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Invited Mini Review

Branched *N*-glycans and their implications for cell adhesion, signaling and clinical applications for cancer biomarkers and in therapeutics

Naoyuki Taniguchi[1,2,3,*] *& Hiroaki Korekane*[1,2]

[1]Disease Glycomics Team, Systems Glycobiology Research Group, RIKEN Advanced Science Institute, and [2]RIKEN Max Planck Institute Joint Center, Wako, Saitama, [3]RIKEN Alliance Laboratory, The Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka, Japan

Branched *N*-glycans are produced by a series of glycosyltransferases including *N*-acetylglucosaminyltransferases and fucosyltransferases and their corresponding genes. Glycans on specific glycoproteins, which are attached via the action of glycosyltransferases, play key roles in cell adhesion and signaling. Examples of this are adhesion molecules or signaling molecules such as integrin and E-cadherin, as well as membrane receptors such as the EGF and TGF-β receptors. These molecules also play pivotal roles in the underlying mechanism of a variety of disease such as cancer metastasis, diabetes, and chronic obstructive pulmonary disease (COPD). Alterations in the structures of branched *N*-glycans are also hall marks and are useful for cancer biomarkers and therapeutics against cancer. This mini-review describes some of our recent studies on a functional glycomics approach to the study of branched *N*-glycans produced by *N*-acetylglucosaminyltransferases III, IV, V and IX (Vb) (GnT-III, GnT-IV, V and IX (Vb)) and fucosyltransferase 8 (Fut8) and their pathophysiological significance, with emphasis on the importance of a systems glycobiology approach as a future perspective for glycobiology. [BMB reports 2011; 44(12): 772-781]

INTRODUCTION

As compared to the genome, transcriptome and proteome, the glycome is much more complex and heterogeneous and glycosylation is one of the most abundant protein modification reactions. In fact, over 50% of proteins have undergone glycosylation (1). Moreover structural studies of glycans are much more difficult because there are no methods for sequencing or synthesizing them, like DNA and proteins and no cloning techniques such as PCR are currently available. Even though capillary electrophoresis, Mass-spectrometry and NMR techniques have been extensively developed for the analysis of glycans, problems remain in terms of sensitivity and the quantitative analysis of glycans.

For the last 20 year or so, our group has been interested in the functions of *N*-linked glycans, especially glycosyltransferases that are involved in the *N*-linked glycan branching (2-8). Over time, we purified a series of glycosyltransferases to homogeneity using classical but, in fact, unique and sophisticated methodology such as affinity chromatography using donor or acceptor substrates. We were able to obtain partial amino acid sequences for these glycosyltransferases and to then successfully clone their cDNAs and genes. These studies made it possible to analyze the structure and function of *N*-linked glycans (8)

This mini-review will introduce our investigations and studies by other group as well in terms of the onset of disease, cancer biomarkers and therapeutics especially antibody therapy.

Biosynthesis of *N*-glycan branching structures

N-glycan branching structures are biosynthesized by various glycosyltransferases such as GnTs (*N*-acetylglucosaminyltransferases), Futs (fucosyltransferases) and GalTs (galacotosyltransfrases) and STs (sialytransferases) in the Golgi apparatus.

N-acetylglucosaminyltransferases (GnTs) I to -VI act on a common core structure of Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn (9, 10) but in this mini-review we will focus on GnT-III, IV, V and IX (Vb) and Fut8 (Fig. 1).

Bisecting GlcNAc and GnT-III

GnT-III catalyzes the formation of a unique structure, namely the "bisecting GlcNAc", and is involved in the biosynthesis of complex and hybrid types of *N*-glycans. The enzyme was first reported by Narasimhan (11), Gleeson and Schachter (12). On the other hand our group found that the enzyme which is involved in the turnover of glutathione, γ-glutamyltranspeptidase, is highly activated in the process of azo-dye induced hepatoma and that the same enzyme from various sources had identical properties, except for the sugar chains (13, 14). Subsequently, in collaboration with Kobata's group, we identified the bisecting GlcNAc

*Corresponding author. Tel: +81-48-467-8094; Fax: +81-48-462-4692; E-mail: tani52@wd5.so-net.ne.jp
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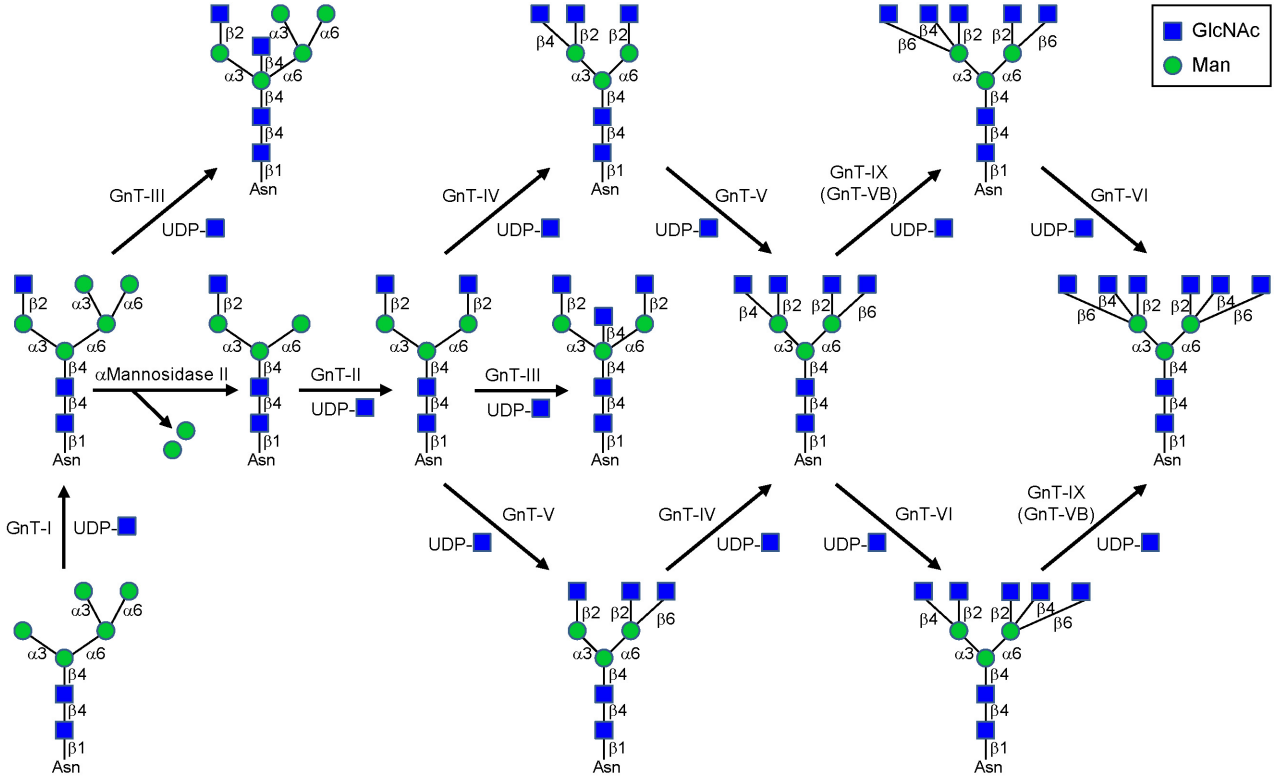

Fig. 1. Branched *N*-glycans and their relevant glycosyltransferases. Each GlcNAc branch may be elongated with galactose, poly-*N*-acetyllactosamine, sialic acid and fucose. GnT-VI is not found in humans.

structure in a γ-glutamyltranspeptidase purified from ascites hepatoma cells but this structure was not present in the enzyme from a normal liver (15). Our group developed an assay method for GnT-III that involved the use of pyridylaminated sugars as a fluorescent probe which was developed by Hase's group (16-18). GnT-III activity was found to be very high in those tissues, including hepatoma tissues, kidney and brain of rats, whereas in normal liver tissue, the activity is almost negligible (19). We then purified this enzyme to homogeneity from rat kidney using an affinity column to which a biantennary sugar chain was immobilized. The enzyme was purified to homogeneity and a partial amino acid sequence was obtained and the cDNA and its gene was then cloned (20). Using a functional glycomics technique (21) we found various target proteins toward GnT-III on which bisecting GlcNAc was attached *in vitro* and *in vivo*. The GnT-III gene suppressed cancer metastasis *in vivo* in a model of lung cancer metastasis (22). We then found that one of the target proteins toward GnT–III was E-cadherin (23) which plays a key role in the suppression of cancer metastasis in an experimental rat model. The addition of bisecting GlcNAc to E-cadherin alters its distribution in the cell and most of the glycosylated E-cadherin is recruited to the cell surface of tumor cells and enhances cell-cell contacts of tumor cells (6, 23). Very recently Pinho *et al*. also reported the similar data (24) and also found that GnT-III induced a stabilizing effect on E-cadherin at the cell membrane by inducing a delay in the turnover rate of the protein that promotes the formation of a stable and functional adherens-junction, and further prevents clathrin-dependent E-cadherin endocytosis (unpublished). The other major glycoprotein on the cell surface is integrin. In fact, the overexpression of GnT-III resulted in a reduction in β1, 6 GlcNAc branching structures, along with an increase in the bisected *N*-glycans on integrins (6, 25, 26). This resulted in the inhibition of integrin- mediated cell spreading and migration, and the level of cellular phosphorylation. Conversely, the knockdown of endogenous GnT-III expression resulted in an increased cell migration, concomitant with an increase in β1-6GlcNAc-branched *N*-glycans on integrins. Thus, *N*-glycans can be considered to be either a positive or negative regulator of the biological functions of integrin. Zhao *et al*. reported a similar type of regulation in α3 integrin (27), α5 integrin (28) and laminin 332 (29). Therefore GnT-III and GnT-V have adverse effects on cancer invasion and metastasis by adding bisecting GlcNAc or β1-6GlcNAc branching (Fig. 2) to a glycan structure. Detailed information on GnT-V will be described below.

GnT-III also regulates various signaling molecules or signaling pathways by adding the bisecting GlcNAc to these mole-

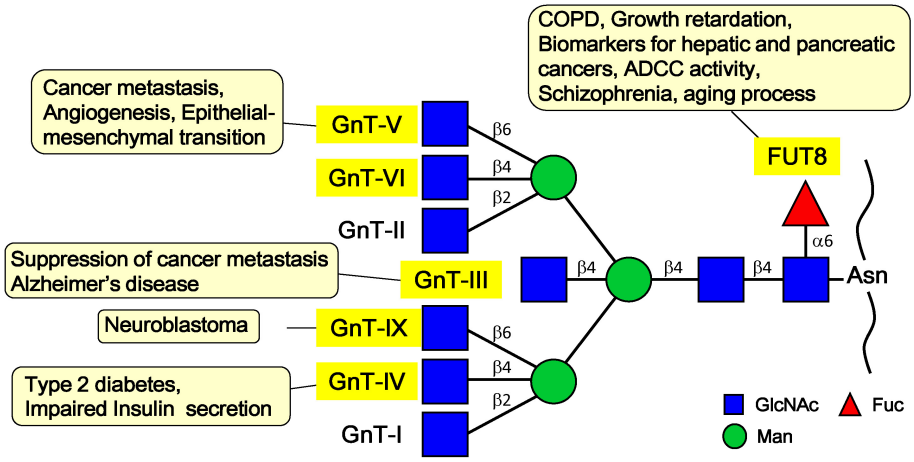

Fig. 2. Role of patho-physiological significances of *N*-glycans and their relevant glycosyltranferases and their genes. Items in each box indicate the patho-physiological significances observed in each glycan and/or related glycosyltransferases.

cules such as EGFR (30-32), nerve growth factor receptor (33), Wnt/β catenin (34), PKC δ (35), adenylyl cyclase III (36), PI-III kinase (37), Ras/Erk signaling (38), and retinoic acid (39) and many others.

Bisecting GlcNAc also regulates the expression of the α1-3 Gal epitope (40-42). Bisecting GlcNAc also plays a key role in the metabolism of glycans because the addition of a bisecting GlcNAc inhibits the elongation of glycans by inhibiting GnT-IV, V and Fut8 (9, 10). Therefore GnT-III controls a variety of important steps under physiological and pathological conditions.

β1-4GlcNAc branch and GnT-IV

UDP-*N*-acetylglucosamine:α1,3-D-mannosideβ1,4-*N*-acetylglucosaminyltransferase IV (GnT-IV) catalyzes the transfer of GlcNAc from UDP-GlcNAc in β1-4 linkage to α1,3-D-mannoside on GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAc β1-4GlcNAcβ1-Asn, which is the common substrate for GnT-III, -V and β1,4-GalT (9, 10, 12) (Fig. 1). The enzyme was identified for the first time in the hen oviduct by Schachter's group (10). A simple assay method was established by our group and the enzyme was successfully purified from bovine small intestine using affinity chromatography, in collaboration with our group in 1997 (43) and Minowa *et al*. subsequently cloned its cDNA (44). The regulation of GnT-IV expression is particularly interesting because enzymatic activity increases during oncogenesis and differentiation. Nishikawa *et al*. (19) showed that a solid ascites hepatoma cell line, AH-130, has a higher GnT-IV activity than normal liver tissue. A structural analysis of *N*-glycans on γ-glutamyltranspeptidase from human hepatoma tissue also suggests that hepatocarcinogenesis increases GnT-IV activity (45). A similar increase in GnT-IV activity may occur during choriocarcinogenesis because aberrant triantennary sugar chains containing GlcNAcβ1-4Manα1-3branch are present in human chorionic gonadotropin from patients with choriocaricinomas (46). The upregulation of GnT-IV activity has also been reported during the differentiation of human myelocytic cells (47, 48). There are two isozymic forms of GnT-IV and human GnT-IVb exhibits the same acceptor substrate specificities as human GnT-IVa, although GnT-IVb has a lower affinity for donors and acceptors than GnT-IVa. This suggests that GnT-IVa is more active than GnT-IVb under physiological conditions and that its primary function is in the biosynthesis of *N*-glycans. In pancreatic cancer patients, the expression of GnT-IVb was dominant in tumor tissues and the expression of GnT-IVa was dominant in normal, surrounding tissues (49). The expression of GnT-IVa was increased in all 3 cell lines that had been treated with 5-aza-c and butyrate, suggesting that the down-regulation of GnT-IVa in pancreatic cancer cells is due to an epigenetic abnormality in the gene.

GnT-IVa-deficient mice showed signs of metabolic disease, including hyperglycemia, impaired glucose tolerance, hyperinsulinemia, hepatic steatosis and a diminution in insulin action in muscle and adipose tissues (50) similar to type 2 diabetes (51). Protection from disease was conferred by enforced β cell-specific GnT-IVa protein glycosylation and is involved in the maintenance of glucose transporter expression and the preservation of glucose transport. This pathogenic process was also found to be active in human islet cells obtained from donors with type 2 diabetes; suggesting a pathway to the disease is associated with the diet- and obesity-associated component of type 2 diabetes mellitus. The phenotype of GnT-IVa/-IVb double deficiency largely overlapped that of a GnT-IVa single deficiency and showed induced glycomic compensation, indicating that mammalian organs have highly organized glycomic compensation systems for preserving *N*-glycan branching complexity (52).

β1-6GlcNAc branch and GnT-V

GnT-V catalyzes the transfer of GlcNAc from UDP-GlcNAc to the 6-OH position of α-linked Man residues in GlcNAcβ1-2Manα1-6Man-β1-4GlcNAc (10). The enzyme was purified from rat kidney tissue by Michel Pierce's group (53) and from a human cancer cell line by our group (54). cDNA clones were subsequently obtained by both groups from rat and human tissue. Both groups made both cDNAs available to Dennis's group and

his group used our sequence and developed KO mice (54). The product of GnT-V is β1-6GlcNAc structure which may further elongate to a polylacotosamine structure and can play a role in cancer invasion and metastasis.

GnT-V and cancer metastasis has been reported by Kobata (54) and Dennis's group reported various interesting phenotypic changes of GnT-V KO mice (55-57) including the suppression of tumor growth in these mice.

The implication of GnT-V in cancer metastasis has been reported by many researchers (see in review 58). However, the issue of how the levels of glycosyltransferase genes are regulated remains unclear. We reported on the direct implication of the transcriptional factor, Ets-1 in the underlying mechanism of activated GnT-V in various cancer cell lines (59, 60) and down regulation by TGF-β (61). The upregulation of src kinase (62) was subsequently reported. Human cancer tissues in which Ets-1 is highly expressed also express high GnT-V levels and a dominant negative of Ets-1 actually suppressed GnT-V expression. This confirms that the GnT-V gene is regulated by Ets-1. Recently several glycosyltransfrase genes have been reported to be regulated by Ets-1 and, actually, some of the glycosyltransferases appear to be regulated by a similar mechanism (50, 63, 64). These results may provide us with a new route to cancer metastasis via Ets-1. A different type of underlying mechanism for cancer metastasis may exist but the acquisition of β1-6GlcNAc branching of a specified glycoprotein(s) may undergo functional changes and may cause changes in metastatic potential (23, 55). We obtained a GnT-V-expressed cell line and identified a matriptase, a metal dependent serine proteinase, as a target glycoprotein for GnT-V in culture media (65, 66). Matriptase was independently cloned by two groups, since the enzyme is involved in cancer progression, invasion and metastasis and is also involved in the activation of Hepatocyte Growth Factor and tissue plasminogen activator (67, 68). Matriptase contains 4 potential *N*-glycosylation sites one of which, Asn 772, is an actual target residue for glycosylation by GnT-V, as evidenced by site-directed mutagenesis studies. Matripatase, to which β1-6GlcNAc branching had been added by GnT-V, was found to be resistant to auto-digestion (self digestion), as well as to tryptic digestion. Thus, the aberrant glycosylation of matriptase by GnT-V at *N*-glycosylation sites such as Asn 772 caused the constitutive activation of this proteinase and this may enhance cancer invasion and metastasis. We have also found that GnT-V is a bifunctional protein that functions as a glycosyltransferase as well as an angiogenesis inducing factor (69). GnT-V contains a heparin biding domain, KKKRK which is very similar to those of other angiogenic factors. Deletion mutants of GnT-V, which lacks catalytic activity, also contains the angiogenic factor releasing activity against FGF2, indicating that the angiogenic releasing factor of GnT-V is not involved in the catalytic domain. These data suggest that GnT-V has bifunctional properties and that the angiogenesis factor releasing activity is not mediated by the catalytic function of glycosyltransferases. Moreover, very recently, we found that GnT-V is cleaved by proteinase(s) including γ secretase and is then released as a soluble form that functions as an angiogenesis inducing factor (70). Miyo shi recently found that the soluble form of GnT-V is implicated in endothelial mesenchymal transition. (71). Ko's group reported that TIMP-1 is also a target molecule against GnT-V and plays a key role in cancer metastasis. They also found that TIMP-1 was aberrantly glycosylated by GnT-V showed a weaker inhibition with respect to both matrix metalloproteinase (MMP)-2 and MMP-9, and this aberrancy was closely associated with cancer cell invasion and metastasis *in vivo* as well as *in vitro* (72). As described above, GnT-V and GnT-III have adverse effects in cancer invasion and metastasis (5, 6, 23-26).

Our group identified a new enzyme designated GnT-IX, β1-6 *N*-acetylglucosaminyltransferase, a homolog of GnT-V (73). A novel β6-*N*-acetylglucosaminyltransferase (β1-6GnT) cDNA was identified as the result of a BLAST search using the amino acid sequence of human GnT-V as a query. Piece's group subsequently reported the same enzyme (74). Our group showed that GnT-IX acts on the *O*-mannosylglycan. GnT-IX may be a novel β6-*N*-acetylglucosaminyltransferase that could be responsible for the formation of the 2, 6-branched structures in brain *O*-mannosyl glycans (75). The enzyme is highly and specifically expressed in brain tissues and neuroblastoma cells (76, 77). Very recently, we found that the genomic region around the transcriptional start site of the GnT-IX gene was highly associated with active chromatin histone marks in a neural cell-specific manner, indicating that brain-specific GnT-IX expression is under the control of an epigenetic "histone code". This study may explain, for the first time, how the organ specific or cell specific expression of glycans are controlled by epigenetic mechanisms (78). This report was very important because the enzyme is specifically expressed in the brain. Moreover, there is an enzyme designated POMGnT-1 that catalyses the addition of a β1-2 GlcNAc to *O*-mannosylglycans and the mutation of which causes MEB(muscle eye brain) disease with an impairment in the glycosylation of α-dystroglycans (79-81). Our results suggest that the GnT-IX and PomGnT1 enzymes are involved in the *O*-mannosyl glycosylation pathway, and play an active role in modulating integrin and laminin-dependent adhesion and the migration of human neuronal cells. Pierce's group in a human neuronal model, showed that GnT-Vb expression enhances neurite outgrowth on laminin. GnT-Vb has been shown to be involved in both *N*-linked and *O*-mannosyl-linked glycosylation. They also reported that GnT-Vb acts as a regulator of RPTPβ signaling and influences cell-cell and cell-matrix interactions in the developing nervous system. Moreover, the GnT-Vb-mediated glycosylation of RPTPβ promotes galectin-1 binding and the retention of RPTPβ levels on the cell surface, showing that galectin-1 binding contributes to the increased retention, after GnT-Vb expression (82).

Core fucose and Fut8

Core fucose is a product of α1-6 fucosyltransferase, designated as Fut8, even though Fut8 has very few homologies with other fucosyltransferases such as α1-2, 1-3, 1-4fucosylatransferases

(83). The enzyme was first identified and reported by Schachter' group in 1980 (10). Glick's group (84) subsequently attempted to purify the enzyme but our group finally successfully purified it from a human cancer cell line as well as porcine brain tissues by affinity chromatography using core fucosylated biannetennary sugar chain as a solid ligand (85, 86).

KO mice were successfully produced by our group and we found that most Fut8 KO mice die within 3 days and the survivors show growth retardation and finally eventually develop emphysematous changes of the lung at 3 weeks after birth (87). The underlying mechanism of the lung emphysema was found to be due to the lack of the core fucose of TGF-β receptor which otherwise acts as a negative regulator of expression of the matrix metalloproteinase gene via Smad3. However without a core fucose, the TGF-β receptor does not function very well and the phosphorylation of Smad decreased, with the result that MMP genes such as MMP-1, MMP 19 and MMP12 were highly expressed in the lung which led to the degradation of the alveolar matrix, resulting in emphyematous changes in the lung. The exogenous addition of TGF-β rescued the phenotypic changes.

Very recently we found that when heterozygous KO Fut8 mice were exposed to cigarette smoke 4 times per day for 5 days per week for 3 months they developed emphysema, as compared to wild mice which show signs of emphysema only after a 6 month exposure to cigarette smoke, indicating that, compared to wild mice, these mice are genetically sensitive to developing emphysema (unpublished data). The persistent elevation in the expression level of Smad7 resulted in a significant reduction in Smad2 phosphorylation that controls MMP-9 expression, in Fut8+/- mice and in Fut8 mutant embryonic fibroblast cells. In patients with COPD, which involves both emphysema and chronic bronchitis, when they develop severe emphysematous changes, Fut8 enzyme activity in serum/plasma is decreased (unpublished data). At this moment the issue of why serum Fut8 enzyme activity decreases in those patients is unclear, but one possibility is that serum Fut8 is derived from platelets which reflects serum Fut8 activity.

Yamada *et al*. reported very recently that in the case of Fu8 polymorphism, Thr267Lys is highly associated with the onset of COPD (88), suggesting that Fut8 is also involved in the development of COPD in humans.

The mice also show a schizophrenic type of behavior and actually serotonin metabolism was deregulated, compared to the corresponding data for control mice The Fut8-/- mice exhibited multiple behavioral abnormalities with a schizophrenia-like phenotype (89).

Fut8 plays a key role in the discovery of cancer biomarkers. A well known tumor marker for hepatocarcinomas in humans is α-fetoprotein (AFP) but is also increased in hepatitis and chirrhosis of the liver. However, core fucosylated AFP is a much better tumor marker for hepatocarcinoma (90, 91) and has been approved by the FDA. Our group analyzed glycans of fucosylated AFP from a patient with a hepatoma and actually found that fucosylated AFP contains core fucose (92). We also reported that fucosylated haptoglobin is a marker for pancreatic cancer (93). Mass spectrophotometric analysis data indicate that there are four putative glycosylated sites on the haptoglobin b chain and some that contain core fucose and Lewis type fucose were found to be glycosylated. Approximately 65% of patients with pancreatic cancer showed positive values (94). The mechanism by which fucosylated proteins are found in cancer patients is still unclear but Nakagawa *et al*. found that most of fucosylted glycans are found in bile ducts, suggesting that fucosylation is a possible signal for the secretion of glycoproteins into bile ducts in the liver. A disruption in this system might involve an increase in fucosylated AFP and fucosylated haptoglobin in the serum of cancer patients (95).

Core fucose also plays a key role in glycans in terms of antibody dependent cellular cytotoxicity (ADCC) which is the main mechanism of antibody therapy against cancer and asthma. Deletion of the core fucose from IgG1 enhances ADCC activity by up to 50-100 fold (96, 97) and several trials using this antibody were successful for the treatment of adult T cell leukemia and asthma which used the antibody against chemokines CCLR and the IL-5 receptor, respectively. In the US, over 20 antibodies using this technique are in clinical phase 1 or 2 trials. The underlying mechanism by which ADCC is highly activated is probably due to the implication of carbohydrate interactions between FcγIIIa receptor and IgG1 (98, 99).

CONCLUSIONS AND PERSPECTIVES

Branching of *N*-glycans play a pivotal role in various patho-physiological situations and these structural glycan changes *in vivo* as well as *in vivo* are applicable for elucidating the mechanism of disease, biomarker discovery and in therapeutics. As shown in Fig. 2, the roles of these branching *N*-glycans and their related glycosylatransferases are summarized. In order to investigate more integrated analysis of glycan changes, a systems glycobiology approach, including chemical biology and bioinformatics and other related areas of research fields (100) would be essential for understanding glycan functions of branching *N*-glycans and glycan functions in general.

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