

Review

# Implication of *N*-acetylglucosaminyltransferases III and V in cancer: gene regulation and signaling mechanism

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Received 30 November 1998; received in revised form 23 April 1999; accepted 23 April 1999

## Abstract

*N*-Acetylglucosaminyltransferases III (GnT-III) and V (GnT-V) play a pivotal role in the processing of N-linked glycoproteins, and are highly involved in cancer progression and metastasis. Expression of GnT-III and GnT-V in the liver is enhanced during hepatocarcinogenesis, although they are not expressed in the normal liver. Gene expression of GnT-V is regulated by a transcriptional factor, *ets-1*, which is involved in angiogenesis and invasion of tumor cells. When the formation of the product of GnT-V, GlcNAc- $\beta$ 1-6 branches, is inhibited by overexpression of GnT-III, lung metastasis of melanoma cells is suppressed. Modification of glycoprotein receptors such as the receptors for epidermal growth factor and nerve growth factor by GnT-III sense transfection changes an intracellular signaling pathway, which may lead to a variety of biological alterations in tumor cells. In this review, we focus on cancer progression and metastasis in relation to GnT-III and GnT-V. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** *N*-Acetylglucosaminyltransferase V; *N*-Acetylglucosaminyltransferase III; Gene regulation; Signaling; Cancer

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## 1. Introduction

It is well known that the structures of complex carbohydrates are altered in cancer and that these changes are highly associated with invasion and metastasis. The mechanism by which those changes occur are still unknown, but in most cases the activation of glycosyltransferases plays a major role and this leads to so-called ‘aberrant glycosylation’ in cancer tissues [1]. The activities of the glycosyltransferases are mainly controlled by their gene expression levels, substrate specificity toward donor and acceptor molecules, and localization in organelles [2]. Recent data obtained from the manipulation of glycosyltransferase genes have been utilized to explore the mechanisms associated with malignant changes of oligosaccharide structures and the signaling pathway. In this mini-review, we will focus on the *N*-acetylglucosaminyltransferases III and V, both of which play a pivotal role in the branching of Asn-linked glycoproteins and their roles in invasion and metastasis of cancer.

## 2. The *N*-acetylglucosaminyltransferases

The processing of *N*-glycans in the Golgi apparatus is the most important step in the biosynthesis of the complex type of Asn-linked glycoproteins. It is known that at least six *N*-acetylglucosaminyltransferases (GnTs), designated as GnT I–VI, are involved in the biosynthesis of a core structure of the complex type of *N*-glycans [2], as shown in Fig. 1.  $\beta$ 1-3 *N*-Acetylglucosaminyltransferases (iGnT) and  $\beta$ 1-6 *N*-acetylglucosaminyltransferases (IGnT) are responsi-

ble for generating peripheral structures, i.e., linear and branched poly-*N*-acetylglucosamine backbones.

## 3. *N*-Acetylglucosaminyltransferase V

GnT-V catalyzes the formation of GlcNAc- $\beta$ 1-6 branches at the Man  $\alpha$ 1-6 side of the trimannosyl core of *N*-glycans. These branches are abundant in cancer tissues, especially in those with high metastatic potential. In viral or oncogene-transformed rodent fibroblasts, branching at the trimannosyl core of complex type *N*-glycans, especially the GlcNAc- $\beta$ 1-6 branch, is increased [3–5]. This increase contributes to the elongation of *N*-glycans including poly-*N*-acetylglucosamine structures, with associated sialyl Le<sup>x</sup> and Le<sup>x</sup> structures known to be involved in cell-cell interactions. Dennis et al. reported that a number of different types of tumor cells which bind to the L-PHA lectin have increased metastatic properties [6]. These cells have an increase in GlcNAc- $\beta$ 1-6

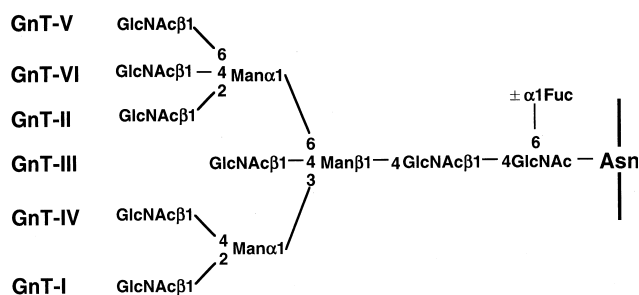


Fig. 1. *N*-Acetylglucosaminyltransferases involved in complex type *N*-glycans. GnT, *N*-acetylglucosaminyltransferase; Man, mannose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Asn, asparagine.

linked branching of *N*-glycans, as judged by the binding to L-PHA lectin which preferentially recognizes the  $\beta$ 1-6 branch structure, due to increased activity of GnT-V. GlcNAc- $\beta$ 1-6 branch formation is well correlated with the metastatic potential of tumor cells such as human breast malignancies as judged by the binding to L-PHA lectin. In addition, swainsonine, an inhibitor of *N*-glycan processing which blocks the pathway prior to the initiation of the  $\beta$ 1-6 branching, also inhibits organ colonization by MDAY-D2 cells and B16 melanoma cells [7,8]. Swainsonine is an inhibitor of  $\alpha$ -mannosidase II and was first isolated from *Swainsona canescens*, an Australian plant that is toxic to animals and produces symptoms resembling those of human  $\alpha$ -mannosidosis upon prolonged ingestion of this plant. Therefore, swainsonine is used for preventing the conversion of high mannose oligosaccharides into those of the complex type in various mammalian cell lines. Swainsonine treatment of either B16-F10 murine melanoma cells or MDAY-D2 murine lymphoreticular tumor cells resulted in a substantial impairment of tumorigenic activity. Tumor cells cultured in the presence of swainsonine for 24–48 h showed reduced organ colonization potential when they were injected into mice. These data also suggest that GlcNAc- $\beta$ 1-6 branching or other complex *N*-glycan structures may play a critical role in cancer metastasis. GnT-V expression is induced by viral and oncogene transformation [9] and during hepatocarcinogenesis in rats [10]. In cases of human hepatoma, the GnT-V activity is well correlated with the severity of the disease [11]. In addition GnT-V activity is also augmented by phorbol esters and transforming growth factor  $\beta$ 1 [12]. Our group and other workers independently purified the enzyme from human lung cancer cells and rat kidney, respectively, and cloned the cDNA [13,14]. Messenger RNA was detected as two bands in HepG2 and MCF7 cells [13], and LEC rat liver [10], but the level was not always consistent with enzymatic activity. To determine the details in the up-regulation of GnT-V, analysis of the 5'-upstream regions of the GnT-V gene as well as post-transcriptional modification were required.

### 3.1. Structure of the human GnT-V gene

We have isolated genomic DNA clones encoding

human GnT-V and reported that the human GnT-V gene is divided into 17 exons, and that the open reading frame is encoded by exons 2–17, spanning 155 kb [15]. Analysis of the 5'-untranslated regions of mRNA from various cells showed multiple sequences depending on the cell types. Exon 1, which was obtained by 5'-RACE using GOTO cell RNA as a template, only contained a 5' non-coding region. Exon 17 contained both coding and 3' non-coding regions in the RNA, but the size of exon 17 is not known because the GnT-V cDNA clone isolated did not contain a poly(A) tail at its 3' end. The promoter region of the GnT-V gene was characterized by searching for any consensus sequences matching those for transcription factor binding.

### 3.2. Identification of potential cis-regulatory DNA sequences

Regions upstream from exon 1 and intron 1 appear to function as promoters. On sequence analysis of the upstream region, a TATA box was found at nucleotide position –27 from the start site of exon 1, but a CCAAT motif was not found. A putative binding site for AP-1 which is a complex of proto-oncogene products Jun and Fos was observed at nucleotide –535. It is noteworthy that putative binding sites for products of the Ets family (C/AGGAA/T) and c-Myb (AACNG) were located at nucleotides –66, –565 and –728 and –138 and –287, respectively, because GnT-V expression has been thought to be related to malignant transformation. These results suggest that tissue-specific expression of the human GnT-V gene may be regulated by activation of tissue-specific transcription factor binding sites identified in these 5'-upstream regions. Recombinant plasmids containing variable lengths of putative promoter and CAT reporter gene were transiently expressed in COS-1 cells, which is a mammalian cell line expressing a large T antigen necessary for activation of the SV40 enhancer. The promoter activities of pCAT-GV3 and pCAT-GV4 were lower than that of the pCAT control vector, but approx. 13-fold and 1-fold higher than that of the pCAT enhancer vector, respectively. Thus promoter activities of the 5'-upstream regions of exon 1 and of intron 1 were demonstrated [16]. The consensus sequences for a TATA box, AP-1, AP-2 and some other transcription fac-

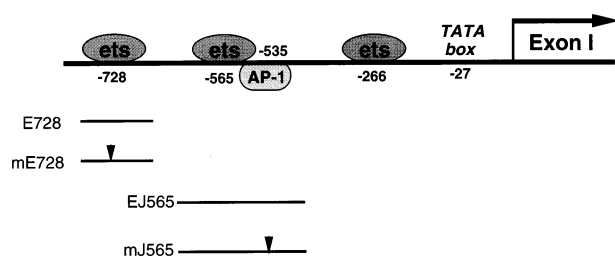


Fig. 2. Schematic drawing of the consensus sequence of the 5' upstream region of GnT-V gene. Ets and AP-1 sites on the GnT-V 5' flanking region are shown. Number indicates nucleotide upstream from exon 1. The oligonucleotide probes used for electrophoretic mobility shift assay are also indicated. An arrow shows the position of the mutation on the nucleotide.

tors were found in the 5' upstream region of exon 1, and consensus sequences for LF-A1, HNF1-HP1, Ets-1 and liver-restricted transcription factors and other factors were found in intron 1. The GnT-V gene employs a multiple promoter system for its transcription, and gene expression may be regulated in a tissue-specific and cell type-specific manner as shown in Fig. 2.

### 3.3. Identification of a functional regulatory element within the 5'-untranslated regions of the human GnT-V gene in HuCC-T1 cells

Saito et al. reported that the 5'-upstream regions of both exon 1 and intron 1 exhibited promoter activity when they were transiently expressed in COS-1 cells [15]. HuCC-T1 cell, a human bile duct carcinoma cell line, was also found to transcribe two types of messages on 5'-rapid amplification of cDNA end analysis (5'-RACE). To elucidate the transcriptional mechanism for the human GnT-V gene in more detail, in particular to identify functional elements necessary for this cell line-restricted expression, GnT-V activity was measured and two types of transcripts of GnT-V were confirmed.

### 3.4. Regulation of the GnT-V is mediated by Ets-1

Deletion analysis indicated that the -1460/-710 and -362/-243 regions of this promoter act as *cis*-acting elements for HuCC-T1 restricted transcription. We have shown that several putative binding sites for LBP-1 (positions -1334, -1038, -932), AP-2 (positions -1264 and -227), nuclear factor-

interleukin-6 (positions -1385, -1005, -965, and -255), c-myc (position -287) and Ets-1 (positions -728, -565 and -266) are included in these two elements [15]. We chose to focus our analysis on three putative binding sites for transcription factor Ets-1, which is a product of proto-oncogenes related to the malignant transformation and metastasis of tumors as described below. To determine whether or not the Ets-1 protein is able to bind to these putative binding sites in the GnT-V gene regulatory regions, gel mobility shift experiments were performed using 23 bp GnT-V promoter-derived oligonucleotides E266, E565, and E728, corresponding to the three putative Ets binding sites in the 5' untranslated regions of the GnT-V gene [16]. At first we confirmed that the *in vitro* transcribed/truncated Ets-1 protein and nuclear extracts of HuCC-T1 cells indeed bind to the Ets-1 consensus sequence. Specific and high affinity binding of the Ets-1 protein to the Ets binding sites of the GnT-V regulatory regions was demonstrated in competition experiments using labeled E266 and E728 probes. This finding suggests that the Ets-1 protein recognizes the Ets consensus sequence at positions -266 and -728. A gel mobility shift assay using the same GnT-V promoter-derived Ets binding sequence incubated with nuclear extracts prepared from HuCC-T1 cells showed retarded protein-DNA complexes and, again, specific binding was identified on competition analysis. The addition of anti-Ets-1 IgG resulted in competition with the protein-DNA complex whereas the mutated Ets binding sequences did not compete. These results again revealed the binding of an Ets-related factor to the 23 bp Ets binding site of the HuCC-T1 restricted promoter region of the GnT-V gene.

### 3.5. Ets-1 as a transcription factor

The Ets transcription factor family shares a common DNA binding domain which interacts specifically with sequences containing a common core trinucleotide sequence, GGA [17]. Ets binding sites have been identified in the regulatory regions of human T cell receptor- $\alpha$  [18] and - $\beta$  [19], and interleukin-2 $\beta$  receptor [20], as well as other cellular and viral enhancers, and these Ets binding sites regulate their transcriptional activities [21–26]. Ets-1 was first described as a cellular homologue of v-ets in replica-

tion-defective retrovirus E26 and is thought to be associated with tumorigenesis and embryogenesis [27]. The gene encoding ets-2 appears to be important in cartilage/bone development and is one of the major factors of Down's syndrome [28]. The expression of c-ets-1 is associated with the invasion of tumor cells in both in vitro and in vivo systems [29,30]. One possible mechanism by which Ets-related proteins promote invasion of tumor cells is that they enhance the transcription of matrix metalloproteinase genes [31,32]. In contrast, Suzuki et al. [33] reported that overexpression of the Ets-related protein in colon cancer cells reversed the transformed phenotype and tumorigenicity. The presence of Ets binding sequences in the promoters of c-myc, and cdc-2 has also been reported. The c-myc and cdc-2 oncoproteins have been implicated in the control of normal cell growth and their deregulation is associated with neoplasia. From the above reports, expression and regulation of these proteins are highly associated with cancer invasion, metastasis and tumor cell growth.

### 3.6. Regulation of the GnT-V promoter by Ets-1 in various cancer cell lines

When the GnT-V gene was transfected into a lung epithelial cell, the transfectant showed an increased tumorigenicity, as evidenced by an assay involving the subcutaneous injection of the cells into nude mice [34]. This cell showed an altered 'transformed cell' morphology as is often observed for oncogenically transformed cells. In addition, the overexpression of GnT-V decreased the serum growth requirements of the contact-inhibited parental cells, but increased the migration rates of controls by 3–10-fold, and the cells adhered less well to fibronectin or collagen type IV. Ko et al. have investigated whether the expression of GnT-V is correlated with that of ets family mRNAs in 16 human and murine cancer cells, and the effects of overexpression of ets-1 and dominant-negative ets-1 on GnT-V expression, as well as the coordination of AP-1 and GnT-V genes [67]. Northern blot analyses were performed on various cancer cell lines using ets-1, GnT-V, ets-2, erg and fli-1 cDNAs as probes. Kato-III, HepG2, Huh7, Colo201, Colo205 and B16-F1 cells showed higher levels of ets-1 expression than the other cells.

The expression of GnT-V mRNA showed a pattern similar to that for ets-1 expression. In contrast, A549, Lu65A, Lu99B, MKN45, PacaII, MB231, Huh7, Hep3B and AH66 showed lower levels of both GnT-V and ets-1 mRNA expression. Expression levels of GnT-V and ets-1 mRNA, quantitated by densitometry, were plotted. A positive correlation was found between these mRNAs expressed in cancer cell lines. This suggests that the GnT-V gene expression is controlled by ets-1 in various cell lines. Although ets-2 has a motif similar to ets-1, the ets-2 expression pattern was different from the expression of ets-1 and GnT-V. Erg, another protein of the ets family, showed low expression levels in various cell lines and did not correlate with GnT-V expression. Expression of fli-1 which is known to be 98% homologous to erg showed a very similar pattern to erg.

To investigate expression of ets-1 and ets-2 at the protein level, Western blot analysis was performed using A549, B16-F1, HepG2, Huh7, Kato-III and MKN45 cells. A high level of expression of ets-1 and ets-2 was observed in HepG2, Huh7 and Kato-III cells. In contrast, their expression was quite low in A549 cells, but high expression of ets-2 was observed in MKN45 cells. These data were quite consistent with their mRNA expression.

Nuclear extracts from MKN45, HepG2 and Colo201 cell lines were subjected to electrophoretic mobility shift assay (EMSA) analysis. This was performed using a 24 bp GnT-V promoter-derived oligonucleotide E728 (–741/–718), which was shown to have moderate binding affinity among the three ets binding sites located in the 5' untranslated regions of the GnT-V gene [16]. When the radiolabeled oligonucleotide was incubated with nuclear extracts prepared from MKN45, HepG2 and Colo201, retarded protein-DNA complexes were detected. The intensities of the complex of HepG2 and Colo201 were higher than that of MKN45. Specificity of binding was identified with a mutant oligonucleotide. In order to confirm specific binding, the supershift assay was performed by the addition of anti-ets-1 and anti-ets-2. While both antibodies were shown to supershift in the HepG2 cells, a supershifted band by the ets-2 antibody was more clear than that of ets-1 antibody in MKN45 cells which express higher levels of ets-2 than those of ets-1. The supershifted band by ets-1 showed a competitive pattern in Colo201 cells.

These results strongly reflect the importance of *ets-1* and *ets-2* mRNAs in each cell.

### 3.7. Is AP-1 cooperative with *ets-1* in *GnT-V* gene expression?

Ets is already known to cooperate with AP-1 in the transcriptional regulation of genes such as IL-2 [35], collagenase [36], and TNF [37]. To investigate the cooperative *trans*-activation of c-Jun on the *GnT-V* promoter throughout the c-Jun binding element and the ets binding element, we first performed Northern blot analysis in various cancer cell lines. Expression of c-Jun mRNA was observed in nearly all cells and was not correlated with *GnT-V* expression. To investigate directly the cooperation of AP-1 and ets binding elements on *GnT-V* expression, EMSA was performed using a 58 bp *GnT-V* promoter-derived oligonucleotide EJ565 (–578/–522), which contained both ets and AP-1 sites. When the radiolabeled EJ565 oligonucleotide was incubated with nuclear extracts prepared from HepG2, the formation of retarded protein-DNA complexes was observed. When the radiolabeled MJ565, which contained a mutant AP-1 site, and was designed to detect the specificity of the AP-1 site, was incubated with the nuclear extracts, the retarded protein-DNA complex pattern was the same as when EJ565 was used. Ets-specific binding was confirmed using competition analysis and the supershift assay. These results indicate that the DNA-protein complex with E565 nucleotide was independent of AP-1 binding. To further confirm that the AP-1 and *ets-1* sites do not cooperate, antisense c-Jun was transfected to A549 and PacaII cells. Although these cells expressed low levels of both *ets-1* and *GnT-V*, the transfection of a vector alone to these cells brought a slight enhancement of expression of *GnT-V* mRNA. When antisense c-Jun was transfected into the cells, the *GnT-V* mRNA expression was not changed, suggesting that c-Jun is not linked to *GnT-V* expression.

### 3.8. The dominant negative *ets-1* down-regulates *GnT-V* gene expression

To demonstrate the enhancement of *GnT-V* by *ets-1* proteins by a different approach, the expression of *ets-1* was inhibited by the transfection of domi-

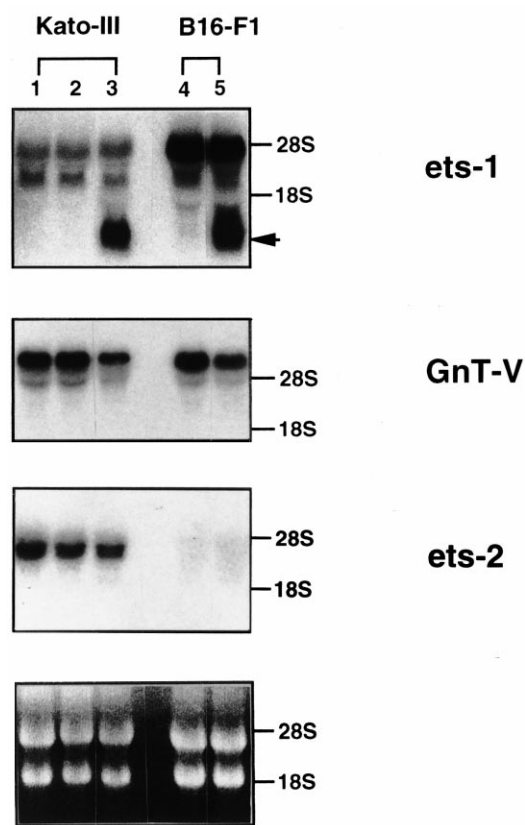


Fig. 3. A dominant negative *ets-1* gene suppressed the expression of *GnT-V* gene. A dominant negative mutant of *ets-1* was transfected into Kato-III and B16-F1 cells which showed high levels of *GnT-V* and *ets-1* expression. 30 µg of total RNA extracted from these cells were analyzed by Northern blotting. Lane 1 indicates control plasmid pEGFP-N1, lanes 2 and 4 indicate mock transfectants, and lanes 3 and 5 indicate transfectants of the negative dominant mutant of *ets-1*. The mRNA of the negative dominant *ets-1* is indicated by an arrowhead. The ethidium staining of the gel for equal amounts of RNAs is shown in the bottom panel.

nant negative *ets-1* in Kato-III and B16-F1 cells, which showed high levels of intrinsic *ets-1* and *GnT-V* expression. When the dominant negative mutant of *ets-1* was transfected into Kato-III and B16-F1 cells, the expression of *GnT-V* was decreased in comparison with a mock transfection, suggesting that *ets-1* regulates *GnT-V* expression in these two cell lines (Fig. 3).

### 3.9. Activation of *GnT-V* gene

As described above, *GnT-V* gene expression in oncogenic transformation is probably mediated by

the ets family including ets-1, a transcriptional factor which also regulates several enzymes associated with cell invasion and metastasis. This may up-regulate the signaling pathway of cellular proliferation. This pathway usually starts with growth factor receptors with tyrosine kinase activities on the cell surface and then proceeds through src, ras and raf signals, but direct evidence for this is not available. The GnT-V gene is activated by transfecting Ha-ras into NIH3T3 cells and EGF and insulin did induce the enzyme in rat hepatocarcinoma cells [38]. Src also induces enzyme activity [39]. Very recently, Pierce's group reported that *her-2/neu* oncogene actually stimulates the transcription of GnT-V in breast carcinoma cells [40] and neu is mediated by the Ras-Raf-Ets signaling pathway; this group previously reported that src showed similar stimulation. From these data the GnT-V gene is probably regulated by different receptor tyrosine kinases.

### 3.10. Overexpression of GnT-V and GnT-V null mice

Dennis's group reported that overexpression of GnT-V in non-transformed Mv1Lu cells, an immortalized lung epithelial cell line, results in loss of contact inhibition of cell growth, and this was totally blocked by swainsonine, an inhibitor of  $\alpha$ -mannosidase II [34]. In serum-deprived and high density monolayer cultures, the transfectants formed foci, and typical characteristics of proliferating cells such as maintained microfilaments and also accelerated cell death by apoptosis. Injection of the GnT-V transfectants into nude mice produced a 50% incidence of benign tumors and progressively growing tumors as compared to the control group. In adhesion assays, the GnT-V overexpressing cells in which their sugar chains were modified, were less adhesive on surfaces coated with fibronectin and collagen type IV. The target proteins glycosylated by GnT-V gene transfection were found to be LAMP-2 (lysosome associated membrane protein 2),  $\alpha 5$ ,  $\alpha V$  and  $\beta 1$  integrins. These data suggest that GlcNAc- $\beta 1$ -6 branching of *N*-glycans contributes directly to relaxed growth controls and reduced substratum adhesion in premalignant epithelial cells.

Very recently Dennis's group generated GnT-V null mice by homologous recombination in ES cells [41]. GnT-V activity and L-PHA binding were absent

in all tissues examined. The mice had alterations in T cell receptor (TCR) function, increased frequency of intestinal crypts and decreased apoptosis in the intestine. This is consistent with the finding that the GnT-V expression is known to be unregulated following T cell activation. In the null mice, T cell surface glycoproteins underwent aberrant glycosylation and this may result in the changes of physical association of TCR, thus affecting a change in the threshold of agonist required for intracellular signaling. More interestingly, when the GnT-V null mice were crossed with polyoma virus middle T transgenic mice, the frequencies of experimental breast cancer and tumor metastasis were markedly reduced as compared to controls [41]. These data also support the view that GnT-V and its enzymatic product, the GlcNAc- $\beta 1$ -6 branch, play a pivotal role in cancer progression and metastasis.

## 4. N-Acetylglucosaminyltransferase III

### 4.1. Competition of GnT-III and GnT-V in vivo leads to suppression of cancer metastasis

GnT-III catalyzes the addition of GlcNAc via  $\beta 1$ -4 linkage to the  $\beta$ -mannose of the mannosyl core of *N*-glycans, and was originally identified in the hen oviduct membrane [42]. It is well known that the bisecting GlcNAc structure affects the conformation of sugar chains and after GnT-III acts on the biantennary sugar chains other glycosyltransferases such as GnT-II, GnT-IV and GnT-V are no longer able to act on the biantennary sugar chains [43–45]. Therefore GnT-III is a key enzyme in the biosynthesis of *N*-glycans. In normal rat tissues, GnT-III activity is abundant in kidney and brain but in the adult liver, especially in hepatocytes, the enzyme activity is almost lacking. However, once the rat develops a primary hepatoma, the enzyme activity is rapidly increased up to 100-fold or more. The same phenomenon was observed in the case of ascites hepatoma cells [46]. A typical plasma membrane protein,  $\gamma$ -glutamyltranspeptidase, is a glycoprotein and this enzyme is also very low in adult rat liver but, again, the activity is extremely high in the primary hepatoma or ascites hepatoma [47]. The  $\gamma$ -glutamyltranspeptidase catalyzes the degradation of glutathione,

a major thiol antioxidant in the cells and the enzyme belongs to the so-called second phase of drug metabolizing enzymes (see a review in [48]). The enzymes were purified from adult rat kidney, adult rat liver and ascites hepatoma and their carbohydrate structures were compared. The  $\gamma$ -glutamyltranspeptidase purified from normal rat liver contains no bisecting GlcNAc but the enzyme purified from primary hepatoma, ascites hepatoma or kidney contains a considerable level of such residues in the enzyme [49]. These data are consistent with those of high activity of GnT-III in rat primary hepatoma and ascites hepatoma as well as rat kidney and suggested that one of the target glycoproteins in those tissues is  $\gamma$ -glutamyltranspeptidase.

Nishikawa et al. then purified the enzyme from normal rat kidney using several chromatographic procedures including substrate affinity chromatography and obtained a partial amino acid sequence of the purified protein [50]. Oligonucleotide primers were designed using amino acid sequences and a cDNA was obtained. The mRNA of GnT-III is highly expressed in rats with primary hepatoma [10] and also in leukemia cells of patients [51]. As described above, GnT-III regulates the further processing of *N*-glycans and GnT-V expression is very high in several tumor cells with high metastatic potential.

We have studied whether or not GnT-III can modulate the expression of GnT-V activity in melanoma B16 cells [52]. The experimental strategy was as follows. First we have isolated the high metastatic clone of B16 melanoma cells and obtained B16 melanoma F1hm cells. The enzymatic activity and mRNA expression of GnT-V were very high whereas those of GnT-III were negligible if any. Then we made nine transfectants of GnT-III which showed high GnT-III gene expression. When we performed lectin blot analyses on those transfectants and parental B16-F1hm cells, L-PHA staining was very intense and E-PHA staining was negligible in parental cells. On the other hand, E-PHA staining was very strong and L-PHA staining was negligible in the GnT-III transfectants.

In order to know whether the transfectants did affect tumor metastasis and invasion, we have injected both cells into the tail vein of mice and after 3 weeks we counted the number of colonies in the

lung due to metastasis. We found that lung metastasis occurs very frequently in the parental cells, but the GnT-III transfectants markedly reduce the metastasis. In order to determine the mechanism by which L-PHA binding in B16-F1hm was reduced by GnT-III transfection, the molecular model of core mannose in the presence and absence of bisecting GlcNAc was made on an INDIGO workstation using Insight II/discover software on the basis of nuclear magnetic resonance data [53]. The biantennary structure of a core mannose was twisted in the presence of bisecting GlcNAc. These conformational changes may be responsible for the substrate inaccessibility to GnT-V to form the GlcNAc- $\beta$ 1-6 structure, which raised the possibility that  $\beta$ 1-6 branching may be suppressed by the introduction of the GnT-III gene. We also analyzed the substrate specificity of GnT-V purified from human lung cancer cell lines and confirmed that the bisected biantennary sugar chain did not serve as a substrate for GnT-V, indicating that once the GnT-III acts on the biantennary structure of *N*-glycans, the GnT-V will not be able to act.

We focused on important adhesion molecules such as E-cadherin which play a major role in cell-cell contact of cancer cells and contribute to invasion of target tissues. When we examined the expression of E-cadherin in the GnT-III transfectants, no difference in the mRNA levels was observed, but protein levels of E-cadherin and its localization were dramatically changed [54]. In the parental cells, E-cadherin was weakly expressed at the cell-cell contacts, while positive transfectants showed elevated expression at cell-cell contacts. Moreover, pulse chase experiments indicated that the half-life of E-cadherin in the positive transfectants is prolonged, that it was not cleaved by proteolysis and was retained on the cell surface membranes after a long period of time. This indicates that the glycosylation of E-cadherin by GnT-III occurs and this aberrant glycosylation of E-cadherin may confer resistance to proteolysis, resulting in the accumulation of E-cadherin at cell-cell contacts. Overexpression of E-cadherin due to the presence of the bisecting GlcNAc structures will make tumor cells more tightly aggregate with each other and prevent detachment from the lung metastatic colony thereby promoting invasion into lung tissues.



#### 4.2. Growth factor signaling and GnT-III transfectants

GnT-III is highly expressed in brain and kidney tissues. To examine the intracellular role of GnT-III and its product in neural cells, the GnT-III gene was overexpressed in a rat pheochromocytoma cell line, PC-12 [55]. The GnT-III transfectants showed a significant increase in E-PHA binding on lectin blot analysis, indicating that some glycoproteins contained elevated levels of bisecting GlcNAc structures. The parental cells were differentiated into sympathetic neurons upon treatment with nerve growth factor (NGF) but the GnT-III transfectants showed neither morphological response nor changes in cell growth rate. In addition upon NGF treatment, tyrosine phosphorylation of the Trk/NGF receptor could not be detected in GnT-III transfecting PC-12 cells, although the degree of NGF binding to control and GnT-III transfectants remained unaltered. The above study also showed that the modification of Trk/NGF receptor as the result of the overexpression of GnT-III in PC-12 cells, blocked ligand-induced receptor dimerization. These results indicate that the overexpression of the GnT-III gene in PC-12 cells results in some aberrant glycosylation of the Trk receptor which, in turn, affects NGF-induced signaling in PC-12 cells. Rebbaa et al. reported that the binding of E-PHA lectin to the EGF receptor in U373 MG human glioma cells blocks EGF binding and receptor autophosphorylation [56]. In addition, it has also been reported that overexpression of the GnT-III gene in the U373 MG cells inhibits EGF-R functions [57]. EGF-R is a glycoprotein and the oligosaccharide residues play an important role in ligand binding, receptor clustering and the phosphorylation of its tyrosine residues. As described above, we reported that overexpression of GnT-III in mouse melanoma B16-F1 cells suppresses the metastatic potential in vivo. The oligosaccharide structure of EGF-R in GnT-III transfecting B16-F1 mouse melanoma cells was modified. However, in contrast to U373MG glioma cells, ligand-induced tyrosine kinase activity of EGF-R was similar in both parental and GnT-III transfecting B16-F1 melanoma cells. Further analysis of EGF-R specific substrates showed a significant phosphorylation of oncoprotein c-cbl (J. Suresh et al., in preparation). Oncoprotein c-

cbl is a novel 120 kDa adapter protein which is implicated in the regulation of PI3-kinase and MPK/ERK pathways of signal transduction. We have also shown that EGF-induced MEKs1 and 2 phosphorylation is inhibited in GnT-III transfecting B16-F1 cells. Since MEKs are upstream kinases which phosphorylate and activate ERKs, we also observed the down-regulation of EGF-induced ERK activation in GnT-III transfecting B16-F1 melanoma cells. The MAP kinases are central transducers of transcytoplasmic signaling from hormones, growth factors, cytokines and environmental stresses to the nucleus. MAP kinase isoforms ERKs1 and 2 have been implicated in the regulation of cellular proliferation and differentiation via the phosphorylation of transcription factors and cytoskeletal proteins.

#### 4.3. Transgenic mice and null mice of GnT-III genes

Cell specific targets for GnT-III modification and their differential functions are so numerous that GnT-III can be called a 'multifacial glycosyltransferase'. In the normal rat liver GnT-III activity is nearly undetectable, but during liver regeneration and hepatocarcinogenesis its activity is markedly increased [10,58]. To determine the biological significance of GnT-III in hepatocytes, transgenic mice which specifically express GnT-III in the liver were established [59]. The hepatocytes from transgenic mice showed a swollen oval-like morphology with accumulation of lipid droplets. In addition, circulating levels of triglycerides,  $\beta$ - and pre- $\beta$ -lipoprotein fractions (LDL and VLDL) and apolipoprotein B100 were significantly reduced in the serum of transgenic animals, compared to controls. However, apolipoprotein B which contained an increased level of bisecting GlcNAc accumulated in the transgenic hepatocytes. Decreased levels of triglycerides in transgenic mice plasma may also be related to the impairment of microsomal triglycerides transport protein which has two putative *N*-glycosylation sites. Collectively, these data demonstrate that aberrant glycosylation, as a direct result of the formation of bisecting GlcNAc, disrupts certain functions of apolipoprotein B such as its transport or lipoprotein complex formation, leading to the generation of a fatty liver.

Marth and his colleagues developed GnT-III deficient mice by homologous recombination in ES cells

and Cre-mediated gene deletion. The mice were found to have no GnT-III activity as well as no reactivity to E-PHA lectins. However, the GnT-III deficient mice were viable and reproduced normally. In addition no abnormalities were found in brain and kidney in which the GnT-III is highly expressed in the wild mice [60]. However, very recently, Stanley and her group have reported that GnT-III knockout mice are resistant to diethylnitrosamine-induced tumorigenesis [61]. Stanley's group investigated the functional role of the bisecting GlcNAc in the development of liver cancer. The GnT-III gene (*Mgat3*) was inactivated by targeted gene disruption, and the susceptibility of null mice (*Mgat3*<sup>-/-</sup>) to tumor induction was examined. After a single injection with diethylnitrosamine and subsequent treatment with phenobarbital for 6 months, the livers of the *Mgat3*<sup>-/-</sup> mice were normal in size and only 50% of mice had one to four small tumors, while the *Mgat3*<sup>+/+</sup> or *Mgat3*<sup>+/-</sup> mice had enlarged livers that contained numerous tumors. These data and histological analyses indicated that tumor initiation occurred in *Mgat3*<sup>-/-</sup> mice but tumor progression was severely retarded.

These data suggest that GnT-III plays an important role in the progression of preneoplastic foci to primary hepatoma. However, hepatic tumor in *Mgat3*<sup>+/+</sup> or *Mgat3*<sup>+/-</sup> mice showed low activity of GnT-III. The authors suggested that a glycoprotein factor with bisecting GlcNAc structure facilitates tumor progression in liver. Although such glycoproteins have not yet been found, cytokines or growth factors produced by other cells may be responsible. Another possibility arises that in the case of GnT-III transgenic mice, sensitivity to a carcinogen such as diethylnitrosamine may be increased and the animals which overexpressed the GnT-III gene in the liver are prone to have primary cancer in the liver. This experiment is now underway in our laboratory.

#### 4.4. *Implication of GnT-III gene expression in various cancers*

GnT-III activity is also elevated in lymphoma cells and in human leukemia cells especially in the state of blast crisis [51]. A high level of activity of GnT-III was observed in fetal liver but not in adult liver. These data suggest that GnT-III may be linked to

undifferentiation. Treatment of hepatoma cells with forskolin, an adenylyl cyclase activator, increases GnT-III gene expression while other agents or several cytokines failed to induce GnT-III [62]. Interestingly, induction of endogenous GnT-III by forskolin treatment inhibited the sorting of membranous proteins, lysosomal membrane glycoprotein 1 and  $\gamma$ -glutamyltranspeptidase. This indicates that bisecting GlcNAc impairs the sorting toward the cell surface in a rat hepatoma cell M31.

It is interesting to note that  $\gamma$ -glutamyltranspeptidase purified from rat hepatoma contains bisecting GlcNAc [49], while the enzyme purified from human hepatoma contains very little. Moreover, during hepatocarcinogenesis in rats, the enzyme activity in serum is not markedly increased even though very high activity is found in hepatoma tissue. On the other hand, in serum and hepatoma tissues of patients with primary hepatoma, enzyme activity is high (unpublished). This suggests that once the bisecting GlcNAc is attached to  $\gamma$ -glutamyltranspeptidase, as in the case of rats, the enzyme could not anymore undergo sorting and will not be released to the plasma.

Since GnT-III inhibits further processing of sugar chains by other glycosyltransferases, the transfection of GnT-III brought about tremendous biological changes. For example, transfection of GnT-III into K562 cells (a human erythroleukemia cell line) increases their resistance to lysis by natural killer cells and enhances their spleen colonization ability [63]. Transfection of GnT-III into a human hepatoma cell line, HB611, which expresses hepatitis B virus suppresses the expression of hepatitis B virus [64]. The mechanisms underlying these results require further study. Aberrant glycosylation influences adhesion molecule functions such as E-cadherin and CD44, a ligand to hyaluronate. When GnT-III transfected B16-F1 melanoma cells were injected into a synergetic mice via tail vein, lung metastasis was suppressed [52] because of increased E-cadherin levels on their cell surface [54]. In contrast, when the same cells were subcutaneously injected into the back of mice, splenic colonization was promoted through oligosaccharide remodeling of CD44 [65]. The spleen contains abundant amounts of hyaluronate. The GnT-III transfected cells had an increased affinity to immobilized hyaluronate and FITC labeled hya-

luronate increased binding to the cell surface. The enhanced adhesion in the cells was suppressed by the treatment with  $\beta$ -*N*-acetylhexosaminidase, indicating that *N*-acetylglucosamine residues were responsible for the enhanced adhesion. These data demonstrate the multifacial functions of GnT-III in tumor metastasis. Moreover, the role of bisecting *N*-acetylglucosamine residues are quite different according to the tissue and cell types. The reason why the bisecting GlcNAc has multifactorial properties, is that the regulation of the GnT-III gene expression is controlled in a tissue- and cell-specific manner.

## 5. Perspectives

In this review the implication of the GnT-III and GnT-V genes and their roles in cancer including invasion and metastasis were discussed. The earlier works on structure and lectin binding assays for the identification of GlcNAc- $\beta$ 1-6 branches and also bisecting GlcNAc structures in the complex *N*-glycans actually opened a new field of glycobiology. The recent success in the purification of GnT-III and GnT-V proteins and their cDNA cloning and gene analyses could explain the pathophysiological significance of these genes. The analysis of upstream regions of these genes prompted us to identify the transcriptional factors and signaling molecules [66]. Moreover, the gene targeting technique has added new insights into this field. The next step for elucidating the actual function of these genes would be to identify the target proteins when we have overexpressed the gene or knocked out the gene in vitro or in vivo because the overexpression or knockout of the glycosyltransferase genes would definitely change the carbohydrate structure of target proteins. These issues suggest the possibility that the phenotypic changes observed in the gene targeting animals were due to the secondary effects, via the modification of carbohydrate structures in the target proteins.

## Acknowledgements

We thank Drs. M. Feather and J.F. Gutteridge for editing this manuscript and Drs. Tomohiko Taguchi

and Suresh Jain for their valuable suggestions to this manuscript.

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