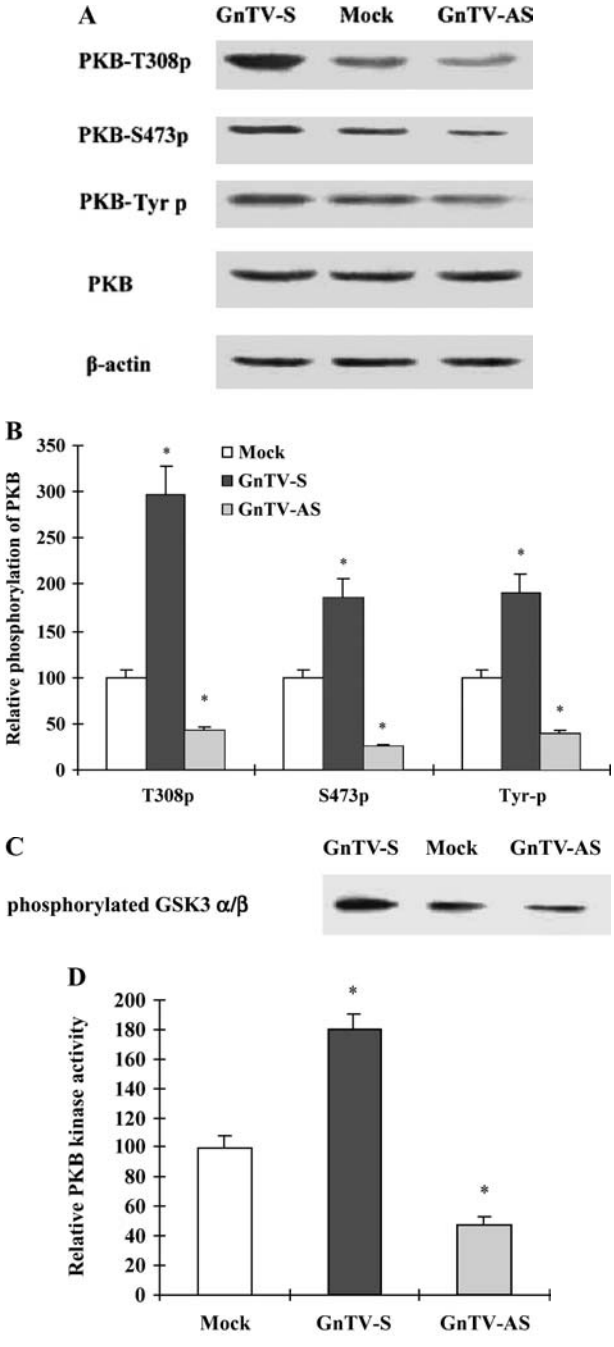

Figure 3. PKB phosphorylation at T308, S473, tyrosine residues and PKB activity in GnTV-S- and GnTV-AS-transfected H7721 cells. Mock, GnTV-S, GnTV-AS, same as in figure 1. PKB-T308p, phosphorylation at T308 of PKB protein, PKB-S473p; phosphorylation at S473 of PKB protein, PKB-Tyr p: phosphorylation at tyrosine residue(s) of PKB; PKB, protein of PKB; β-actin, protein of β-actin (loading control). Three reproducible experiments were performed. * $p < 0.01$ compared to Mock. The experimental procedure is described in Materials and methods. (*A*) Western blot profiles after staining with specific antibodies to phosphorylated PKB at T308, S473, or tyrosine residues (PT66) and antibody to PKB and β-actin (loading control), followed by HRP labeled 2nd antibody (phosphotyrosine of PKB was detected after immuno-precipitation of PKB). (*B*) Densitometric analysis of A for the quantification of phosphorylated T308, S473, or tyrosine residues of PKB. (*C*) Determination of PKB activity with GSK3α/β as substrate. (*D*) Densitometric analysis of C for the quantification of PKB activity

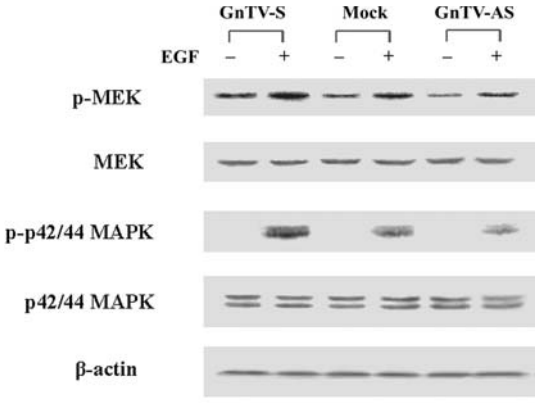

Figure 4. Phosphorylation of MEK and p42/44 MAPK before and after stimulation with EGF. Cells were cultured in FCS-free medium for 24 h. EGF (–), before EGF stimulation; EGF (+), 100 ng/ml EGF treatment for 5 min. Mock, GnTV-S, GnTV-AS, same as in figure 1. MEK, MEK protein, p-MEK; phospho-MEK; p42/44 MAPK, 42/44 MAPK protein, p-p42/44 MAPK. phospho-42/44MAPK; β-actin; protein of β-actin (loading control). Three reproducible experiments were performed. The experiment procedure is described in Materials and methods.

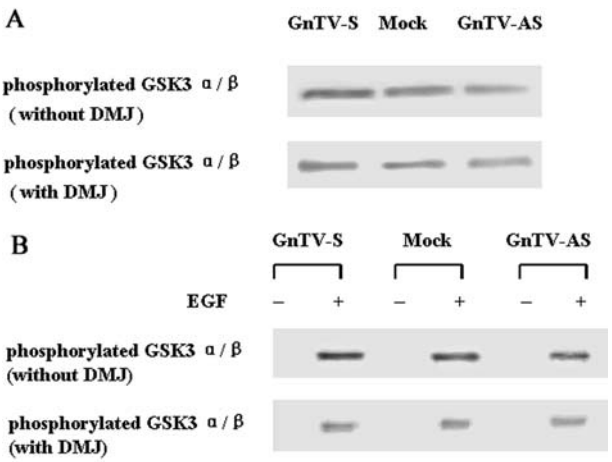

Figure 5. Activity of PKB in GnTV-S/H7721 and GnTV-AS/H7721 cells in the absence and presence of DMJ before and after EGF stimulation. Mock, GnTV-S, GnTV-AS, same as in figure 1. With DMJ, 5.0 µM DMJ treatment for 48 h; EGF (–), before EGF stimulation; EGF (+), 100 ng/ml EGF treatment for 5 min. Three reproducible experiments were performed. The experiment procedure is described in Materials and methods. (*A*) Determination of PKB activity with GSK3α/β as substrate (without serum starvation). (*B*) PKB activity before (–) and after (+) stimulation with 100 ng/ml EGF for 5 min (cells were precultured in FCS-free medium for 24 h).

GnT-AS/H7721 cells, respectively. After 100 ng/ml EGF stimulation for 5 min, p-MEK was elevated in all three cell lines, the relative phosphorylation of MEK being increased 114.5%, 170.1% and 84.0% in mock, GnTV-S/H7721 and GnT-AS/H7721 cells, respectively (all p values < 0.01 compared with the levels before EGF treatment), and the differences among GnTV-S/H7721, mock and GnTV-AS/H7721 cells became more obvious (GnTV-S or GnTV-AS vs 'mock', $p < 0.01$). Therefore, GnTV-S/H7721 cells were more, but GnTV-AS/H7721 cells less sensitive to EGF stimulation than the mock cells. On the other hand, p42/44 MAPK was phosphorylated only after EGF stimulation, the relative phosphorylation of this protein (p-p42/44 MAPK/p42/44 MAPK) being higher (178.4%, $p < 0.01$) in GnTV-S/H7721 cells but lower (60.4%, $p < 0.01$) in GnTV-AS/H7721 cells when compared with the mock ones.

PKB activity and p42/44 MAPK phosphorylation in GnTV-S/H7721 and GnTV-AS/H7721 cells in the presence and absence of DMJ

To study whether the above-mentioned alteration of EGFR signaling was mediated by the structural change of N-glycan on EGFR resulting from the increase or decrease of the GlcNAcβ1,6Manα1,6- branch, the activity of PKB and the phosphorylation of p42/44 MAPK were measured after the cells had been treated with DMJ. DMJ specifically inhibits the Golgi α-mannosidase I, and blocks the processing of N-glycans at the high mannose-type stage [23, 24], but it does not cause the misfolding of the glycoproteins [25]. After the cells had been incubated in the medium containing 5.0 µM DMJ for 48 h, the PKB activity was very obviously decreased, and the difference in GnTV-S/H7721, mock and GnTV-AS/H7721 cells was significantly attenuated ($p > 0.05$) (fig. 5A). In addition, after the cells had been serum starved and treated with EGF in the absence of DMJ, the difference in PKB activity among the three cell lines was significant (GnTV-S/H7721 or GnTV-AS/H7721 vs 'mock', $p < 0.05$). However, in the presence of both DMJ and EGF, the difference among the three cell lines became very slight ($p > 0.05$), far less than that in the samples without DMJ treatment (fig. 5B).

The result of p42/44 MAPK phosphorylation was similar to that of PKB activity. Before DMJ treatment, the relative phosphorylation of p42/44 MAPK (p-p42/44 MAPK/p42/44 MAPK) was significantly increased in GnTV-S cells, up to 231.0% of the 'mock' value ($p < 0.01$), while it was apparently decreased in GnTV-AS cells, down to 37.5% of the 'mock' level ($p < 0.01$). After DMJ treatment, the expression of p42/44 MAPK was slightly decreased but its phosphorylation was significantly reduced and the difference in the relative phosphorylation among GnTV-S/H7721, mock and GnTV-AS/H7721 cells was also remarkably reduced ($p > 0.05$) (fig. 6).

PKB activity and p42/44 MAPK phosphorylation in GnTV-S/H7721 and GnTV-AS/H7721 cells in the presence of EGFR antibody

The antibody against the extracellular glycan-rich domain of EGFR was used to block the receptor-ligand binding in order to study further the relationship between

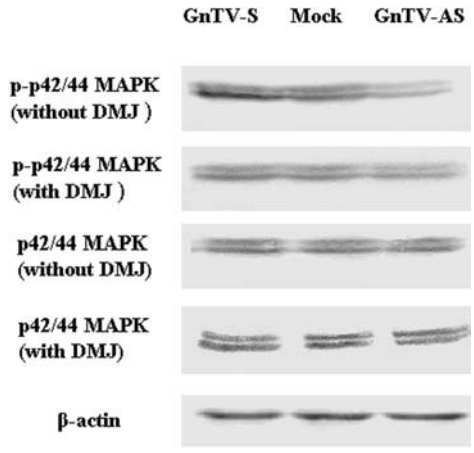

Figure 6. Expression and phosphorylation of p42/44 MAPK in the absence and presence of DMJ. Mock, GnTV-S, GnTV-AS, same as in figure 1. p42/44 MAPK, p-p42/44 MAPK, β-actin, same as in figure 4. With DMJ, 5.0 µM DMJ treatment for 48 h. Three reproducible experiments were performed. The experiment procedure is described in Materials and methods.

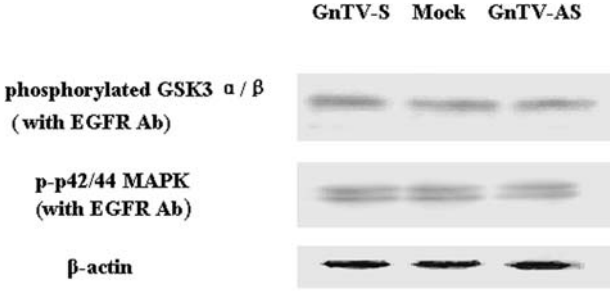

Figure 7. Activity of PKB and phosphorylation of p42/44 MAPK in the presence of EGFR antibody. Mock, GnTV-S, GnTV-AS, same as in figure 1. p-p42/44 MAPK, β-actin, same as in figure 4. With EGFR Ab, treatment with 50 µg/ml of EGFR antibody for 2 h. Three reproducible experiments were performed. The experiment procedure is described in Materials and methods.

EGFR signaling and its extracellular glycans. The results are shown in figure 7. Both PKB activity and the phosphorylation of p42/44 MAPK were reduced when compared with the results from cells without any treatment shown in figures 3C, D and 6. In addition, the difference among GnTV-S/H7721, mock and GnTV-AS/H7721 cells was apparently decreased and became statistically insignificant ($p > 0.05$).

Discussion

The H7721 cell line was established on 1 February 1977 at the Shanghai Second Military Medical College from a human hepatocellular carcinoma, which was positive in α-fetal protein and negative in hepatitis B virus. It expresses a moderate amount of GnT-V, but only low and slight amounts of GnT-III and GnT-IV, respectively [26]. In the present investigation, both GnTV-S- and GnTV-AS-transfected H7721 hepatocarcinoma cells were used to study the alteration of EGFR function and signal transduction. The bidirectional changes of EGF function and signaling may provide substantial evidence to elucidate the relationship between the N-glycan structure and function of EGFR.

Our results using monoclonal antibody against EGFR combined with flow cytometry and immunoprecipitation/Western blot methods demonstrated that the expression of EGFR both on the cell surface and in the cells was not changed by the transfection with GnTV-S. This finding was similar to that reported by Guo et al. [27] that the overexpression of GnT-V in human fibrosarcoma HT1080 cells did not affect the amount of surface N-cadherin. However, in the present studies, the GlcNAcβ1,6Manα1,6- branch on the N-glycans of EGFR was found to be increased in GnTV-S-transfected H7721 cells. These findings revealed that the promotion of binding affinity to EGF and tyrosine autophosphorylation of EGFR on GnTV-S cells was due to the increase in the GlcNAcβ1,6Manα1,6- branch on EGFR N-glycans, but not related to the amount of EGFR protein. They suggest that there is a relationship between the glycan structure and function of EGFR. Concomitantly, after the transfection with GnTV-S, the signal transduction from EGFR to downstream molecules was also enhanced or upregulated. These included (i) the phosphorylation of PKB at T308, S473 and tyrosine residue(s) as well as PKB activity; (ii) the phosphorylation of MEK before and after EGF stimulation; (iii) the phosphorylation of p42/24 MAPK after EGF stimulation. The opposite results were observed in the GnTV-AS-transfected cells. One can assume that the altered signaling of EGFR is a consequence of the structural change of EGFR glycans, since the different intensities of EGFR signaling in the three cell lines were greatly attenuated after the treatment with DMJ. DMJ blocks N-glycan processing at the high mannose-type stage [23, 24], leading to the accumulation of high mannose-type N-glycan on EGFR and the reduction of biantennary and $C_2C_{2,4}$ triantennary complex-type N-glycan. Hence, the glycan structure of EGFR on the three cell lines became similar and the responses of this receptor to EGF no longer differed. The observation using EGFR monoclonal antibody against the extracellular glycan-rich domain (29.1.1) instead of DMJ gave similar results, further implicating EGFR N-glycans in the modification of EGFR signaling. The above findings also suggest that signal transduction from EGFR to PKB, MEK and p42/44 MAPK may be more and less effective in GnTV-S and GnTV-AS cells, respectively. Conceivably, the GlcNAcβ1,6Manα1,6- branching structure on complex-type N-glycans is beneficial to EGFR for its ligand binding and signaling. However, the reason why DMJ significantly inhibits the PKB activity and p42/44 MAPK

phosphorylation is not well known. DMJ may inhibit the sorting of EGFR to the cell membrane, or the complex-type but not high mannose-type N-glycan is essential for EGFR signaling. This speculation is being investigated.

The mechanism for the changed function and signaling of EGFR by the GlcNAcβ1,6Manα1,6- branching structure is unknown. Addition or deletion of this branch very likely alters the conformation of EGFR protein [1], causing the changes in the ligand-binding affinity, tyrosine autophosphorylation and subsequent signaling of EFGR. In addition, the GlcNAcβ1,6Manα1,6- branch is a preferred substrate for the synthesis of [GlcNAcβ1,4Galβ1,3-]$_n$ (poly-*N*-acetyllactosamine) repeat sequences at the outside of this branch [25], resulting in the enlargement of EGFR N-glycans. These structural changes are sufficient to alter the conformation of EGFR. Consequently, the changed conformation of EGFR may alter the recruitment of the adaptor proteins or downstream signaling molecules to EGFR, leading to enhancement or abatement of EGFR signaling.

The phosphorylation signals at T308 of PKB are transferred from phosphoinositide-3-kinase (PI-3K)/phosphotidylinositide dependent kinase-1 (PDK-1) [20, 21], but the signal for S473 phosphorylation is reported to come from the integrin/integrin-linked kinase (ILK) signaling pathway [28]. One can therefore speculate that the transfection of sense and antisense GnT-V may also affect the signaling of the integrin/ILK pathway. Another finding in our laboratory supports this speculation that the transfection of GnTV-S or GnTV-AS also increases or decreases the GlcNAcβ1,6Manα1,6- branch on the β1 subunit of integrin, respectively [11]. Very recently, Isaji et al. [29] reported that introduction of bisecting GlcNAc on integrin α5, α2 and α3 subunits by transfection with GnT-III led to a significant reduction in the binding affinity of integrin $\alpha 5\beta 1$ to fibronectin and the inhibition of cell spreading and migration on fibronectin. Furthermore, Guo et al. [27] reported that the transfection of GnT-V to human fibrosarcoma HT1080 and NIH 3T3 cells upregulated the GlcNAcβ1,6Manα1,6- branch on N-cadherin and subsequently modified the signal transduction pathway of MAPK (also called ERK). Finally, the calcium-dependent cell-cell adhesion mediated by N-cadherin was decreased. These findings provided strong evidence that the integrin or N-cadherin signaling pathway could be modified by the alteration of their glycan structure. Interestingly, the signal function of surface Notch receptor during the development of *Drosophila* was modified by the mutation of a β1,3GnT, called Fringe, which added a β1,3GlcNAc to the O-linked fucose [30]. This discovery reveals that the signaling of a surface receptor can be modified by a single sugar change on the receptor glycans.

The tyrosine phosphorylation signal from EGFR to PKB was possibly passed through the Src tyrosine kinase family, including Src, Yes and Fyn [22, 31]. Of interest will be to study whether the signal from EGFR to the Src family is also modified by the transfection with GnTV-S or GnTV-AS.

In our previous paper, we reported that PKB activity is regulated by both MEK/MAPK and PI-3K/PKB signaling pathways [13], hence, the modification of these two pathways by over- or downexpression of GnT-V may function as a feedback control between GnT-V and these two pathways. This feedback control was suggested to be involved in the amplification of the oncogene signal, which increased the expression of GnT-V [32].

The modification of EGFR signaling to PKB and MEK/MAPK will further affect the phophorylation of the downstream molecules, including transcription factors or regulators, and consequently cause changes in gene expression. As we reported previously, the expression of integrin subunits and some other glycosyltransferases, such as α1,3fucosyltransferase III, VI and VII and α2,3 sialyltransferase IV, were significantly altered after the transfection by GnTV-S and GnTV-AS [11, 12].

Saito et al. [33] reported that a secreted type of GnT-V without glycosylation activity could induce tumor angiogenesis [33]. This indicates that GnT-V has other novel biological activities unrelated to its glycosyltransferase activity. However, the present results indicate that the alteration of EGFR signaling is closely associated with the glycosyltransferase activity of GnT-V and results from the increased or decreased GnT-V product on EGFR.

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