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ORIGINAL ARTICLE

Blocking of N-acetylglucosaminyltransferase V induces cellular endoplasmic reticulum stress in human hepatocarcinoma 7721 cells

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N-acetylglucosaminyltransferase V (GnT-V) is an important tumorigenesis and metastasis-associated enzyme. To study its biofunction, the GnT-V stably suppressed cell line (GnT-V-AS/7721) was constructed from 7721 hepatocarcinoma cells in previous study. In this study, cDNA array gene expression profiles were compared between GnT-V-AS/7721 and parental 7721 cells. The data indicated that GnT-V-AS/7721 showed a characteristic expression pattern consistent with the ER stress. The molecular mechanism of the ER stress was explored in GnT-V-AS/7721 by the analysis on key molecules in both two unfolded protein response (UPR) pathways. For ATF6 and Ire1/XBP-1 pathway, it was evidenced by the up-regulation of BIP at mRNA and protein level, and the appearance of the spliced form of XBP-1. As for PERK/eIF2α pathway, the activation of ER eIF2α kinase PERK was observed. To confirm the results from GnT-V-AS/7721 cells, the key molecules in the UPR were examined again in 7721 cells interfered with the GnT-V by the specific RNAi treatment. The results were similar with those from GnT-V-AS/7721, indicating that blocking of GnT-V can specifically activate ER stress in 7721 cells. Rate of ^{3}H-Man incorporation corrected with rate of ^{3}H-Leu incorporation in GnT-V-AS/7721 was down-regulated greatly compared with the control, which demonstrated the deficient function of the enzyme synthesizing N-glycans after GnT-V blocking. Moreover, the faster migrating form of chaperone GRP94 associated with the underglycosylation, and the extensively changed N-glycans structures of intracellular glycoproteins were also detected in GnT-V-AS/7721. These results supported the mechanism that blocking of GnT-V expression impaired functions of chaperones and N-glycan-synthesizing enzymes, which caused UPR *in vivo*.

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Abbreviations: GnT-V (N-acetylglucosaminyltransferase V); ERAD (ER-associated degradation); DTT (dithiothreitol); TM (tunicamycin); dsRNA (double-stranded RNA); siRNA (small interfering RNA); Man (mannose); Leu (leucine); DSA (Datura stramonium agglutinin); ConA (concanavalin A); BIP (immunoglobulin heavy chain binding protein); ATF6 (activating transcription factor 6); PKR (double-stranded RNA-activated protein kinase); PERK (PKR-like ER kinase); XBP-1 (X-box binding protein-1); GRP94 (glucose-regulated protein 94)

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Introduction

N-acetylglucosaminyltransferase V (GnT-V) is an important cancer-associated enzyme. It catalyzes the formation of β-1,6-GlcNAc linkage in the complex-type N-glycans of C2 C2, 6 tri-antenna or C2, 4 C2, 6 tetra-antenna. It is not an ubiquitous enzyme, of which expression is regulated in a tissue-specific manner, that distributes mainly in intestine, brain and lung [1, 2]. GnT-V as well as its product β-1,6-GlcNAc branch were reported to overexpress in many cancers such as human mammary, colon and hepatic tumor, and it was essential for tumor metastasis as well as for tumor

growth [3-9]. However it was unchanged in some human renal carcinomas [10], so the role of GnT-V for the transformation of animal cells also may be the tissue-specific. In previous studies, GnT-V biofunction was extensively studied with the GnT-V-AS/7721 cell line obtained by stably transfecting 7721 human hepatocarcinoma cell line with antisense GnT-V cDNA [11-14]. In this paper, gene expression profile of GnT-V-AS/7721 cells was studied by cDNA array analysis, and the expression pattern consistent with ER stress was found.

ER stress is induced by the conditions affecting the function and homeostasis of ER. During the ER stress, unfolded protein response (UPR) is triggered by the activation of the sensor proteins, such as ATF6, Ire1, and PERK. In mammalian cells, UPR comprises two signaling pathways with the aim to up-regulate ER chaperones and folding enzymes, and to repress the protein synthesis respectively [15, 16]. The ATF6 and Ire1/XBP-1 dependent signaling pathway is specific to ER stress and generates critical transcription factors that activate UPR responsive genes. The protein synthesis inhibition is mediated by the activation of PERK followed by eIF2α phosphorylation. This UPR pathway is shared with other cellular stresses and is essential for the induction of BIP [17, 18]. Additionally, during the ER stress, the unfolded proteins, accumulated and aggregated in ER, are targeted to be degradated by the ubiquitin-proteasome pathway, a process known as ERAD whose efficiency is increased by the UPR [19, 20].

To date, ER stress is well known to be induced in cells with the specific stimulant agents or conditions, such as tunicamycin (glycosylation inhibition), DTT (reductive stress), thapsigargin (ER Ca^{2+} depletion) and glucose starvation (metabolism stress), and it has been also implicated in health and diseases [15, 16, 21]. Here, a chronic and persistent ER stress was observed in human hepatocarcinoma cells with shut-down of GnT-V, and was also supported by the transient inhibition of GnT-V by RNAi in the same cell line.

Oligosaccharides on glycoproteins are altered in tumorigenesis, and they often play a role in the regulation of the biological characteristics of tumors. Each oligosaccharide is synthesized by a specific glycosyltransferase, Among many glycosyltransferases, N-acetylglucosaminyltransferase V (GnT-V), a key enzyme in the formation of branching of asparagine-linked oligosaccharides, is the most strongly linked to tumor invasion and metastasis. Our data proved the GnT-V as potential drug target for tumor treatment.

Materials and methods

Cell lines and culture conditions

7721 hepatocarcinoma cell line was from the Institute of Cell Biology, Academic Sinica. GnT-V-AS/7721 cell line was constructed by transfecting the expression plasmid of antisense GnT-V (pcDNA3/GnT-V-AS) into 7721 cells as described earlier [11]. GnT-V-AS/7721 and parent 7721 cells were cultured at 37ºC, 5% CO_2 in RPMI-1640 medium (Gibco) containing 10% FCS, and 1% penicillin and streptomycin.

The GnT-V protein in GnT-V-AS/7721 was down regulated to 44% compared with 7721 cells (Figure 1A), while no significant difference between mock-transfected pcDNA3/7721 and 7721 cells. The mRNA level of GnT-V in GnT-V-AS/7721 was detected to be similar to that in 7721 cells (data not presented), indicating a partial blockage of GnT-V expression in GnT-V-AS/7721.

cDNA array analysis

The cDNA array system was established as the previous method [22]. The PCR products of cDNA clones verified by gel electrophoresis or pUC18 vector DNAs (negative controls) were denatured and spotted on two Hybond-N nylon membranes (Amersham Pharmacia) using an arrayer (BioRobotics). Each cDNA fragment was placed in two different spots (double-offset). After prehybridized with the prehybridization solution at 68ºC for 3 h, the array was hybridized with denatured ^{33}P-labeled probe in hybridization solution at 68ºC overnight. After washed extensively, the membranes were exposed to a Phosphor S creener (Molecular Dynamics) overnight and then scanned. Data came from three individual experiments.

Western blot analysis

The 7721 cells treated with 10 mM dithiothreitol (DTT, sigma) for 2 h (BIP and XBP-1) or 0.5 h (PERK) were used as positive control of ER stress. To detect the XBP-1, 1 μM MG132 (Sigma) was used to stabilized the proteins by pretreating cells for 3 h, which was not removed when DTT was used for positive control [23]. The cellular proteins were separated and electrophoretically transferred as the previous methods [24], then probed with indicated primary antibodies (goat anti-GnT-V, goat anti-BIP, rabbit anti-XBP-1 and goat anti-GRP94 were from Santa Cruz, rabbit anti-PERK were from Cell Signaling.). For the detection of GRP94, the gel electrophoresis time was increased to 6 h. The signal was revealed using an enhanced chemiluminescence (ECL) detection kit (Pharmacia) after reacting with second antibody.

RT-PCR analysis

With the positive control of 7721 cells treated with 10 mM DTT for 2 h, 5 μg of the total RNA from GnT-V-AS/7721 cells was reversely transcribed into cDNA using MMLV (Promega). The primer pairs specific for GnT-V (5'-ggc aga aaa gca gaa cct tg-3' and 5'-agc atg cac tgg taa tga acc-3') [25], BIP (5'-ctg ggt aca ttt gat ctg act gg-3' and 5'-gca tcc tgg tgg ctt tcc agc cat tc-3') [26], β-actin (5'-acc ttc aac cca gcc atg tac-3' and 5'-ctg atc cac atc tgc tgg agg gtg g-3') [27], and XBP-1 (5'-cct tgt agt tga gaa cca gg-3' and 5'-ggg gct tgg tat ata tgt gg-3') [23] were used. The PCR reaction consisting of 1 cycle of 10 min at 94ºC, 22 cycles of 1 min at 94ºC, 1 min at 64ºC and 110 s at 72ºC, followed by 10 min at 72ºC. The products were separated on agarose gels and quantified by fluorescent imaging.

RNA interference

The RNAi expressing vector was constructed by ligation of following annealed oligonucleotide pairs into the pSUPER plasmid (OligoEngine) as described [28]: GnT-V interfering sequence, P1: 5'-gat ccc cTT CAT TGG CGG AAA TTC GTt tca aga gaA CGA

ATT TCC GCC AAT GAA ttt ttg gaa a-3', P2: 5'-agc ttt tcc aaa aaT TCA TTG GCG GAA ATT CGT tct ctt gaa ACG AAT TTC CGC CAA TGA Agg g-3'; nonspecific control sequence, P1: 5'-gat ccc cAC TAC CGT TGT TAT AGG TG t tca aga gaC ACC TAT AAC AAC GGT AGT ttt ttg gaa a-3', P2: 5'-agc ttt tcc aaa aaA CTA CCG TTG TTA TAG GTG tct ctt gaa CAC CTA TAA CAA CGG TAG Tgg g-3'. The specific interfering sequence (5'-ttc att ggc gga aat tcg t-3') corresponding to positions 672-690 of GnT-V mRNA in human hepatic cell (gi4545221) and the nonspecific control sequence (5'-act acc gtt gtt ata ggt g-3') was determined to be unique by BLAST search of GenBank™ database. The constructed plasmids were transfected into 7721 cells by using GeneJamer™ Transfection Reagent and the peak GnT-V suppression was observed at 48 h after transfection.

^{3}H-Man and ^{3}H-Leu incorporation rate

The 7721 cells treated with 1 μg/ml TM (Sigma) for 24 h were used for control. ^{3}H-Leu and ^{3}H-Man incorporation rate were measured as described [29]. Briefly, cells were incubated with RPMI1640 medium containing 4 μCi/ml ^{3}H-Leu or 10 μCi/ml ^{3}H-Man (Shanghai Atomic Energy Research Institute) for 12 h before harvested. After incorporation, cells were lysed by ultrasound. After precipitated with 10% trichloroacetic acid, the proteins were dissolved with 2% SDS and their radioactivities were determined by scintillation counting.

Intracellular glycoproteins staining with HRP-lectin complexes

The 7721 cells treated with 1 μg/ml TM for 24 h were also used for control. The glycoproteins on cell surface were digested by incubating cells with PBS containing 100 μg/ml pronase E (Sigma) for 5 min at RT [30]. The reaction was terminated by aspirating the enzyme solution and washing the cells with cold PBS for four times. Then the intracellular glycoproteins were stained with HRP-DSA or HRP-conA as previously described [14]. The details were as follows. The cells were treated with HEPES buffer (pH 7.2) containing 2% TritonX-100 at 0ºC for 5 min. After shattered with ultrasound, the samples were centrifuged for 10 min at 1,000 × g, 4ºC and the supernatant was collected. To remove terminal sialic acid residues, samples were added 10 μU neuraminidase (Sigma) and incubated for 1 h at 37ºC before electrophoresed. 80 μg proteins from each sample were electrophoresed by SDS-PAGE and then transferred to a PVDF membrane. After blocking with PBS containing 5% BSA overnight, the membrane was incubated with HRP-DSA or HRP-ConA for 4 h. The membrane was then developed using the ECL detection system.

Results

The gene expression pattern corresponding to ER stress was identified in GnT-V-AS/7721 through cDNA array

To explore the global effects of GnT-V blocking on gene expression in 7721 cells, cDNA array was performed with the mRNA from GnT-V-AS/7721 and 7721 cells. After filtered for the genes whose background subtracted intensity was lower than 10, the hybridization signals from GnT-V-AS/7721 and 7721 were compared in pair. The differentially expressed genes with absolute ratio larger than 2.0 between GnT-V-AS/7721 and 7721 parent cells

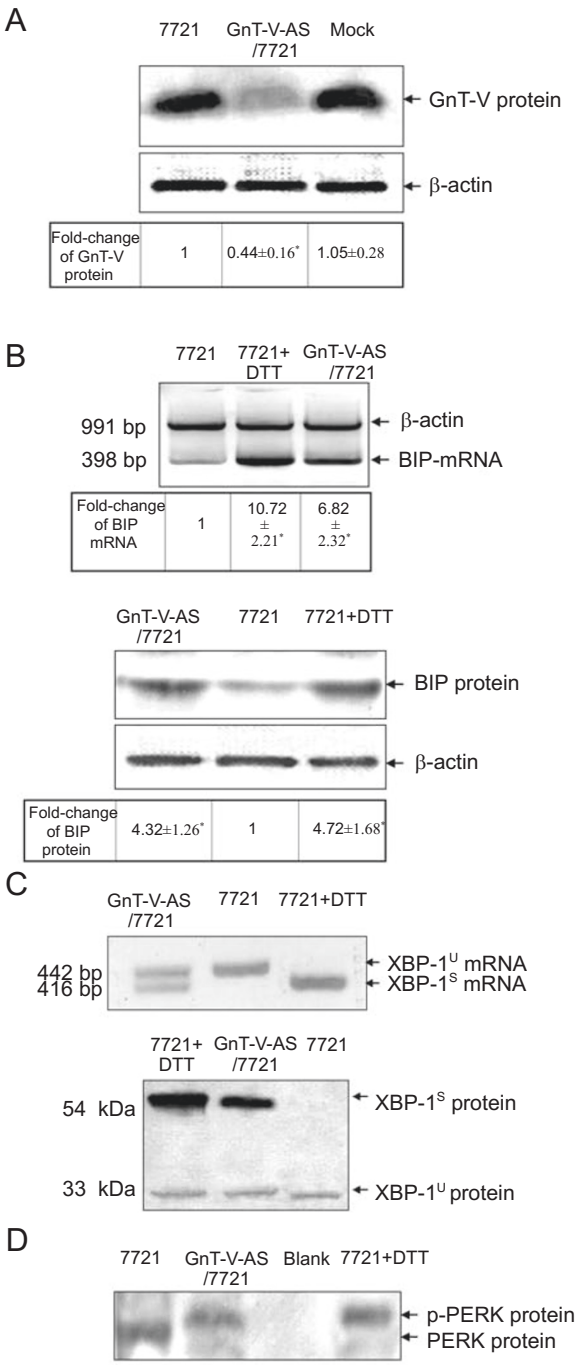

Figure 1 Features of key molecules associated with ER stress in GnT-V-AS/7721. **(A)** Western blot analysis of GnT-V in GnT-V-AS/7721. **(B)** RT-PCR (above) and western blot (lower) analysis of BIP in GnT-V-AS/7721. **(C)** RT-PCR (above) and western blot (lower) analysis of XBP-1 mRNA in GnT-V-AS/7721. **(D)** Western blot analysis of PERK in GnT-V-AS/7721. β-actin was used as an internal control in A, B and * represented $p<0.05$ vs 7721($n=3$). mock: 7721 transfected with pcDNA3 vector; GnT-V-AS/7721: 7721 transfected with pcDNA3/GnT-V-AS plasmid; 7721+DTT: 7721 treated with 10 mM DTT was used as positive control; XBP-1^U: unspliced forms of XBP-1; XBP-1^S: spliced forms of XBP-1.

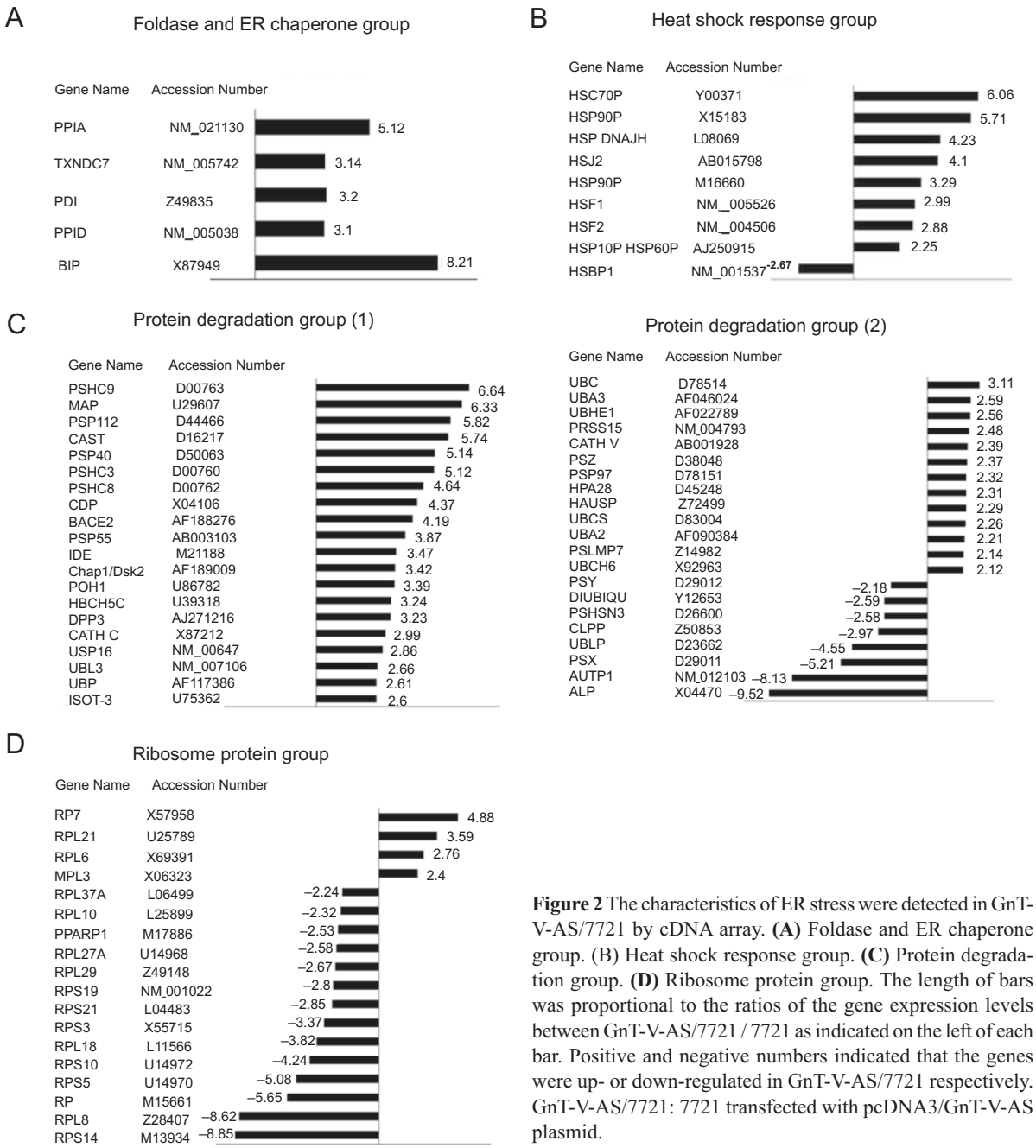

Figure 2 The characteristics of ER stress were detected in GnT-V-AS/7721 by cDNA array. **(A)** Foldase and ER chaperone group. (B) Heat shock response group. **(C)** Protein degradation group. **(D)** Ribosome protein group. The length of bars was proportional to the ratios of the gene expression levels between GnT-V-AS/7721 / 7721 as indicated on the left of each bar. Positive and negative numbers indicated that the genes were up- or down-regulated in GnT-V-AS/7721 respectively. GnT-V-AS/7721: 7721 transfected with pcDNA3/GnT-V-AS plasmid.

were selected as the consequence of GnT-V blocking, and the consistent tendency in three individual experiments from separate cell clones was also required in above gene selection. Based on above criteria, 506 of the 3501 genes spotted on cDNA array were selected, of which 278 genes were up-regulated and 228 genes were down-regulated in GnT-V-AS-7721. The 506 of differentiated genes were clustered into 16 groups according to their functions referenced with the Gene Ontology database and literature searches (Supplemental Material). Four of the 16 groups were closely related to ER stress, such as the genes of foldase and ER chaperone, heat shock response and protein degradation, which were up-regulated in 5/5, 8/9, and 33/41 respectively, while genes encoding ribosome proteins were down-regulated in 14/18. In foldase and ER chaperone gene group, the expression levels of PPIA, TXNDC7, PDI, PPID and BIP in GnT-V-AS/7721 cells were over 3.1 times higher than those in 7721, particularly BIP was 8.21 times higher

(Figure 2A). For the heat shock response group, HSC70P, HSP90P, HSJ2, HSF1, HSF2, HSP10P and HSP60P in GnT-V-AS/7721 cells were over 2.25 times higher than those in 7721 (Figure 2B). The compositions facilitating protein degradation in ubiquitin and proteasome pathway, including UBA2, UBA3, UBCH6, UBCH5C, UBP, UBHE1, HPA28, and proteasome subunits P40, P55, P97, P112, HC3, HC8, HC9, LMP7, Z in GnT-V-AS/7721 had a 2.12~6.64 times higher expression level than those in 7721 cells (Figure 2C). In GnT-V-AS/7721 cells, the expression levels of ribosome protein genes, such as S3, S5, S10, S14, S19, S21, L8, L10, L18, L27A, L29, L37A, and PPARP1 were down-regulated to -2.24 fold ~ -8.85 fold of those in 7721 (Figure 2D). Therefore, GnT-V-AS/7721 cells showed the representative gene expression pattern of ER stress defined with the up-regulation of chaperones assisting protein folding, and the down-regulation of genes associated with protein synthesis and group enhancement of ERAD which functioned with ubiquitin and proteasome pathway [15, 16].

Key molecules in both Ire1/XBP-1 dependent and PERK dependent UPR signaling pathways were activated in GnT-V-AS/7721

The misfolded proteins are retained in the ER and induce an ER stress response that initiates the so-called UPR pathway. BIP, XBP-1 and PERK are key molecules in UPR and they were investigated in this paper as the mediators and/or markers of ER stress response.

BIP is the key chaperone for folding and maturation of protein in ER and its up-regulation is the usual marker of ER stress. The RT-PCR and Western blot analyses indicated that BIP in GnT-V-AS/7721 was markedly up-regulated at both transcriptional and translational levels, which were about 7 and 4.3 times higher than those in 7721 severally and the similar results were observed in the positive control of 7721 cells treated by DTT (Figure 1B).

XBP-1 was a substrate of an unconventional mRNA splicing system in mammalian cells, and the splicing form of XBP-1 mRNA acted as a transcription factor which induced the expression of ER-resident molecular chaperones during ER stress [16, 23, 31]. To evaluate the possible role in GnT-V blocking induced ER stress, the XBP-1 was examined by RT-PCR with the primers designed according to the report by Yoshida *et al* [23]. The expected fragments amplified encompassed the overlapping region of two open reading frames existing in XBP-1 mRNA before and after splicing. The amplified fragments obtained from 7721 and positive control were of 442 bp and 416 bp respectively as expected. Differently, these two bands with 26 bp difference, representing the spliced and unspliced mRNA of XBP-1 (XBP-1^S and XBP-1^U), were observed in GnT-V-AS/7721 (Figure 1C). The proteins, translated from the XBP-1^S and XBP-1^U, were visualized as the bands of 54 kDa pXBP-1^S and 33 kDa pXBP-1^U respectively. When cells were stabilized by pretreating with MG132, a proteasome inhibitor [23, 32], the dominant pXBP-1^S and marginal pXBP-1^U were simultaneously detected in both GnT-V-AS/7721 and the positive control, but only marginal pXBP-1^U was detected in 7721 cells (Figure 1C).

PERK plays a particularly important role in mediating the global cellular response to ER stress. The ER kinase PERK, which undergoes transphosphorylation in response to ER stress, can attenuate protein synthesis through phosphorylating eIF2α. Therefore, the state of PERK as an index of ER stress was investigated in three types of cells as indicated in Figure 1D. The phosphorylated PERK was detected in positive control cells and GnT-V-AS/7721, while only unphosphorylated in 7721. Since the kinase served as a proximal effector of the UPR, it indicated the activation of the UPR pathway of repressing protein synthesis in GnT-V-AS/7721. The findings mentioned above showed that UPR signaling pathways were activated in GnT-V-AS/7721, which confirmed the ER stress characteristics of GnT-V-AS/7721 revealed by cDNA array and that the blocking of GnT-V expression might be the initial trigger of ER stress.

Transient suppression of GnT-V by RNAi induced ER stress

In GnT-V-AS/7721 cells, the plasmid containing antisense cDNA of GnT-V may be integrated into genome at random, and the dsRNA may activate the kinase PKR, which phosphorylated eIF2α and repressed the synthesis of proteins [33, 34]. To exclude the above nonspecific effects beyond GnT-V blocking, the key signaling molecules of UPR pathways were examined again after RNAi induced GnT-V suppression.

The data documented that GnT-V were reduced to 22% and 18% at the mRNA and protein levels respectively in 7721 treated with GnT-V RNAi for 48 h (Figure 3A). The results of UPR examination in 7721 treated with RNAi were similar to those from GnT-V-AS/7721. For example, the BIP expression level was up-regulated for its amounts of mRNA and proteins were about 7.2 and 3.9 folds respectively in RNA interfered 7721 (Figure 3B). In the respect of XBP-1 mRNA, both XBP-1^S and XBP-1^U existed in RNAi treated 7721, but only XBP-1^U was observed in intact 7721 and control cells. For the detection of the XBP-1 protein, dominant pXBP-1^S and marginal pXBP-1^U were found simultaneously in 7721 with GnT-V RNAi (Figure 3C). Furthermore, the phosphorylated PERK was detected in the RNAi treated cells while only unphosphorylated PERK in control and 7721 (Figure 3D). The results indicated that the UPR was activated in 7721 treated with

GnT-V RNAi, and further supporting the findings from GnT-V-AS/7721.

Inhibition of N-glycans synthesis, N-glycans structure alteration of intracellular glycoprotein and the faster migrating form of GRP94 were detected in GnT-V-AS/7721

The GnT-V catalyzes the addition of β-1,6-GlcNAc to N-glycan intermediates found on newly synthesized glycoproteins transiting the medial Golgi. Glycosylation of glycoproteins with N-acetylglucosamine (GlcNAc) is a dynamic protein modification of intracellular glycoproteins. We proposed that reduced expression of GnT-V might induce imperfect glycosylation, resulting in the accumulation of misfolded or unfolded (glyco) proteins in ER followed by UPR. To check the hypothesis, the N-glycans synthesis and structure of intracellular glycoproteins were examined.

In GnT-V-AS/7721 cells, the rate of ^{3}H-Man incorporation was decreased by 44% compared with 7721 transfected with pcDNA3 vector (mock cells) (Table 1A). Given that the repression of proteins synthesis may contribute to the decrease of N-glycans synthesis, the rate of ^{3}H-Leu incorporation, which represented the state of protein synthesis, was also investigated, and the rate of ^{3}H-Leu incorporation

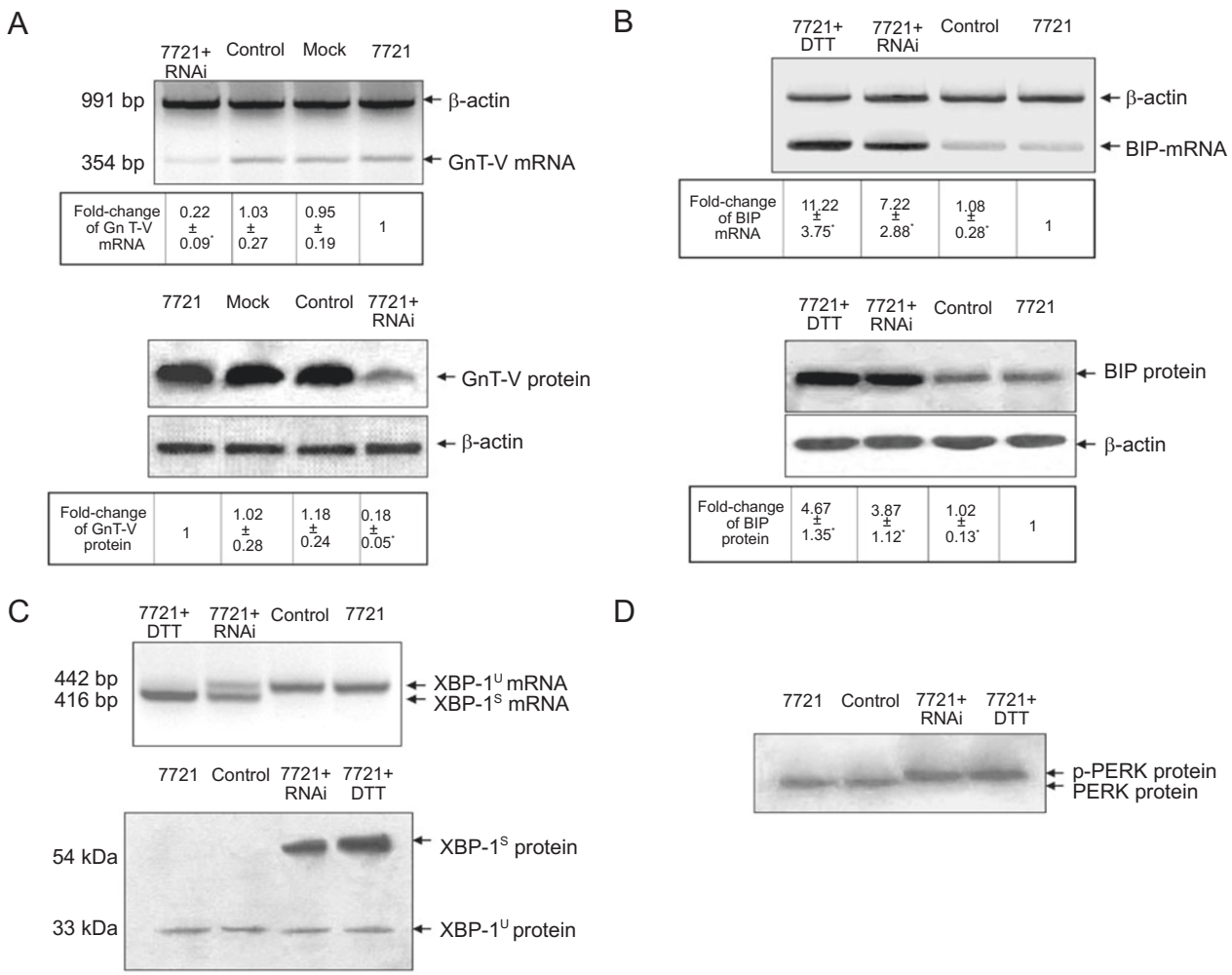

Figure 3 RNA interference to GnT-V induced ER stress in 7721 cells. **(A)** RT-PCR (above) and western blot (lower) analysis of GnT-V in 7721 treated with RNAi. **(B)** RT-PCR (above) and western blot (lower) analysis of BIP in 7721 treated with RNAi. **(C)** RT-PCR (above) and western blot (lower) analysis of XBP-1 in 7721 treated with RNAi. **(D)** Western blot analysis of PERK in 7721 transfected with RNAi. b-actin was used as an internal control in A, B and * represented $p<0.05$ *vs* 7721(n=3). 7721+RNAi: 7721 transiently transfected with RNA interfering sequence of GnT-V for 48 h; control: 7721 transiently transfected with nonspecific RNA interfering sequence; mock: 7721 transiently transfected pSUPER vector; 7721+DTT: 7721 treated with 10 mM DTT was used as positive control; XBP-1^{U}: unspliced forms of XBP-1; XBP-1^{S}: spliced forms of XBP-1.

in GnT-V-AS/7721 was reduced about 30% (Table 1B), the substantial reduction of N-glycan synthesis was about 14%. The results revealed that the functional deficiency of enzymes synthesizing N-glycans was induced after GnT-V blocking.

To examine the structural change of intracellular N-glycans after blocking expression of GnT-V, the intracellular proteins from three types of cells (GnT-V-AS/7721, 7721 treated with TM and mock) were stained using HRP-DSA and HRP-ConA. It was well known that DSA bound to tri- and tetra-antennary glycans, especially that containing β-1,6-GlcNAc branch, while the ConA bound to biantennary glycans without β-1,6-GlcNAc and bisecting branch [35, 36]. The results showed that the intensity of HRP-DSA staining in GnT-V-AS/7721 cells was significantly decreased to 53%, while the intensity of HRP-ConA staining in GnT-V-AS/7721 cells was significantly increased to154% (Figure 4A, and 4B). It suggested that the β-1,6-GlcNAc branches on intracellular glycoproteins were decreased and the biantennary N-glycans without the structures of β-1,6-GlcNAc and bisecting GlcNAc were increased after GnT-V blocking. The result indicated that the structure of N-glycans in intracellular glycoproteins was altered generally due to the repression of GnT-V. It was consistent with the change of structure of N-glycans on GnT-V-AS/7721

Table 1 The rate of ^{3}H-Man incorporation into GnT-V-AS/7721 corrected by the rate of ^{3}H-Leu incorporation was decreased

A

Cell type	Radioactivity (cpm/mg protein)	Incorporation rate of ^{3}H-Man (%)	Inhibition rate (%)
mock	13579±1569	100	0
7721(TM)	3582±977**	26	74
GnT-V-AS/7721	7620±1101**	56	44

B

Cell type	Radioactivity (cpm/mg protein)	Incorporation rate of ^{3}H-Man (%)	Inhibition rate (%)
mock	9079±1277	100	0
7721(TM)	6800±1107¶	75	25
GnT-V-AS/7721	6380±1158*	70	30

(A) The comparison of the ^{3}H-Man incorporation rate among mock, 7721 treated with TM and GnT-V-AS/7721. **(B)** The comparison of the ^{3}H-Leu incorporation rate among mock, 7721 treated with TM and GnT-V-AS/7721. ¶, * and ** represented $P<0.01$, $P<0.005$ and $P<0.001$ vs mock respectively ($n=6$). mock: 7721 transfected with pcDNA3 vector; 7721(TM): 7721 treated with 1 μg/ml TM for 24 hours; GnT-V-AS/7721: 7721 transfected with pcDNA3/GnT-V-AS plasmid.

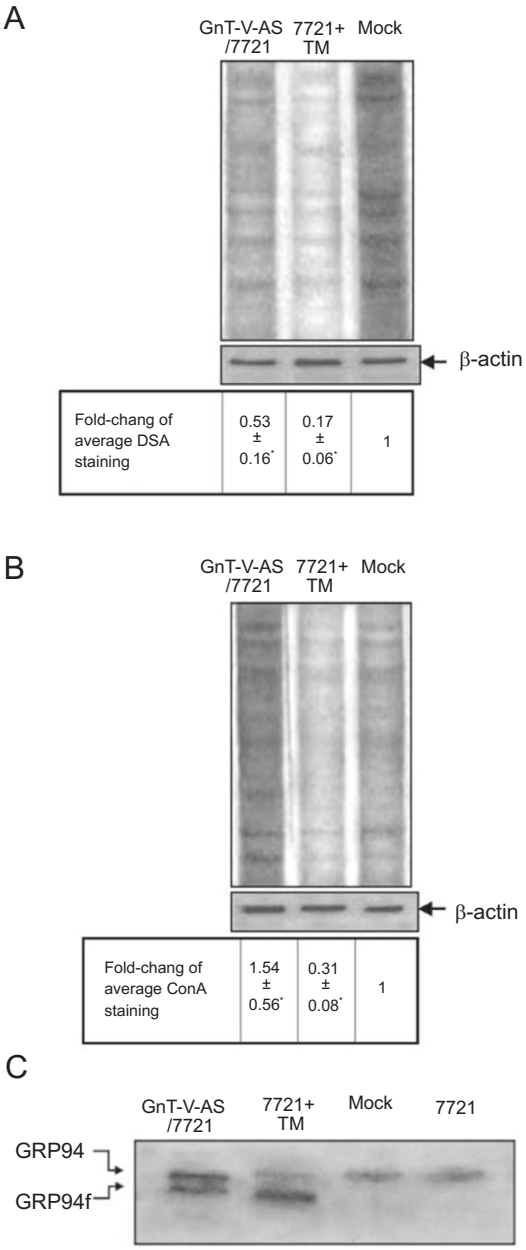

Figure 4 The N-glycans structures of intracellular glycoproteins in GnT-V-AS/7721 were changed using the staining of HRP labeled DSA and ConA. **(A)** HRP-DSA staining of intracellular glycoproteins in GnT-V-AS/7721. **(B)** HRP-ConA staining of intracellular glycoproteins in GnT-V-AS/7721. **(C)** Western blot analysis of GRP94 in GnT-V-AS/7721. β-actin was used as an internal control in A and B. * represented $p<0.05$ vs mock in A and B ($n=3$). The gel electrophoresis time of GRP94 in C was 6 h. mock: 7721 transfected with pcDNA3 vector; 7721+TM: 7721 treated with 1 μg/ml TM for 24 hours; GnT-V-AS/7721: 7721 transfected with pcDNA3/GnT-V-AS plasmid; GRP94f: faster migrating form of GRP94.

cell surface in the previous studies [11-13].

Glucose regulated protein 94 (GRP94), an N-gly-

coprotein of HSP90 family, is a resident protein of the endoplasmic reticulum (ER) and is up-regulated by the accumulation of unfolded proteins [37]. The N-glycosylation status is associated with chaperone activity of GRP94, and also influences the electrophoretic mobility of glycoprotein for more glycan structures retard the migrating velocity of PAGE [38, 39]. To further investigate the mechanism between ER stress and GnT-V blocking, the ER chaperone GRP94 was detected in GnT-V-AS/7721, 7721 treated with TM, 7721 transfected with pcDNA3 vector (mock) and 7721 cells by Western blot analysis. The long electrophoresis time (6 h) separated the protein of GRP94 in GnT-V-AS/7721 and 7721 treated with TM into two distinct bands, but only the slower migrating band was seen in the 7721 and mock cells (Figure 4C). The faster migrating form of GRP94 in GnT-V-AS /7721 suggested the underglycoylation of the chaperone after GnT-V blocking in 7721 cells. These evidences suggested an existing linkage between the loss of GnT-V activity and ER stress in hepatocarcinoma 7721 cells.

Discussion

In this study, both UPR pathways were activated in GnT-V stably and transiently suppressed 7721 cells. However, GnT-V is located in trans Golgi, which does not directly assist the N-glycans synthesis and protein folding in ER, so the mechanism is complicated.

The up-regulation of GnT-V is always accompanied by the increase in the amount of its product, β-1, 6-GlcNAc branch of N-glycans, indicating that the effect of GnT-V is very likely mediated by the structure. The previous studies also found that the fuction of the glycoproteins on cell surfaces was changed for abnormal quantity of GnT-V products, which influenced the pathways or mechanisms associated with cancer progress and metastasis potential [8, 9, 12, 13]. However the effects of altering GnT-V expression or activity on glycans structures as well as functions of intracellular N-glycoproteins have not been reported. It was reported that the enzymes and chaperones assisting the N-glycans synthesis and proteins folding were glycoprotein resident in ER and many of them were N-linked [40]. Furthermore, the amino acid sequences of considerable enzymes and cheperones resident in ER were found to possess N-glycosylation sequon by searching NCBI database, and 90% of the sites were glycosylated [40]. Based on above documents, the functions of enzymes and chaperones were suspected to be defective after blocking expression of GnT-V in 7721 cells. The rate of ^{3}H-Man incorporation that was corrected by the rate of ^{3}H-Leu incorporation was decreased in 7721 after GnT-V suppression. This indicated that the function of N-glycan synthesis enzymes in ER was down-regulated after GnT-V blocking, and the N-glycosylation status in parent 7721 cells would be interfered with. The abnormal glycosylation is the usual reason of ER stress, such as treatment of TM. The experiment of intracellular glycoproteins DSA and ConA staining indicated that the structures of their N-glycans were extensively changed after GnT-V blocking. So the deficient function of the enzymes synthesizing N-glycans, which caused the ER stress in GnT-V suppressed 7721 cells, was associated with their changed glycans structures.

To further investigate the priming mechanism of ER stress in GnT-V blocking 7721 cells, the specific ER molecules concerning UPR were searched in our lab, and the ER chaperone GRP94 was found to be important to induce ER stress. It is an ER resident N-glycoprotein with six N-glycosylation sites and possesses multifunction such as calcium binding, ATP binding and involving antigen presentation [41]. It was reported that GRP94, resembling BIP, was an important ER stress protein that prevented proteins aggregation and helped nascent protein folding [37, 39, 41]. In this study, it was revealed that the glycosylation of the chaperone GRP94 in GnT-V-AS/7721 was compulsorily altered, reflected by the observation of faster migrating form of GRP94 in GnT-V-AS/7721. The underglycosylation of the GRP94 was consistent with the deficient function of the enzymes synthesizing N-glycans, which detected by the decrease of ^{3}H-Man incorporation rate. The N-glycosylation status is related to GRP94 chaperone activity [39], so GnT-V blocking can lead to the deficient function of folding and aggregation prevention of the protein. Furthermore, the GRP94 in GnT-V-AS/7721 was up-regulated and accumulated in ER in GnT-V-AS/7721 cells, so it acted as not only a direct ER stress promoter but also an ER stress effector.

The detailed mechanism of ER stress in suppressed GnT-V 7721 is not completely understood, and currently being investigated in our lab. Based on the results above and published, the relationship between ER stress and GnT-V blocking in 7721 cells may include followings. Firstly, the changed N-glycosylation status interferes with the activity of chaperones and N-glycan synthesis enzymes, which can not function as them in 7721 cells. The key molecules during the ER quality control can disarrange the whole system of assembly, maturation and selectively degradation, such as GRP94 and may be calnexin which contains two potential N-glycosylation sites and participates in the glycoprotein maturation, and they served as both promoter and effector proteins of the ER stress. Secondly, the defective functions of the newborn enzymes or chaperones concerning synthesis of glycans can amplify the effect of the GnT-V blocking. It could form the cycle to cause disturbance of glycosylation in 7721 after GnT-V

blocking, so the stimulatory effect of unfolded proteins can never disappear. As a result, chaperones expression was up-regulated persistently. Accompanied with this, besides up-regulation of ERAD and down-induction of ribosome protein genes, PERK pathway was activated persistently. In this study, a small quantity of GnT-V protein remained in GnT-V stably and transiently suppressed 7721 cells, and the XBP-1 mRNA was spliced partially but not fully, so the ER stress was possibly chronic and formed the balance with the protection mechanism. Furthermore, the underglycosylation of p90ATF6, the important transcriptional activator of the UPR, was reported to be the sensing mechanism for activation of UPR in recent study [38], so p90ATF6 was conjectured to be the also key molecule for the initiation of ER stress in GnT-V suppressed 7721 cells.

The changed N-glycosylation status of the enzymes or chaperones concerning folding and glycans synthesis in ER may be not the only reason of ER stress in GnT-V-AS/7721, and other mechanism after GnT-V blocking should be considered. The consequence of GnT-V suppression was confirmed opposing to that in the enzyme overexpressed carcinoma cells. The decreased β-1,6-GlcNAc branch on the receptor of some growth factors, such as EGFR on 7721 cells surface, reduced their binding affinity and tyrosine autophosphorylation, and the phosphorylation and/or activity of some key signal molecules in both Ras/Raf/MEK/MAPK and PI-3-K/PKB pathway was sequentially reduced [12]. Besides these, the amount of β-1,6-GlcNAc branch on integrin β1 subunit was decreased after blocking GnT-V in 7721, which conduced to adhesion [13]. The suppression of the enzyme also leads to the effect independent of its function of N-glycan modification. Obvious down-regulation of bcl-2 and up-regulation of specific glycosyltransferases for sialyl Lewis X synthesis were observed in 7721 after blocking GnT-V, and it was also found that insufficiency of the enzyme can influence the expression of integrin subunit α5 and β1 [13, 14, 42]. Furthermore the opposing up-regulation of GnT-III and the decreasing of secreted type enzyme cause the effect of GnT-V blocking more complicated and extensive [43, 44]. It seems that these known changes after blocking GnT-V are not to induce ER stress directly, but serve as coordinated effects and cooperate with ER stress to lessen the malignant phenotype. The further experiments are required to investigate the relationship between them. For GnT-V blocking universally decreases the amount of β-1,6-GlcNAc branch of molecules on the surface, it suggests that the glycans and corresponding function of transporters for both glucoses and amino acids, which are both N-glycoproteins [45, 46], can be changed in GnT-V-AS/7721 cells. The impaired function of two types of transporter in 7721 cells may also induced ER stress for the starvation of glucoses and amino acids are the usual reason of ER stress, which was preliminarily confirmed in our lab. Besides, the possibility that decrease of secreted type GnT-V and the increased bisecting β-1,4-GlcNAc structure formed by GnT-III contribute to ER stress should be considered.

Our experiments also found capase-12, the ER stress specific upstream caspase which is not activated by membrane or mitochondria targeted apoptosis signals [47], was activated in GnT-V-AS/7721 after treated with ATRA (other submitted article). So the ER stress may give the explain for the GnT-V-AS/7721 phenotype of lower growth rate and higher sensitivity to cell death than parent 7721 cells, which was found by the previous study [11]. In summary, in GnT-V-AS/7721 cells, cooperated with other known effect caused by blocking GnT-V, ER stress is a new effect reversing the malignant phenotype, and all these indicate that the regulation function of GnT-V is extensive and essential in hepatocarcinoma cells.

GnT-V overexpression and its up-regulation during the process of cancerous transformation were observed in many cancers, such as human mammary, colon and hepatic tumor [3-9]. For liver, in normal hepatic tissue, the GnT-V activity is low, but increases in human hepatocellular carcinoma (HCC) tissues, which is correlated with its progression, and the enzyme activity in human HCC tissues increases during metastasis *in vivo*[48, 49]. The effect of high expression and activity of GnT-V, which contributes to the potential of tumor metastasis and growth, can be blocked by GnT-V suppression. In this study our researches reveal an important relationship between the GnT-V suppression and the initiation of ER stress, and the latter confronts hepatocarcinoma cells with the possibility of apoptosis derived from ER. Therefore all these results suggest that suppression of GnT-V may be a potential way in the treatment of HCC and other tumors overexpressing the enzyme.

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