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Review

Deciphering Protein O-GalNAcylation: Method Development and Disease Implication

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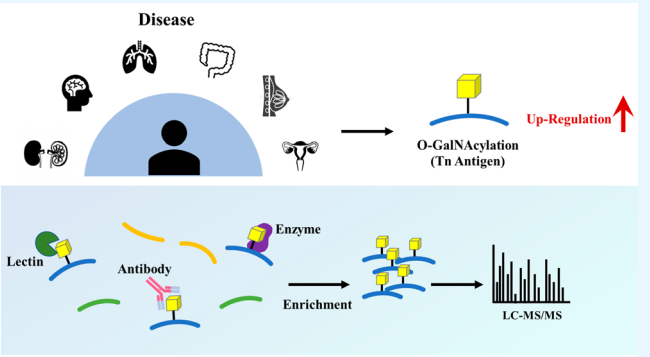
ABSTRACT: Mucin-type O-glycosylation is an important protein post-translational modification that is abundantly expressed on cell surface proteins. Protein O-glycosylation plays a variety of roles in cellular biological functions including protein structure and signal transduction to the immune response. Cell surface mucins are highly O-glycosylated and are the main substance of the mucosal barrier that protects the gastrointestinal or respiratory tract from infection by pathogens or microorganisms. Dysregulation of mucin O-glycosylation may impair mucosal protection against pathogens that can invade cells to trigger infection or immune evasion. Truncated O-glycosylation, also known as Tn antigen or O-GalNAcylation, is highly upregulated in diseases such cancer, autoimmune disorders, neurodegenerative diseases, and IgA nephropathy. Characterization of O-GalNAcylation helps decipher the role of Tn antigen in physiopathology and therapy. However, the analysis of O-glycosylation, specifically the Tn antigen, remains challenging due to the lack of reliable enrichment and identification assays compared to N-glycosylation. Here, we summarize recent advances in analytical methods for O-GalNAcylation enrichment and identification and highlight the biological role of the Tn antigen in various diseases and the clinical implications of identifying aberrant O-GalNAcylation.



1. INTRODUCTION

Glycosylation is one of the most common post-translational modification of proteins, lipids, and RNAs. Protein glycosylation mainly consists of N-glycosylation on asparagine (Asn or N) and O-glycosylation on serine (Ser or S), threonine (Thr or T), or tyrosine (Tyr or Y).[1,2] It is a complex and dynamic non-template-driven biosynthetic process that depends on the presence of glycoenzymes, sugar donors, and precursors, the accessibility of protein substrates, and cell signaling in the cellular microenvironment.[3−6] Aberrant glycosylation is often attributed to altered biosynthesis resulting from abnormal changes in these factors; thus, pathophysiological states of the body are associated with unique glycosylation.[5,7−11] The characterization of N-glycosylation is more mature than that of O-glycosylation due to advances in analytical methods and the availability of N-glycosidases with high specificity for the conserved N-X-S/T, which is a consistent motif for N-glycosylation. The latter still needs further exploration due to its structural diversity, lack of general O-glycosidases, and difficulty in assigning O-glycosylation sites.[12,13] The analysis of O-glycosylation has recently been advanced by the newly discovered O-glycoproteases that can cleave the N- or O-termini of mucin-type O-glycoproteins.[14−17] These O-glycoproteases tend to digest only certain types of O-glycosylated peptides, but are challenging for O-GalNAcylation analysis. Here, we focus on truncated mucin-type O-glycosylation (O-GalNAcylation), as it is an important modification of proteins whose abnormal changes are associated with many diseases.

O-GalNAcylation is a type of O-glycosylation, and its Ser or Thr is covalently modified by N-acetylgalactosamine (GalNAc) to GalNAc-α-O-Ser/Thr. Tn is a truncated O-glycosylation and a neoantigen that is often highly expressed in tissues during inflammation or cancer, and its expression correlates with tumor progression, metastasis, and poor survival.[18,19] O-GalNAcylation is also known as mucin-type glycosylation because most mucins are heavily modified with O-GalNAc.[20] Mucins play an important role in a variety of intercellular signaling and chemical barrier functions[21] and are generally increased in many adenocarcinomas, including

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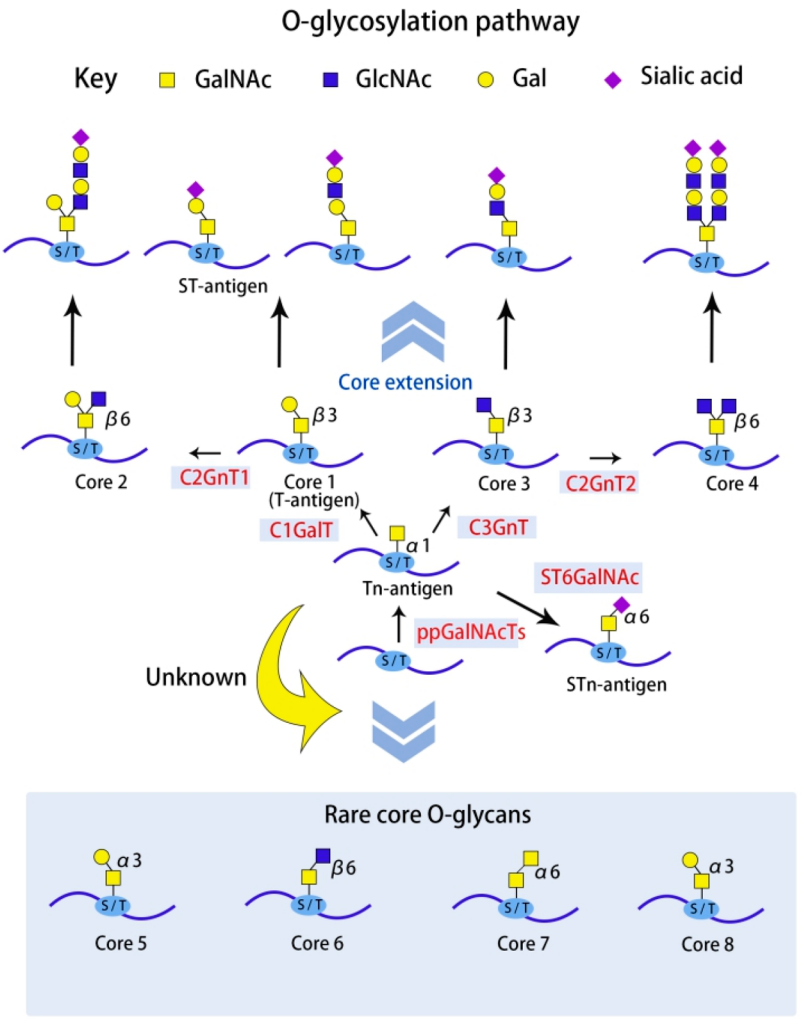

Figure 1. Main synthetic pathway of O-glycosylation. O-glycosylation begins with the formation of GalNAc on serine (S), threonine (T), or tyrosine (Y) by polypeptide GalNAc-transferases (ppGalNAcTs or GALNTs). Further elongation is regulated by different O-glycosyltransferases, including C1GalT, C3GnT, and ST6GalNAc. C1GalT catalyzes β1,3 Gal to GalNAc to generate T or sT antigen and at the same time further adds β1,6 GlcNAc to GalNAc to form the core 2 structure. C3GnT produces a core 3 structure with GlcNAc-β1,3-GalNAc, while C2GnT2 further develops GlcNAcβ1,6-(GlcNAcβ1,3-GalNAc) (core 4). Other rare cores 5−8s have also been identified, but the biosynthetic pathway is unknown. GalNAc = *N*-acetylgalactosamine; Gal = galactose; GlcNAc = *N*-acetylglucosamine; C1GalT = core 1 β1,3-galactosyltransferase; C2GnT1 = core 2 β1,6-*N*-acetylglucosaminyltransferase; C2GnT2 = β-1,6-*N*-acetylglucosamine-transferase; C3GnT = core 3 β1,3-*N*-acetylglucosaminyltransferase; ST6GalNAc = α-*N*-acetylgalactosaminide α-2,6-sialyltransferase 1.

pancreatic, lung, and breast cancers.[22−24] O-Glycosylation is first catalyzed by a polypeptide GalNAc-transferase (GalNAc-T), whose regulation is complex and involves the catalysis of T synthase.[25] The function of T synthase needs the help of specific molecular chaperone *COSMC*.[26] Studies have confirmed that the downregulation of *COSMC* expression is often associated with cancer and various diseases.[27,28]

Understanding the relationship between cancer and Tn antigens can help identify disease biomarkers and drug targets; however, there is much more to decipher Tn antigens.[12] The role of Tn antigen in disease development has yet to be fully elucidated, such as how disease changes the expression of Tn and sTn glycosylation on glycoproteins; O-GalNAcylation analysis methods need to be further improved for complex biological samples, including the distinction of sialic acid isoforms and GlcNAc glycoforms, and low abundance Tn/sTn antigens. To fully characterize O-GalNAcylation, Tn/sTn glycoproteins must be enriched to remove ionization-competed molecules such as peptides, N-glycopeptides, and O-GlcNAc peptides, thus enabling comprehensive quantification of enriched O-GalNAc peptides by tandem mass spectrometry (MS).[29]

2. BIOSYNTHESIS OF PROTEIN O-GalNACYLATION

Mucin-type O-glycosylation is initiated by the GalNAc-T family of enzymes (GANLT). As of today, 20 human GALNT genes have been identified, 15 of which have been shown to be expressed and active in human tissues or cells.[10,30] The schematic diagram of O-glycan synthesis is shown in Figure 1. GalNAc is transferred from the donor UDP-GalNAc to the Ser, Thr, or Tyr of the target protein, while the UDP-GalNAc donor is metabolized by nutrient influx.[31,32] Tn may go on to be catalyzed by ST6GalNAc to generate sialyl Tn antigen (sTn).[27,33] At the same time, Tn without sialic acid modification can be further modified by C1GALT1 (core 1 synthase, glycoprotein-*N*-acetylgalactosamine 3-β-galactosyltransferase 1), which can add a galactose (Gal) to Tn to

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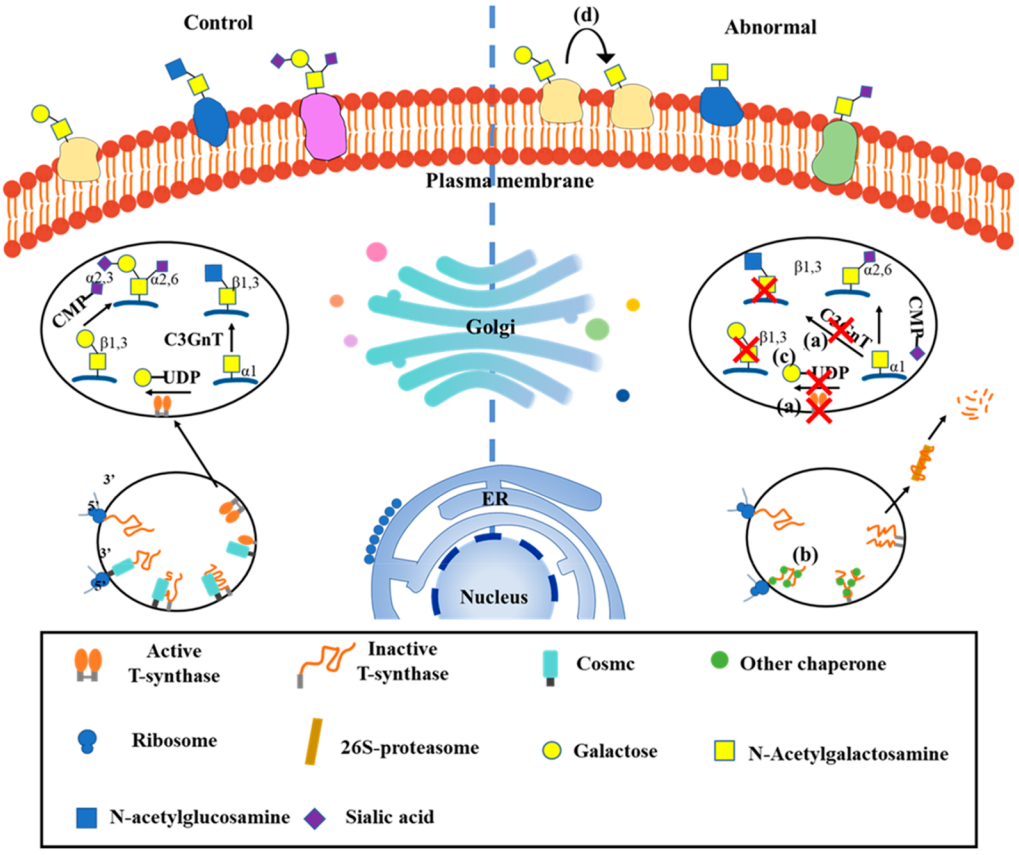

Figure 2. Upregulation of Tn and sTn antigens under abnormal pathophysiological conditions of cells. Under normal circumstances, O-glycosylation synthesis is catalyzed by a variety of O-glycosyltransferases to form core 1 to core 4 O-glycans with complex structures. Upregulation of Tn antigens also occurs under abnormal pathological conditions. Potential mechanisms include (a) differential regulation of O-glycosyltransferases in the Golgi apparatus, especially downregulation of T synthase and C3GnT, resulting in inability to synthesize complex O-glycan structures; (b) activation of the chaperone protein COSMC blocked synthesis, resulting in failure of T synthase to fold and function; (c) lack of UDP-Gal transporter; and (d) degradation of normal O-glycans.

form the T antigen. However, Tn, sTn, T, and sT antigens are not commonly found in normal tissues but are highly expressed in cancer or autoimmune diseases.[34,35] Tn or T usually continue to synthesize complex O-glycans under the catalysis of glycoenzymes in normal tissues. *N*-Acetylglucosamine (GlcNAc) can be added to Tn to form core 3 or core 4 O-glycan structures (Figure 1).[36] The GlcNAc can also add to T antigen and generate the core 2 structure.[37] Other less common O-glycan structures, such as cores 5, 6, 7, and 8, exist in organisms, but their enzymatic synthesis is largely unknown.[38]

Several different mechanisms have been proposed to explain why certain diseases lead to upregulation of Tn expression (Figure 2).[30] These can include the following: Deregulated changes in glycoenzyme expression during O-glycan elongation,[39] relocation of GalNAc-T from the Golgi apparatus to the endoplasmic reticulum (ER),[40] general reorganization of glycoenzyme topology,[41] somatic mutation or hypermethylation of *COSMC* genes,[42] and pH fluctuations in the cellular environment.[43] These may not be completely exclusive, and some of them play a dominant role in specific cases,[19] but there is a consensus on the common mechanism or biological significance of Tn increase in clinical or experimental studies.

Since Tn structures are considered precursors for the synthesis of complex O-glycans, changes in O-glycan structures may be due to the addition of different carbohydrate donors, which often depend on cell signaling, cell metabolism, and receptor function.[44] Therefore, it is expected that the accumulation of Tn antigens may result in or adapt to the pathophysiological state of the cells. Most complex O-glycans are synthesized based on core 1 and core 2 structures, which are commonly found on cell surface proteins, such as erythropoietin (EPO),[45,46] low density lipoprotein receptor (LDLR),[47,48] transferrin receptor (TFRC),[49] von Willebrand Factor (vWF),[50] and human chorionic gonadotropin (HCG).[51,52] The best known are O-glycans with a core 2 structure that recognize selectins such as P-selectin glycoprotein ligand 1 (PSGL-1).[53] Downregulation of *COSMC* chaperones results in a loss-of-function PSGL-1 ligand, which plays important biological roles in leukocyte trafficking, inflammation, thrombosis, and hemostasis.[54]

Mucins are a class of highly O-glycosylated proteins that carry a large portion of the Tn antigen in disease.[21] More than 21 MUC genes have been identified, including *MUC1*, 2, 3A, 3B, 4, 5AC, 5B, 6, 7, 8, 9, 11, 12, 13, 15, 16, 17, 18, 19, and 20, among which many are intrinsically associated with tumors.[55] Mucins have been found in body fluids as tumor markers for ovarian or pancreatic cancer,[56] such as CA125 that is encoded by *MUC16*.[57,58] In many cancers such as breast, colon, or liver cancer, Tn or sTn is present in high abundance in MUC1, MUC2, MUC4, or MUC6.[59] *MUC1* and *MUC2* also carry

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other tumor-associated carbohydrate antigens such as sialyl Lewis X (sLe^X) or sialyl Lewis A (sLe^A).[60,61]

3. BIOLOGICAL ROLES OF O-GalNACYLATION IN DISEASE

3.1. O-GalNAc Highly Expressed in Tumors. High levels of Tn antigen are found in breast cancer (90%) and to a lesser extent (70−90%) in colon, lung, bladder, cervix, ovary, stomach, and prostate cancers.[18,28,62−64] In contrast, little or no expression was observed in normal adult tissues.[65] Overexpression of Tn antigens has been shown to induce multiple oncogenic features, including extensive cell proliferation, decreased apoptosis, and increased migration and invasion.[66] The level of Tn antigen can predict the prognosis of ovarian cancer, and the survival rate of Tn-negative patients is significantly higher than that of Tn-positive patients.[67] O-Glycans on the large mucin may have an antiadhesion function and thus indirectly support tumor cell metastasis by promoting cell detachment from the extracellular matrix (ECM).[62] It was observed that Tn antigen was detected in 86% of primary or metastatic colon cancer tissues, and *COSMC* hypermethylation was detected, more importantly, after restoring *COSMC* function, carcinogenic features such as antiapoptosis and easy metastasis also disappeared, which may provide valuable ideas for cancer treatment.[66]

Tn antigen promotes tumor growth, especially in the late stage of tumor development, and exhibits an inhibitory effect on the immune system, which is also a research direction that has attracted widespread attention. Abnormal glycosylation on the surface of tumor cells can be recognized by some ligands on the surface of immune cells, thereby conducting immune escape. Cornelissen et al. found that Tn antigen can alter the immune microenvironment in a mouse model of colon cancer, with reduced infiltration of CD8+ T cells in the tumor.[19] Immunosuppressive cells were increased, suggesting a new possibility that Tn antigen promotes tumor development by affecting the immune microenvironment, especially in the later stages of tumor development. In addition, knockdown of *C1GALT1C1* or *COSMC* genes also altered cell identity and affected MAPK signaling pathway, cell migration, angiogenesis, and immune regulation. Knockdown of the *COSMC* gene in human keratinocytes was also observed to reduce the expression of immune-related genes.

3.2. Tn Antigens in Autoimmune Disorders. While the Tn antigen as a popular target for tumor markers is currently a hot topic, it was discovered more than half a century ago when Thomsen-Friedenreich (TF) stumbled upon a sialidase-producing microorganism in his laboratory that caused red blood cell agglutination. Later, Dausset et al. identified a phenomenon called Tn syndrome in cases of hemolysis caused by Tn autoantibodies.[68] Tn syndrome is a severe blood disorder caused by glycogen mutations such as *C1GALT1* or *COSMC*, which truncates the synthesis of O-glycans and produces large amounts of Tn antigen. This leads to polyagglutination of red blood cells, hemolysis, and thrombocytopenia, leading to the development of a rare autoimmune disease.[69] This has been demonstrated in mouse models of severe thrombocytopenia (<5−40% of normal) and even embryonic death.[70]

3.3. Tn Antigen Expression in Neurodegenerative Diseases. Alzheimer's disease (AD) is a common neurodegenerative disorder (ND) and the leading cause of dementia. O-GlcNAcylation, sialylation, and N-glycosylation have been reported to be associated with the development of AD. In 2013, when studying the changes of O-glycosylation during the development of AD, it was found that the expression of Tn antigen in the cerebral cortex of AD patients increased.[71] Subsequently, a 2017 study again showed that serum Tn expression was higher in AD patients than in normal controls.[72] In vitro studies of human Hippodrome cell lines, *COSMC*, a differentially expressed protein, was used to assess changes in patients with mild cognitive impairment as they progressed to AD or remained stable.[73] Later, it was also reported that in the later stage of AD, the *COSMC* gene mutation led to a significant decrease in T synthase activity and an increase in the expression of Tn antigen.[71] However, the role of Tn or T antigen, *COSMC* and T synthase in AD needs to be further evaluated, and the correlation between AD and *COSMC* mutation needs further large-scale study.

3.4. O-GalNAcylation Associated with IgA Nephropathy and Infection. IgA nephropathy (IgAN), a primary glomerulonephritis first described in 1968, has a high incidence in adolescents and can lead to kidney failure if persistent. The main clinical manifestations of IgAN are hematuria and proteinuria. The main difference between IgAN and normal nephritis is the deposition of immune complexes mainly composed of IgA1 (one of IgA subtypes) in the mesangium. About 85% of the IgA circulating in the blood is IgA1, and the hinge region of IgA1 has an O-glycosylation site. Current studies have found that the pathogenesis of IgAN may be related to insufficient galactosylation of IgA1, resulting in the production of truncated Tn antigens.[74] Moldoveanu et al. used Tn-specific lectins to detect serum IgA1 and observed significantly elevated serum lectin-bound IgA1 levels in 117 of 153 adult IgAN patients with a sensitivity of 76.5% and a specificity of 94%.[75] Therefore, they believe that the increased expression of Tn antigen may be related to IgAN. However, the researchers later found through lectin separation, western blot (WB) and mass spectrometry analysis that the Tn antigen also existed in the IgA1 of normal people, and the expression level was the same as that of the IgAN group. Interestingly, they found that IgA1 was only elevated in IgAN patients. Therefore, the role of Tn antigens in the development of IgAN is controversial and needs to be reassessed.[76]

In addition to the above-mentioned major diseases, Tn antigen can also be detected in the serum of patients with parasitic infection, which can be used as an indicator of Echinococcus granulosus infection.[77] Meanwhile, anti-Tn antibodies were found to block the infection of lymphocytes by the human immunodeficiency disease (HIV) virus,[78] but the mechanism and generality thereof are unclear. It is worth mentioning that Tn antigen production may be regulated by multiple processes, so different types of disease must be examined separately to distinguish which underlying pathways are affected. Figure 2 shows the four biosynthetic pathways. (1) Down-regulation of COSMC or T synthase expression: COSMC gene mutations are more common in cells with high Tn expression, usually open reading frame (ORF) point mutations,[79] gene deletion, and promoter hypermethylation.[80] (2) C3GnT deletion: This condition is common in colorectal cancer. In the normal gastrointestinal tract, O-GalNAc normally synthesizes core 3 structures under the action of C3GnT, but in cancer, the expression of C3GnT is downregulated, leading to the upregulation of Tn and sTn.[81,82] (3) Lack of UDP-Gal transporter: Chinese hamster ovary cell (CHO) is a kind of cell with high expression of Tn.

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Table 1. List of Lectin Affinity Enrichment Studies for Tn and sTn Antigens[a]

lectin	specificity	specificity	characteristic	ref
ALL	T/Tn	A	unable to recognize aggregated Tn antigen clusters	125
BfL	Tn	A	selective cytotoxic effects on breast cancer cells	126
BPA	T/Tn cluster	A, B, O (-SA)	high density polyvalent T/Tn > Tn glycopeptide > T monomer > Tn monomer > Gal	127
CFT	T/Tn, Forssman	A, B, O	recognizing α-GalNAc; inactive on β-GalNAc	128
CGB	Tn of O-mucin	A	highly homologous to Jacalin	129
DBA	Tn	$A_1 \gg A_2$	N/A	87
Gleheda	T/Tn	A > B	highly responsive to the polyvalent Tn antigen cluster	130
GS I-A4	Tn	A	recognizing the Tn antigen expressed in colorectal cancer and inhibiting the growth of tumor cells	131, 132
Jacalin	sT/T/Tn	O (+SA)	T-lymphocyte activator	133
LAL	T/Tn	A_1 > O > A_2, B	primarily used to study A1 antigen	134
McL	Tn	nonspecific	strongly inhibiting the proliferation of human cancer cells	135
MLL	Tn, Forssman	A, O	binding to the Tn antigen depending on the negative charge carried by neighboring sialic acid	136
Morniga-G	Tn/T cluster	N/A	inducing the death of Tn-positive tumor cells	137
MPA	T/Tn	A, B, O (-SA)	recognizing mainly T antigen	138, 139
PNA	sT > T > Tn	M, N	strong response to the polyvalent T antigen, but weak response to the polyvalent Tn antigen	140, 141
Riproximin	Tn cluster	N/A	significant cytotoxicity to cells expressing cluster Tn structure	142
SBA	Tn/mucin	A > O > B	α-GalNAc > β-GalNAc	143
SBL	Tn	N/A	N/A	144
SHL	Tn	N/A	N/A	145
SNA	Tn cluster	nonspecific	N/A	146
SNA-II	Tn	nonspecific	N/A	147
SRL	Tn cluster	A, B, O	recombinant variant SSR_1 with a higher affinity for Tn, sTn, but SRL and the recombinant variant SSR_2 only recognizing T antigens	148
SSL	Tn	N/A	activity of binding to the Tn antigen dependent on the density of the antigen exposure	149
VML	T/Tn	N/A	more sensitive to the aggregated Tn antigen	150
VVA B4	Tn	N/A	identification of individual Tn antigens	89
WBL	Tn	A > B	affinity: GalNAc > Gal	151
WFA	Tn	N/A	does not recognize terminal GalNAc with sialic acid or fucose	152
XCL	Tn	N/A	inhibiting cell proliferation	153

[a]ALL = *Amaranthus leucocarpus* lectin; BfL = *Bauhinia forficata* Lectin; BPA = *Bauhinia purpurea* agglutinin; CFT = Codium fragile subspecies Tomentosoides; CGB = Champedak galactose binding; DBA = *Dolichos biflorus* agglutinin; GS I-A4 = *Griffonla simplicifolia* I-A4; LAL = *Laelia autumnalis* lectin; McL = *Myrsine coriacea* lectin; MLL = *Moluccella laevis* lectin; MPA = *Maclura pomifera* agglutinin; PNA = peanut agglutinin; SBA = soybean agglutinin; SBoL = *Salvia bogotensis* lectin; SHL = *Salvia horminum* lectin; SNA = *Sambucus nigra* agglutinin; SRL = *Sclerotium rolfsii* lectin; SSL = *Salvia sclarea* lectin; VML = *Vatairea macrocarpa* lectin; VVA B4 = *Vicia villosa* isolectin B4; WBL = winged bean lectin; WFA = wisteria floribunda agglutinin; XCL = *Xerocomus chrysenteron* lectin; SA = sialic acid.

It lacks the UDP-Gal transporter and thus lacks Gal in N-glycan and O-glycan synthesis.[83] Therefore, loss of UDP-Gal transporter or UDP-Gal may lead to high expression of Tn antigen. (4) This could also be due to the degradation of normal O-glycans.

4. METHOD DEVELOPMENT FOR ANALYSIS OF O-GalNACYLATION

4.1. Lectin Affinity Enrichment. Lectins are plant or animal proteins that recognize cell surface carbohydrate structures and can trap glycoproteins via glycoconjugates on the cell surface.[84,85] Human tumor cells expressing Tn antigen were first described in 1969 as binding *Helix pomatia* agglutinin (HPA).[86] So far, a variety of lectins have been identified as T/Tn-specific lectins, which are effective means to identify T/Tn antigens (Table 1). The B4 iso-lectins of *Dolichos biflorus* lectin (DBA),[87] Maclura Pomifera Lectin (MPL),[88] and Viola Lectin (VVA-B4) can capture T/Tn antigen.[89] Lectin-specific targeting of Tn antigens is a common approach to study tumor occurrence and progression.[90−93] For example, in studies of colon cancer, the binding patterns of lectins were found to be different in normal and neoplastic colonic mucosa due to changes in the expression of associated Tn antigens during malignant transformation.[64,94]

Although various lectins with Tn specificity have been reported, there are subtle differences in carbohydrate substrate recognition between different lectins. Lectins recognize α-D-galactose and have a stronger affinity for galactose than GalNAc, so these lectins cannot distinguish between Tn and T antigens.[95] Several oligosaccharides and glycopeptides can reduce the affinity of lectins for Tn antigens, such as *N*-acetylgalactosamine, methyl-α-galactoside, *p*-nitrophenyl-α, or β-galactopyranoside, The most potent glycopeptide inhibitors are those containing two α-N-acetylgalactosamine units.[89] Lectins have limited specificity for distinguishing fine structures that often require further identification through orthogonal experiments.[92]

4.2. Anti-Tn Antibodies. Monoclonal antibody (mAb) therapeutics have made great strides over the past few decades, especially in the field of cancer diagnosis and treatment, but specific antibodies need to be developed. However, few monoclonal antibodies are available for diagnosis due to limited tumor markers with high specificity and sensitivity, including carcinoembryonic antigen (CEA) for colon cancer

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Table 2. Enrichment of Tn/sTn Antigens Using Different Antibodies

antibody	specificity	cancer	classification	characteristic	ref
B72.3	sTn (TAG-72)	adenocarcinomas (breast, colon, lung)	IgG1	bound strongly to consecutive sTn antigens (sTn2 and sTn3), but weakly to monovalent antigens (sTn1)	154
CC49	sTn (TAG-72)	adenocarcinomas (breast, colon, lung)	IgG1	usually more effective in combination with B72.3	155, 156
TKH2, HB-sTn1	sTn	colorectal and other epithelial neoplasms		stronger recognition of aggregation sTn antigens than that of individual antigen	157
MLS102	sTn (α2,6)	cancers of intestine, esophagus and ovary	IgG	identifying clusters of sTn antigens	158
LLU9B4	sTn	colonic adenocarcinoma	IgG	highly sensitive to colon cancer	159
3P9	sTn	colonic adenocarcinoma	IgM	effective inhibition on tumor growth with therapeutic potential	160
NCC-LU-35, NCC-LU-81	Tn	adenocarcinomas (lung)	IgM	in addition to Tn antigen, the cross-reaction with blood group A and AB antigen, but the cross-reaction with NCC-LU-81 weaker, and the two are similar in other aspects	103
83D4	Tn	breast, ovarian, endometrial, pancreatic and colonic adenocarcinomas.	IgM	recognizing Tn antigen, but unclear on tumor cell toxicity	161
CU-1	Tn	squamous cell carcinoma; gastric, colonic, breast, and lung carcinoma; leukemia	IgG3	no cross-reaction with blood group antigens	106
MLS 128	Tn	colonic adenocarcinoma	IgG3	recognizing primarily the aggregated Tn antigens (Tn2, Tn3), which inhibits the growth of some cancer cell lines and has therapeutic potential	105, 162
BRIC 66	Tn	adenocarcinoma	IgM	recognition pattern similar to NCC-LU-35, which preferentially responds to the blood group A antigen	109
BRIC 111	Tn	adenocarcinoma	IgG1	recognition pattern similar to CU-1 and does not react with the blood group A antigen	109
PMH1	Tn	N/A	IgM	reacting with a specific peptide sequence modified by GalNAc, rather than relying solely on GalNAc or peptide sequence recognition	163
KM3413	Tn	colonic adenocarcinoma	IgG1	recognizing the aggregated Tn 2 and Tn 3 antigens but not the individual Tn antigen. Promising therapeutic antibody	110
2154F12A4	Tn	breast cancer	IgM	inhibiting the adhesion of tumor cells and lymphatic endothelial	111
GOD3-2C4	Tn	breast, colon, lung, ovarian and pancreas cancer	IgG1	does not cross-react with GalNAc-β-O epitope, A blood group antigen, and therapeutic potential	112
237	Tn	fibrosarcoma	IgG2	relying on multiple and specific weak interactions between the antibody and both the sugar and peptide moieties to ensure that only the intact glycopeptide will be recognized.	164

and α-fetoprotein for hepatocellular carcinoma.[96,97] Ideally, the differential expression of Tn antigens in tumor patients provides new ideas for the development of new tumor-specific biomarkers.[98] Scientists have designed therapeutic vaccines against the Tn antigen, but success rates have been low due to low immunogenicity.[99,100] Therefore, no vaccine for clinical trials has been developed yet, and better products need to be explored.

Table 2 lists the specificities of common antibodies used in the analysis of various cancers. MAbs against the Tn antigen were first reported by Springer,[101] while the first murine monoclonal antibodies against the sTn antigen were subsequently generated by Hakomori.[102] Anti-Tn mAbs were designated NCC-LU-35 and NCC-LU-81.[103] However, these mAbs are less specific and cannot accurately distinguish between GalNAc-α-O-Ser/Thr and terminal GalNAc-α-. Therefore, they will cross-react with A blood group antigens, interfere with monitoring, and have better curative effect on B and O blood group individuals.[104,105] This limitation makes it more urgent to design antibodies that do not cross-react with the A antigen. This challenge was overcome in 1988 when the IgG mAb CU-1 was reported to fail to react with glycolipids containing the A antigen by multiple assays.[106] Other antibodies have also been developed to overcome cross-react.[106] It was found that the recognition effect of Cu-1 antibody could be inhibited by GalNAc monosaccharide, but not by GlcNAc or Gal. New Tn antigens were subsequently developed, such as MLS 128,[107] BRIC 66 (IgM),[108] BRIC 111 (IgG1),[109] and more recently mAbs KM3413,[110] 2154F12A4,[111] and GOD3-2C4.[112] These antibodies are widely used to recognize the distribution of Tn antigens on tissues and cells associated with cancer and other diseases. However, the results reported by different experiments are controversial, possibly due to the diversity of antibody structures. It has been shown that the binding ability of Tn antigen to antibody is mainly determined by the peptide background of Tn antigen, the antigen specificity of antibody and the type of immunoglobulin.[113]

The advantages of antibodies are high specificity and strong interaction, but even purified antibodies may cross-react, resulting in false positive results. At the same time, their preparation process is relatively complicated, and it usually takes at least three months to manufacture. Using lectins instead of antibodies can solve these problems.[84] Significant efforts are still required to develop antibodies for Tn antigen analysis.

4.3. Chemoenzyme Enrichment and Identification of O-GalNAc Peptides. Lectins and antibody affinities are less specific for the analysis of Tn antigens. At the same time, Tn analysis is more difficult because O-GalNAcylation and O-GlcNAcylation may occur on Ser, Thr or Tyr. O-GalNAc has the same molecular weight as O-GlcNAc, making these modifications indistinguishable by MS. Chemoenzymatic methods may hold promise to address these issues and improve specificity. It has been proposed to enzymatically or chemically preisolate GalNAc or GlcNAc glycosylation prior to MS analysis, or to combine metabolic labeling with different labels for GalNAc and GlcNAc (Figure 3a).[114] Galactose oxidase (GAO) is widely used and can specifically oxidize Gal or GalNAc, but has no effect on Glc and GlcNAc (isomers of

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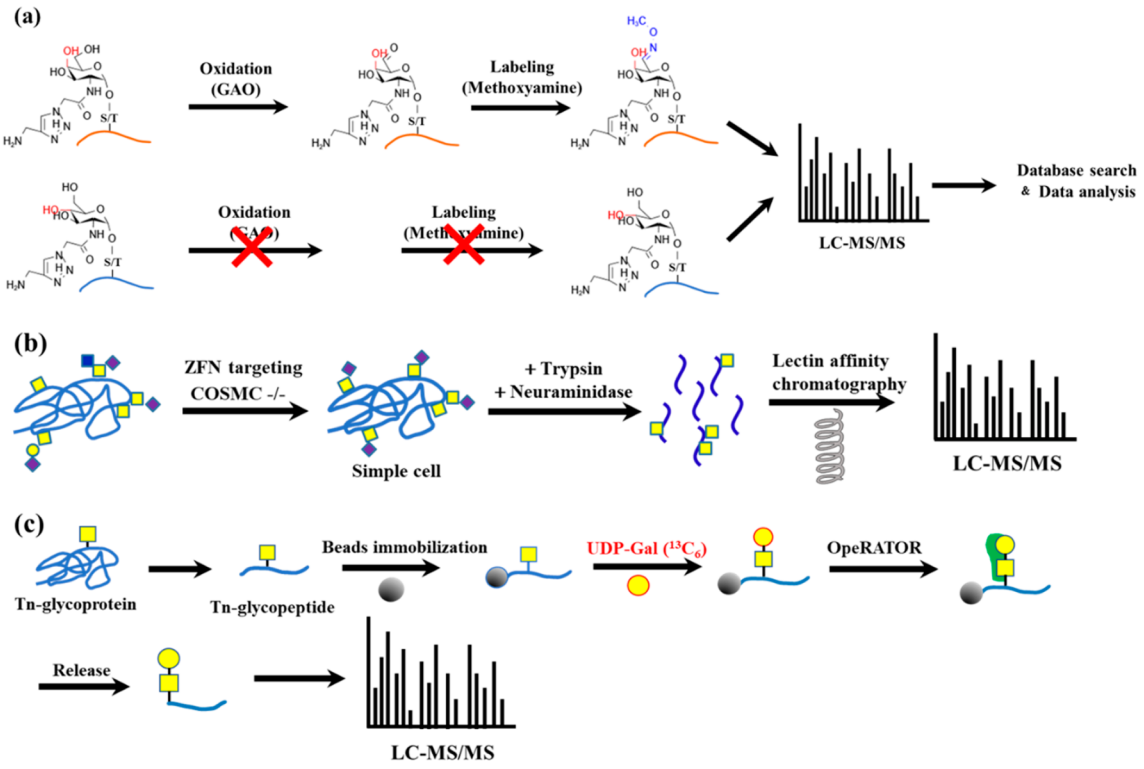

Figure 3. Schematic of the chemoenzymatic workflow for the enrichment and elucidation of O-GalNAc peptides in biological complex. (a) Enzymatic reaction with galactose oxidase oxidizes only O-GalNAc but not O-GlcNAc, resulting in differential labeling between O-GlcNAc and O-GalNAc glycopeptides for MS identification. (b) Homogeneous truncated O-glycosylation was achieved by "SimpleCell" technology for high-throughput identification of O-GalNAc sites. (c) GalNAc is modified by the glycosyltransferase *C1GalT1* and isotopically labeled with UDP-Gal (13C6) to label Tn and convert it to T antigen to carry a unique glycan mass. Tn glycosylation sites can be analyzed by LC-MS/MS. GAO = galactose oxidase; ZFN = zinc finger nuclease technique.

Gal or GalNAc).[115,116] The hydroxyl groups of GalNAc are oxidized to form aldehydes, which can react with amines immobilized on the solid support. Oxidized GalNAc can also be derivatized by methoxamine to generate a mass-shift tag, while GlcNAc remains intact and does not react with methoxyamine. MS can easily identify O-GalNAc with a mass tag.[115]

The enormous heterogeneity of glycans and the lack of O-glycosidases that can universally release all O-glycans hinder the progress of O-glycosylation analysis, not to mention the limitations of analytical techniques. This problem can be overcome by using Simple Cell lines (Figure 3b).[117−119] Through genetic engineering, simplify intracellular O-glycosylation, block the extension of O-glycans as much as possible, and generate truncated and uniform O-glycans, so that lectins can effectively enrich Tn antigens for LC-MS/MS analysis.[119] The feature of this method is the conversion of complex and diverse O-glycosylation to O-GalNAcylation, which facilitates the enrichment and detection of diverse O-glycosylation attached only to O-GalNAc.

Emerging O-glycoproteases have advanced the field of O-glycoproteomics. OpeRATOR is an O-glycoprotease that digests the N-terminus of peptide backbones carrying mucin-type core 1 O-glycans at O-glycosylation sites (Ser, Thr, or Tyr).[17] Therefore, it is often used to map O-glycosites, which require the removal of negatively charged sialic acid for better digestion. However, OpeRATOR can only recognize T antigen, but not Tn antigen. Tn must be converted to T antigen so that OpeRATOR can digest the modified O-glycosylation sites, as shown in Figure 3c. Isotope-labeled galactose is transferred to the Tn antigen to form a glycoform that can be digested by OpeRATOR. Using this method, Yang et al. showed that the identification of Tn antigen in Jurkat cells was significantly improved compared to other methods, which they named EXoO-Tn.[120]

However, these methods still have some deficiencies and need to be improved. In the GAO oxidation method, O-GalNAc and O-GlcNAc can be distinguished by the strict regioselectivity of the GAO enzyme, but free radicals generated during GAO oxidation can lead to glycopeptide degradation (Figure 3a). Although experiments have shown that the addition of DMSO and horseradish peroxidase (HRP) can scavenge free radicals and increase oxidation efficiency,[121] the identification of the Tn antigen may still be limited; In a Simple Cell line approach, all mucin type O-Glycosylation can be converted to Tn or sTn antigens, allowing large-scale, high-throughput identification of which proteins are O-glycosylated, as well as identification of O-Glycosylation site (Figure 3b). The disadvantage is that O-glycans lack complexity and site-specific heterogeneity, while EXoO-Tn can distinguish Tn from other O-glycans by isotope labeling. However, there are many steps involved and the disadvantages are obvious. For example, C1GALT1 is sequence-specific and may affect the efficiency of Gal ($^{13}C_6$) binding to Tn.[122] Second, the sequence specificity of OpeRATOR is not fully understood. Third, the 6 Da difference between Gal-GalNAc and Gal ($^{13}C_6$)-GalNAc could also be the result of mutations of the

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Table 3. Truncated O-GalNAcylation Is Predominantly Expressed in Epithelial Tumors but Barely Expressed in Healthy Tissues

tissue	antigen	% tumor positive	% normal positive	notes	ref
breast	Tn	14/15 (93%)	1/5	lectin	165
	sTn	13/21 (62%)		B72.3	166
	T	47/52 (90%)	2/21 (10%) two positives premalignant	adsorption	167
colon	Tn	21/29 (72%)	14% (n = 22)	VVA	168
	sTn	40/60 (67%) 29/46 (63%)	7/46 (15%)	HBSTn-1	169
	T	21/29 (72%)	0% (n = 22)	AH9-16	168
stomach	Tn	96/163 (59%)		HPA	170
	sTn	53/85 (62.3%)		TKH2	171
	T	18/87 (20.7%)	0/58 (0%)		172
lung	Tn	84/93 (90%)		HPA	173
	sTn	26/27 (96%)		B72.3	174
ovarian	sTn	40/40 (100%)		B72.3	174
bladder	Tn	27/34 (77%)	0/10 (0%)	BaGs2	175
	sTn	1/34 (3%)	1/10 (10%)	TKH2	175
pancreas	Tn	36/36 (100%)	0/45 (0%)	CU-1	176
	sTn	36/36 (100%)		TKH2	176
	T	29/36 (81%)		PNA	176

amino acids Met to His and Pro to Cys, so it is critical to diagnose the oxonium ion to avoid confusion.

4.4. Tn Antigen Characterization on Glycoproteins by Different O-Glycoproteases. The discovery of multiple O-glycoproteases provides more ideas for differentiating various O-glycosylation types, including Tn antigen. However, each O-glycoprotease has its limitations in O-glycosylation analysis. A combination of different O-glycoproteases is recommended to increase glycoside coverage.[116] For example, the O-glycoprotease BT4244 from Bacteroides theraiotaomicron cleaves the N-terminus of Ser/Thr but is inactive on sialyl O-glycans.[123] C1 esterase inhibitor (StcE) secreted protease from *Escherichia coli* can digest C-terminal O-glycosylated Ser/Thr/Tyr,[124] while IMPa has excellent enzymatic activity on Tn antigen.[15] GalNAcEXO is a newly developed glycosidase targeting O-GalNAc, which can efficiently hydrolyze the glycosidic bond between GalNAc and Ser/Thr/Tyr. The combination of O-glycoprotease and GalNAcEXO may provide an innovative idea for the accurate recognition of Tn antigen. We are developing this method to identify O-GalNAcylation in enteritis and colorectal cancer.

5. SUMMARY

Tn antigen is an O-GalNAcylation that has a simple structure but complex biological functions. Tn antigens are associated with various diseases and are abnormally regulated in pathophysiological states. Tn antigens are widely expressed in a variety of diseases, including cancer, neurodegenerative diseases, autoimmune diseases and viral infections. We show that accurate identification of Tn glycosylation is key to understanding its aberrant biosynthetic pathways in disease and developing therapeutics targeting Tn antigens. The most common method is to use special lectins and monoclonal antibodies to enrich Tn antigens, but the overall specificity needs to be further improved. Combination of multiple chemoenzyme methods is one of the promising directions for enriching Tn antigen. The discovery of O-glycoproteases and O-glycosidases has paved the way for the exploration of disease-specific O-GalNAcylation.

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Notes

The authors declare no competing financial interest.

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