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The *Cosmc* connection to the Tn antigen in cancer

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Abstract

The Tn antigen is a tumor-associated carbohydrate antigen that is not normally expressed in peripheral tissues or blood cells. Expression of this antigen, which is found in a majority of human carcinomas of all types, arises from a blockage in the normal O-glycosylation pathway in which glycans are extended from the common precursor GalNAc α 1-O-Ser/Thr (Tn antigen). This precursor is generated in the Golgi apparatus on newly synthesized glycoproteins by a family of polypeptide α N-acetylgalactosaminyltransferases (ppGalNAcTs) and then extended to the common core 1 O-glycan Gal β 1-3GalNAc α 1-O-Ser/Thr (T antigen) by a single enzyme termed the T-synthase (core 1 β 3-galactosyltransferase or C1GalT). Formation of the active form of the T-synthase requires a unique molecular chaperone termed *Cosmc*, encoded by *Cosmc* on the X-chromosome (Xq24 in humans, Xc3 in mice). *Cosmc* resides in the endoplasmic reticulum (ER) and prevents misfolding, aggregation, and proteasome-dependent degradation of newly synthesized T-synthase. Loss of expression of active T-synthase or *Cosmc* can lead to expression of the Tn antigen, along with its sialylated version Sialyl Tn antigen as observed in several cancers. Both genetic and epigenetic pathways, in addition to potential metabolic regulation, can result in abnormal expression of the Tn antigen. Engineered expression of the Tn antigen by disruption of either *C1GalT* (*T-syn*) or *Cosmc* in mice is associated with a tremendous range of pathologies and engineered expression of the Tn antigen in mouse embryos leads to embryonic death. Studies indicate that many membrane glycoproteins expressing the Tn antigen and/or truncated O-glycans may be dysfunctional, due to degradation and/or misfolding. Thus, expression of normal O-glycans is associated with health and homeostasis whereas truncation of O-glycans, e.g. the Tn and/or Sialyl Tn antigens is associated with cancer and other pathologies.

Keywords

Cosmc; T-synthase; Tn antigen; cancer

1. Introduction

Cancer is a leading cause of mortality and morbidity in the United States and worldwide [1,2], despite declines in death rates over the last decade. Cancers arise in virtually all tissues

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and cell types and can be categorized into different stages based on histologic findings. Prognoses and selection of treatment regimens often rely on imaging, biomarkers, staging, and additional histologic analyses [3–5]. While many cellular changes have been noted as hallmarks of cancer [6], it is often not acknowledged that a change in glycosylation of proteins/lipids is also a hallmark of cancer [7–10]. A number of biomarkers for cancer useful as diagnostic and prognostic indicators, such as CA-125, CA19-9, CA1503/CA27.29, PSA, Tn and Sialyl Tn, are glycoproteins or glycans, whose expression, exposure, and secretion may be altered upon cellular transformation, depending on the origin of the tumor [11–16]. Altered glycans and/or altered expression of glycoproteins and glycolipids in tumor cells may provide advantages to tumor development, progression and metastasis by altering cellular interactions with adhesion molecules, growth factors and growth receptor functions, innate immunity, and angiogenesis, but mechanistically these processes are poorly understood. Given the fact that over one-half of all proteins encoded in the human genome are glycosylated [17], it is likely that changes in glycosylation in cancer cells may impact a variety of biological pathways.

Among all the glycan moieties identified as potential tumor biomarkers and as antigens, as opposed to specific glycoproteins containing the glycans, two related glycans, Tn antigen and its sialylated version Sialyl Tn antigen (STn), have stood out as exceptional. These two antigens have been reported to occur in a majority of human cancers of various types [18–23]. The Tn antigen is comprised of a simple Ser/Thr-linked α -GalNAc occurring as GalNAc α 1-O-Ser/Thr (Fig. 1), the precursor that is typically extended and further modified to normal O-glycans; the STn has the α 2-6-linked sialic acid residue Neu5Ac α 2-6GalNAc α 1-O-Ser/Thr, and cannot be extended. Thus, accumulation of the Tn and STn antigens by tumor cells is unusual and readily demonstrable by specific antibodies and other reagents. This review will summarize our current knowledge of the Tn and STn antigens in tumor cells, the biochemical and molecular basis for Tn antigen expression, and the potential physiological consequences of Tn antigen expression on the tumorigenic process.

2. Normal biosynthesis of O-glycans in vertebrates

O-glycans containing Ser/Thr-linked α -GalNAc are ubiquitous in animal cells and found on many different glycoproteins. Some glycoproteins may have a single O-glycan, as is the case for the transferrin receptor [24–27], less than a few dozen O-glycans, as found in the LDL-receptor [28–31], many dozens, as found in PSGL-1 [32–34], or thousands, as found in epithelial mucins, such as MUC1 [35,36], MUC5B [37,38], MUC5AC, and others [38,39,41]. Abnormal expression of such O-glycans is often associated with dysfunctional activity of glycoproteins, such as altered stability, turnover, or expression, as recently seen in murine platelets expressing truncated O-glycans [42].

The biosynthesis of mucin-type O-glycans is initiated mainly in the Golgi apparatus and perhaps some in the late ER by addition of N-acetylgalactosamine (GalNAc) from the donor UDP-GalNAc to select Ser/Thr residues on polypeptides [20,43–46] through the action of ppGalNAcTs. A large set of approximately 20 different ppGalNAcTs, most of which have a catalytic domain and a separate lectin domain that binds the product of the reaction, leads to

efficient modification of specific Ser/Thr residues [43,47]. Loss of the lectin domain activity of the ppGalNAcTs leads to loss of functional efficiency [48,49]. The product of the ppGalNAcT reaction is the Tn antigen, which can occur as single GalNAc α 1-O-Ser/Thr or multiple, closely spaced Tn antigens in a multivalent presentation (Fig. 2).

The Tn antigen product is normally the substrate for subsequent modification by a single enzyme in mammals termed the T-synthase (core 1 β 3-galactosyltransferase or C1GalT1) (Fig. 1), which is found in all tissue and cell types. This enzyme transfers Gal from the donor UDP-Gal to synthesize the core 1 disaccharide Gal β 1-3GalNAc α 1-O-Ser/Thr, which is also called the T antigen (and is also sometimes called the Thomsen-Friedenreich or TF antigen) [50,51]. The core 1 disaccharide is the acceptor for a wide range of enzymes expressed differentially in cells that can lead to modifications, such as the core 2 O-glycans and the extended core 1 O-glycan. While the T-synthase and generation of core 1 O-glycans are considered ubiquitous in mammalian cells, the formation of the alternative core 3 O-glycans, which also arise from the Tn antigen precursor [52,53], appears to be limited to the GI tract epithelia, where the core 3 β 3-GlcNAcT (β 3GnT6) is expressed in a restricted manner. Deletion of *core 3 β 3-GlcNAcT* in mice leads to a low level expression of the Tn antigen in the GI tract epithelial cells [54]. By contrast, deletion of the *T-synthase*, in either whole mice or any tissue, leads to high level expression of the Tn antigen in all tissues [55–57]. Thus, the major pathway for modification of the Tn antigen in most tissues is through the action of the T-synthase, and substantial evidence discussed below supports this conclusion. Little is known about the potential competitive interactions between the T-synthase and the core 3 β 3-GlcNAcT, or their precise cellular and subcellular distributions in the GI tract.

It is likely that Tn antigen formed by the ppGalNAcTs is synthesized early in the Golgi apparatus in a compartment distinct from that in which its modification by the T-synthase and O-glycan extension reactions occur [47], since co-localization of both the ppGalNAcTs and T-synthase in the same compartment could potentially compromise efficient O-glycosylation. However, there is also evidence that initiation could continue to occur in more distal compartments in some cells [58]. The rationale for the likely subcellular separation of the ppGalNAcTs and the T-synthase is that if these enzymes were to act sequentially in the same compartment, then the Tn antigen would be immediately modified by the T-synthase (or potentially core 3 β 3-GlcNAcT), thus negating the binding of lectin domain of the ppGalNAcTs to the Tn antigen, and potentially interfering with the efficiency of O-glycosylation. However, it should be noted the precise subcellular location of all the reactions in O-glycans by segregations of glycosyltransferases in the ER/Golgi pathway are not defined, and could in fact be dynamically regulated and different among cell types. Interestingly, there is evidence that activation of Src in cells can lead to relocalization of some ppGalNAcTs from the Golgi to the ER [59], resulting in higher density of Tn antigen synthesis on some mucins and resultant accumulation of detectable Tn antigen in the ER [43]. Whether this is relevant to expression of the Tn antigen in tumor cells is not yet known, but is an exciting possibility and discussed further below.

3. Purification and cloning of the T-synthase

Many studies spanning the 1970's to the 1990's were conducted on the activity of the core 1 β -galactosyltransferase(s) (now also called the T-synthase), which was thought to arise from multiple members of the enzyme family [60]. These studies reported that the enzyme activity was present in a variety of tissues and efficiently utilized glycopeptide acceptors with the Tn antigen [61], as well as the simple acceptor GalNAc α 1-benzyl [60]. It was anticipated that the core 1 β -galactosyltransferase(s) that modify the Tn antigen product of these ppGalNAcT reactions would exist in a large family, because the pp-GalNAcT family was known to be large and the general β -galactosyltransferase family, to which the T-synthase(s) was expected to belong, was known to be comprised of a large number of related genes [62,63]. The final purification of the T-synthase, however, led to the unexpected observation that it was encoded by a single unique human gene mapped at 7p14-p13 earlier (now designated on 7p21.3) and a single murine gene at 6A1 [64,65]. Confirmation that all T-synthase activity in cells was encoded in a single gene was further supported by universal expression of that gene in human and animal tissue, along with T-synthase activity. In addition, a key confirmation came with the finding that deletion of *T-synthase* in mice leads to ubiquitous expression of the Tn antigen [55]. Thus, instead of referring to the core 1 β -galactosyltransferase, a cumbersome name that invites confusion with unrelated β -galactosyltransferases, the enzyme designation was abbreviated T-synthase, since it alone is responsible for synthesizing the disaccharide T antigen.

4. Discovery of Cosmc

In studying the activity of the T-synthase we found that the enzyme was very poorly expressed at the protein level in the commonly studied Jurkat cell line [66], which was consistent with an earlier observation that Jurkat cells display the Tn antigen [67]. The Jurkat cell line was initially derived from an adolescent male with T cell leukemia [68]; unlike normal T cells, Jurkat cells express the Tn antigen and are deficient in T-synthase enzyme activity. In addition, we found that Jurkat cells contain normal levels of transcript for the *T-synthase*, although have only low levels of T-synthase protein, implying that the T-synthase protein is degraded. We subsequently identified that the cause of the loss of T-synthase was not due to mutation of the *T-synthase* gene itself, but was rather due to mutation in a single X-linked gene now termed *Cosmc* [66], that encodes a molecular chaperone found to be required for formation of the active T-synthase and for expression of the protein itself after translation. As discussed below, in the absence of functional *Cosmc*, the newly synthesized T-synthase is inactive and rapidly degraded. *Cosmc*, which is the abbreviation for the Core 1 β -galactosyltransferase Specific Molecular Chaperone, is X-linked in humans (Xq24) and mice (Xc3).

Human *Cosmc* is a type II transmembrane protein of 318 amino acids with a short N-terminal domain in the cytoplasm, a single transmembrane domain, and a large C-terminal domain that can independently act as a molecular chaperone for the T-synthase [66]. *Cosmc* is an ER-resident protein [69,70], retained there by its unique transmembrane domain, and exists as a disulfide bonded dimer via Cys residues in the trans-membrane domain [71]. We identified the mutation in *Cosmc* in Jurkat cells as a point mutation leading to a stop codon

and predicted truncation of the Cosmc protein [66]. The Cosmc protein itself lacks enzyme activity as a galactosyltransferase, but is required for expression of the functional T-synthase, a fact that caused some confusion in early studies on its function, where it was thought in error that the *Cosmc* gene may be a second *T-synthase* [72], and the *Cosmc* gene is also referred to as *CIGALTIC1* and the *T-synthase* is referred to as *CIGALT1*. Interestingly, human Cosmc and human T-synthase share 26% homology in amino acid sequence suggesting they have arisen from a common ancestor [66,72]. Cosmc itself is expressed by all vertebrates from zebrafish to humans but is not found in invertebrates [65,73], although invertebrates have one or more genes encoding enzymes with core 1 β -galactosyltransferase activity [65,73,74].

5. Mechanism of Cosmc function as a chaperone

The identification of Cosmc as a potential co-factor required for expression of the T-synthase was unexpected, since no such factor had ever been described previously that was required for folding and expression of a glycosyltransferase. Multiple studies over the past few years have helped to clarify the unique activity of Cosmc as a specific molecular chaperone for the T-synthase (Fig. 3). Cosmc does not bind native T-synthase, but Cosmc can bind to heat- or chemically-denatured T-synthase [75] and promote its refolding *in vitro* independent of other factors or chaperones. This experiment *in vitro* cannot replicate, however, the complex environment of the ER, and other factors in addition to Cosmc might be necessary for complete folding and dimerization of the T-synthase *in vivo*. The complete absence of T-synthase activity in cells and in individuals with Tn syndrome that lack a functional Cosmc, demonstrate that Cosmc is an *essential* chaperone for active T-synthase formation. Unexpectedly, in examining release of refolded T-synthase in *in vitro* reconstitution experiments using recombinant His-tagged soluble human Cosmc (His-sCosmc) conjugated to beads, we found that refolded, active T-synthase remains bound to His-sCosmc. As controls, native T-synthase (NT-syn) and denatured HPC4-sT-syn (DT-syn) did not bind nonspecifically to the beads, as well as denatured BSA did not bind nonspecifically to the Cosmc-conjugated beads [75]. The only factors found to release the active, bound T-synthase from Cosmc-conjugated beads were free native or non-native T-synthase, which act in a dose-dependent manner. Importantly, denatured BSA as a control protein was not able to elute the T-synthase, consistent with our recent study [76]. Thus, we hypothesize that T-synthase has a specific domain that is recognized and bound by Cosmc to initiate productive folding of the T-synthase. After folding and release of the T-synthase from Cosmc, this region is buried such that T-synthase can no longer bind Cosmc. During the folding process we hypothesize that the T-synthase acquires an intermediate folded state, whereby this region is still bound by Cosmc; this partially refolded T-synthase can only be released from Cosmc by native T-synthase to form a functional homodimer or by non-native T-synthase to initiate folding of an additional molecule of T-synthase, thereby creating a cycle of binding/release. Interestingly, although Cosmc has an ATP binding activity [69,76], ATP does not affect these interactions *in vitro*; thus, at this time the potential role of ATP, if any, in the function of Cosmc is unknown. The overall model, first put forward in 2002 [66] and further refined in 2008 [69] and 2012 [75] is depicted in Fig. 3. In this model, Cosmc is required in a co-translational function for interacting with newly synthesized T-synthase,

reducing aggregation of the partly folded T-synthase and allowing efficient formation of the dimeric T-synthase, the typical catalytic form. In the absence of functional *Cosmc*, the T-synthase is efficiently retrotranslocated from the ER back to the cytosol, ubiquitinated, and degraded in a 26S proteasome-dependent fashion. Thus, a consequence of the lack of functional *Cosmc* is that cells express the Tn and/or STn antigen.

An interesting question is whether *Cosmc* acts as a chaperone for any other protein. In embryonic stem (ES) cells lacking *Cosmc* we examined the structures of glycans expressed by the cells and the ability of the cells to grow in culture [77]. We observed no changes in the N-glycome profile, nor were there demonstrable changes in cell growth. In addition, the only identified and specific molecular consequence of a loss of *Cosmc* in humans, such as Tn syndrome patients [78], as discussed below, is the loss of T-synthase activity and expression of the Tn antigen [78–85], and consequences of the lack of expression of the T-synthase. Thus, while such results are certainly not comprehensive in addressing the potential multi-chaperone role of *Cosmc*, all the results so far point to the conclusion that *Cosmc* has a limited and specific role in assisting the T-synthase during its biosynthesis and folding into an active form.

6. Discovery of Tn antigen and relationship to Tn syndrome

While it is now recognized that the Tn antigen is a common marker of human and animal tumors, as discussed below, the original discovery of the Tn antigen was not in tumor cells, but in blood cells from a patient with a previously unidentified hemagglutinin syndrome, permanent mixed field polyagglutinability, now termed Tn Syndrome. The patient was identified as having a rare, acquired hemolytic anemia associated with polyagglutinability of erythrocytes upon cold storage which was unrelated to ABO(H) blood groups [86]; the agglutination likely resulted from cold-agglutinins (IgMs) that bind Tn antigen. Individuals with Tn syndrome are characterized by having a large segment of blood cells of all lineages that are Tn-positive, implying a clonal origin in regard to Tn expression. The term Tn antigen actually derived from the earlier discovery by Friedenreich [87] of the T antigen, which is exposed by storage of erythrocytes, due to endogenous neuraminidase activity. Moreau et al [86,88] named the antigen they identified as the “T antigen nouvelle” or Tn antigen in recognition of its potential relationship to the T antigen. However, it was subsequently shown that the Tn antigen is GalNAc α 1-O-Ser/Thr [89,90]. Of note, Tn and STn antigens are not normally found in mammals, except for STn in the submaxillary glands of cows and sheep [13]. The Tn, STn, and T antigens are designated CD175, CD175s, and CD176, respectively [91–94].

In our studies that led to the discovery of *Cosmc* as described above, we were naturally led to explore expression of *Cosmc* in cells from patients with Tn syndrome. Examination of DNA from blood cells of two male individuals with Tn syndrome revealed that both patients had normal transcripts for the T-synthase, but one patient had a point mutation within *Cosmc* resulting in a premature stop codon and loss of full-length *Cosmc*, whereas the other had a point mutation that allowed full-length recombinant forms of *Cosmc* to be generated, but which inactivated its chaperone activity [82]. Other laboratories have confirmed these findings and identified additional patients with mutations in *Cosmc* [83,95]. Although not

the specific subject of this review, the expression of Tn antigen in these patients is an acquired disease, which is thought to arise from mutations in *Cosmc* in hematopoietic stem cells early in embryogenesis, thus accounting for expression of the Tn antigen in a broad subset of blood cells of all lineages, and the single mutation found in each individual examined. Since Tn syndrome is accompanied by a range of phenotypes [78,84], including potential thrombocytopenia, pancytopenia, anemia, and autoantibodies, it is likely that there are many underlying functions of normal extended O-glycans yet to be identified. Some of these have now been explored using specific deletions of *Cosmc* and/or *T-synthase* in mouse models.

7. Expression of Tn antigen in tumors

In some tumor cells, O-glycosylation is dramatically altered, resulting in expression of incomplete O-glycans, as represented by the Tn and STn antigens (Fig. 1). The Tn and STn antigens are examples of tumor-associated carbohydrate antigens (TACAs) [20–23]. These antigens in particular are typically expressed at relatively high levels, i.e. histologically-detectable, on tumor cell surfaces and provide attractive targets in the development of novel diagnostics and therapeutics. However, our understanding of the cause of expression and functions of such changes in tumor biology has been slowed by observations that some TACAs, such as the Tn and STn antigens, may be under metabolic or even epigenetic control, allowing reversion of expression under therapeutic selective pressure.

The association of the Tn antigen with tumor cells was first noted by Springer and colleagues [96,97], which was an observation that evolved from studies on the human MN blood group antigens. Springer's group developed specific monoclonal antibodies (mAbs) to the Tn antigen [98] and found that the Tn antigen was commonly expressed in many tumor cells [22]. Hakomori and colleagues also soon developed specific mAbs to the STn antigen [99].

The expression of the Tn antigen in tumor cells is highly unusual and should be unexpected for many reasons. As noted above, the normal O-GalNAc O-glycosylation pathway is ubiquitous in cells and thus, the expression of the Tn antigen would be associated with an inability to modify the precursor. The Tn/STn antigens expressed by many types of tumors are found frequently in a variety of glycoproteins and mucins and can be useful diagnostic markers [18,19]. It has been estimated that the Tn antigen may be expressed in > 70% of human carcinomas, including colon [100], and others [101–105]. Tn and STn antigens are also highly expressed in human breast cancers [106,107], where their expression is strongly associated with poor prognoses. STn antigen expression is observed in many epithelial cancers, and its expression correlates with enhanced expression of ST6GalNAc-I [108] and elevated serum levels of STn antigen are associated with poor prognosis [109,110]. Recent studies have shown that for patients with ovarian cancer, evaluating the serum levels of STn in circulating mucins, such as MUC16 (now known to be the CA125 antigen), a mucin produced by ovarian tumors and whose ectodomain is found in blood, may aid in diagnosis, tumor staging, and type [111]. STn expression is also enhanced in patients with ovarian cancer, but not in patients with endometriosis, in contrast to expression of the antigen CA125, which is often overexpressed in both conditions [111]. Expression of STn antigen is

associated with poor prognosis, and in the case of gastrointestinal adenocarcinomas its expression is dependent on the aberrant expression of ST6GalNAc-I [112]. While expression of the Tn antigen is clearly tumor-associated, the molecular mechanisms for its expression are still unclear, though studies described below are beginning to shed light on this topic, as discussed below. In addition, many studies examining the relationship between Tn and STn antigens and cancer are difficult to interpret due to the use of mAbs with poorly defined specificities. Partly for such reasons, the potential of Tn and STn antigens as cancer biomarkers has not yet been realized. The lack of appropriate defined specificities of anti-Tn and STn reagents, the common IgM-based iso-type of the antibodies, their often low affinity binding, and the general lack of stable, commercial availability of such well-defined reagents, have unfortunately hindered the use of Tn and STn as reliable biomarkers of cancer in human studies. It is hoped that future improvements to address all of these concerns will lead to the common use of anti-Tn and STn reagents in cancer diagnostics and prognostics, and even potential therapeutics.

8. Tn and STn antigens in colorectal cancer (CRC)

While the Tn and STn antigens are found in many types of tumors, it is interesting to consider the specific example of colorectal cancer (CRC), including colon and rectum cancer. Although there is limited information about expression of Tn/STn antigens in colorectal cancer, results suggest these antigens are not expressed in normal intestinal tissue, however they are expressed in primary/metastatic tumors. For example, early studies by Itzkowitz et al. [100] found no expression of Tn/STn in cells of normal colonic mucosa; however, in colon cancers, the percentage of cases expressing each antigen were Tn = 72–81% and STn = 93–96%. Similarly, Orntoft et al. [113] reported no expression of Tn in adult colorectal tissue, but found accumulation in colon carcinoma. Many studies have concluded that binding of HPA (*Helix pomatia* agglutinin), a snail lectin that recognizes Tn antigen, to CRC cells is an indicator of poor outcome for patients [114]. In some studies HPA reactivity was equal or superior to other classical markers of the metastatic potential of human colon cancer [115]. In fact, Imada et al. [116] noted that both STn expression and lymph node metastasis were important prognostic factors in patients with advanced CRC. Interestingly, Tn/STn antigens are expressed at early stages of colon carcinogenesis. Wargovich et al. [117] found that CEA and STn were elevated in aberrant crypt foci (ACFs), which are the earliest recognizable histological precursor lesions for colon cancer. Yuan [118] found that both Tn and STn were expressed in cancer and pre-malignant lesions of colorectal tissues. Itzkowitz et al. [119] examined 103 colorectal polyps for expression of Tn/STn and found that Tn antigen was expressed by all of the polyps, whereas STn, on the other hand was expressed weakly in 7 of 24 hyperplastic polyps. In a 1, 2-dimethylhydrazine-induced rat colon carcinoma model, both Tn and STn were expressed by early lesions detected following carcinogen administration, and were constitutively expressed at higher levels during tumor development [120].

These studies, however, have several limitations. Many of the studies rely on identifying Tn expression using the lectin HPA, which is not absolutely specific for Tn antigen, and can also bind to other carbohydrates, including blood group A. Some studies utilized poorly characterized anti-Tn or -STn mAbs. In addition, most prior studies explored either Tn or

STn expression, and few investigated them systematically or together. More importantly, none of the studies have explored the molecular mechanism for the abnormal expression of these tumor antigens.

Some potential carriers of Tn/STn antigen in colon cancer include the family of mucins. The goblet cells in the intestinal epithelium synthesize many different mucins, including MUC1-4, 5AC, 5B, and 6-13 [16, 121], which are either secreted into the bowel or attached to the membrane to form a mucus layer. Secreted mucins MUC2, -3, -13, and -5AC with their O-glycans are the primary components of the intestinal mucus layer that overlies the GI epithelium. The expression of some mucins is associated with the tumor phenotype [16,121,122]. Among the GI tract mucins, MUC2, which is mainly synthesized and secreted by the goblet cells, is an important mucus layer component to protect epithelial cells [123]. *MUC2*-deficient (*MUC2*^{-/-}) mice develop small and large intestinal and rectal tumors, indicating that loss of MUC2 could be an initiating event in intestinal tumorigenesis [124]. Interestingly, tumors from *MUC2*^{-/-} mice can arise independently from alterations of Wnt/ β -catenin/Tcf4 signaling, through an inflammation-related pathway that is distinct from and can complement mechanisms of tumorigenesis in *Apc*^{+/-} mice [125].

There is much interest in the potential contribution of Tn antigen and truncated O-glycans in general to tumorigenesis and metastasis. It has been proposed that high expression of the disaccharide T antigen in metastatic colon tumor cells enhances their binding to galectin-3 and may promote their adhesion to endothelial cells [126]. A separate study also found that proper O-glycosylation by the specific ppGalNAcT termed GALNT14 is required for death receptor signaling [127]. *In vivo*, the presence of short O-glycans is correlated strongly with increased metastasis and poor survival prognosis in cancer [20,128]. Genetic screening of cancer patients is also revealing potential contributions of O-glycosylation pathways to cancer. For example, inherited deleterious variants in GALNT12 are associated with susceptibility to colorectal cancer [129], consistent with earlier studies that expression of GALNT12 seems to be a negative marker especially of metastatic gastric and colorectal cancer [130]. Somatic and germ-line mutations in GALNT12 have been identified in individuals with colon cancer [131]. Although these few examples are intriguing, more research is required to understand how expression of the Tn antigen and truncated O-glycans and/or loss of O-glycosylation could contribute to cancer formation or progression.

9. Mechanisms for Tn antigen expression

There are several factors that may contribute to expression of the Tn and/or STn antigen (Table 1). In the case of the Tn antigen, the major specific factors are altered expression of Cosmc and/or T-synthase, and potentially altered expression or localization of the ppGalNAcTs. For example, it is possible that aberrantly expressed members of the ppGalNAcT family in transformed cells may lead to formation of Tn antigen at sites in glycoproteins not normally modified and this in turn could be associated with altered glycoprotein function and perhaps elevated levels of Tn antigen or STn antigen [132]. It is of course possible that loss of ppGalNAcT(s), as observed for GALNT12 in colorectal patients and as discussed above, may disrupt O-glycosylation pathways in general, without causing the specific generation of Tn or STn antigens.

Other less specific factors that could also impact expression of other classes of glycans include altered expression of nucleotide sugar transporters and/or nucleotide sugars, ectopic expression of unusual glycoproteins, altered routing of glycoproteins in the ER/Golgi axis in tumor cells, GOLPH3-dependent alterations in vesicular and Golgi transport and glycosylation pathways [133], altered Golgi pH and altered glycosyltransferase organization [134,135].

In several human tumor cell lines, including Jurkat T leukemic cells and LSC colon carcinoma cells, the expression of the Tn antigen is associated with acquired mutations in *Cosmc* [66,136]. In the case of the invasive human melanoma LOX cells, there is a genetic deletion of a part of the *Cosmc* locus, while in the other cell lines mentioned there are point mutations resulting in truncation or inactivation of *Cosmc*. Murine tumors, such as mouse fibrosarcoma, and neuroblastoma also have specific mutations in *Cosmc* [137]. Human cervical cancer cells have been shown to have *Cosmc* deletion resulting from loss of heterozygosity (LOH) of the locus [136]. In addition, point mutations in *Cosmc* have been observed in epithelial specimens from some patients with ulcerative colitis [57], but whether this is associated with increased risk for colon cancer is not known, and the results have to be further verified.

Interestingly, hypoxia can result in altered expression of the transporter for UDP-Gal, as seen for up-regulation of UGT-1 transcripts in SW480 colon carcinoma cells upon induction of hypoxia and in cancer tissues from patients [167]. Transfection of cells with UGT-1 cDNA also was previously shown to enhance expression of sialyl Lewis x antigen [168]. ppGalNAc-T13 expression is associated with induction of Tn antigen in specific locations as a trivalent (trimeric) structure on Syndecan-1 [141]. Expression of ppGalNAc-T13 and modification of Syndecan-1 was associated with enhanced metastasis in a mouse lung model. Such results raise the possibility that a combination of altered ppGalNAcT expression along with altered function and activity of T-synthase toward Tn antigen acceptors could lead to expression of Tn antigen in some cases.

It is interesting that recent studies show that newborn infant-associated bifidobacteria residing in the GI tract express an α -N-acetylgalactosaminidase capable of degrading the Tn antigen on glycopeptides and may be part of an alternative mucin degradation pathway [169]. Whether such bacteria might be influential in colorectal cancer conditions where the Tn antigen is expressed has not been explored.

10. Targeting the Tn antigen in cancer

As noted above, the Tn antigen is a non-physiological glycan structure in humans; thus, it is not surprising that the Tn antigen arising through mutation may be recognized as foreign by the immune system. In 1985 Springer reported the first development of murine mAbs to the Tn antigen [98], followed shortly thereafter by the first murine mAbs to STn generated by Hakomori and colleagues [99]. Several groups have now developed or used anti-Tn and STn mAbs in multiple studies [136,170–176]. However, many of these different mAbs to the Tn antigen lack specificity and many cross-react with other GalNAc-containing glycans, such as blood group A [172] while some of these mAbs appear to be more specific [174]. One of the

most useful mouse anti-Tn mAb is an IgM (CA3638, clone 12A8-C7-F5), one of several clones originally developed by Springer and colleagues, and useful in IHC and flow cytometry to detect the Tn antigen [171]. This mAb against the Tn antigen has been used to detect Tn antigen and shown to be relatively specific for glycopeptides expressing multiple Tn antigens [13, 177,178]. It recognizes two or more Tn antigens within the minimal sequence -S/T*-X_n-S/T*-, where S, T, and X are Ser, Thr, and X may be any amino acid except Pro, respectively.

Several studies *in vitro* and in animals have targeted Tn or STn antigens on cancer cells. Studies evaluating anti-Tn mAbs include the use of MLS128 to inhibit the growth of colon and breast cancer cell lines [179], the use of single-chain antibody fragments (scFv) fused to an antibody constant region (Fc) having antibody-dependent cellular cytotoxicity (ADCC) activity [180], and the use of mouse-human chimeric anti-Tn IgG1 to target Jurkat cells, which express Tn antigen on their surface *in vitro* and *in vivo* [181]. Recently, Hubert et al. [182] investigated the mechanism of action of a Tn antigen-specific chimeric mAb (Chi-Tn) and found that Chi-Tn showed no direct toxicity against carcinoma cell lines *in vitro*, but induced the rejection of a murine breast tumor in 80–100% of immuno-competent mice through *in vivo* ADCC mediated by macrophages and neutrophils, when associated with cyclophosphamide [180–182]. Welinder et al. [183] reported that the murine anti-Tn antibody displayed anti-tumor activity in xenograft SCID mice. Human tumor-associated glycoprotein-72 (TAG-72) is a carcinoma mucin molecule expressed in colon [184,185], breast [186], pancreatic [187], ovarian [188], lung, and gastric cancers [189]. Epitopes of TAG-72 recognized by the mAbs B72.3 and CC49 are STn and Sialyl T antigens, respectively [190–192]. CC49 has been analyzed and a humanized antibody [193–195] is under clinical trial for use in radioimmunoguided surgery (RIGS). Although some studies showed that the antibody distributed mainly in the xenografts of human tumors in mice [178,196] and in human CRC [197], Sialyl T antigen is a normal O-glycan and reactivity to normal tissue is a major concern. Clearly, more research is needed using specific anti-Tn or -STn in passive immunization studies to treat Tn (+) cancer. The biggest problem for these studies is the lack of well-characterized and specific anti-Tn mAbs.

A related approach has been to develop anti-Tn targeting vaccines, employing synthetic glycopeptides of mucin-backbones expressing the Tn antigen [13]. One of the first studies in this direction was performed by Springer and colleagues. Patients with breast cancer were administered typhoid vaccine, which elicits anti-Tn antigens due the presence of the Tn-like antigens on *Salmonella typhi*. Anti-tumor efficacy was evaluated and some positive results were observed [198]. More recent studies in this direction have explored specific defined synthetic conjugates [175] containing glycopeptides with one or more GalNAc α 1-O-Ser/Thr modifications [199–201]. While such vaccine trials are encouraging, the design of the proper immunogens and conjugates continue to be major challenges in the field, as well as understanding the specific antibody recognition of Tn-containing antigens [177,183,202–207].

11. Summary

The expression of the Tn antigen in human and animal tumor offers an exciting possibility of developing new diagnostic and prognostic markers for cancer. In addition, expression of the Tn antigen in so many different types of tumors also may offer insight into the potential roles of O-glycosylation in tumor initiation, promotion, and progression. Clearly, while the roles of Cosmc and T-synthase are crucial for expression of normal O-glycans, and their dysfunction can clearly lead to expression of Tn antigen, many other potential pathways of Tn antigen expression should be evaluated. At long last, it now appears that the genetic, molecular, biochemical, chemical, and immunological tools are at hand to finally decipher the mysteries of the Tn antigen and its association with human disease.

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Abbreviations

T-synthase	core 1 β 3-galactosyltransferase or C1GalT1
Cosmc	Core 1 β 3-galactosyltransferase Specific Molecular Chaperone
Tn antigen	GalNAc α 1-O-Ser/Thr
STn	SialylTn antigen (STn) Neu5Ac α 2-6GalNAc α 1-O-Ser/Thr
T or TF antigen	Gal β 1-3GalNAc α 1-O-Ser/Thr
ppGalNAcT	polypeptide N-acetylgalactosaminyltransferase

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O-glycan biosynthetic pathways. Glycoprotein biosynthesis begins in the ER where N-glycans are added co-translationally, but O-glycosylation is initiated normally in the Golgi apparatus, where the T-synthase adds galactose from UDP-Gal to the common precursor GalNAc α 1-Ser/Thr (Tn antigen) to generate a core 1 O-glycan Gal β 1-3GalNAc α 1-Ser/Thr. This is then extended by addition of other sugars to generate the normal O-glycans. In the absence of function of the molecular chaperone Cosmc, which is required for formation of active T-synthase, glycoproteins express the Tn antigen. This can lead to glycoprotein misfolding, abnormal oligomerization, instability, proteolysis, loss-of-function, and abnormal recognition by glycan-binding proteins (GBPs). (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/CBM-130375>)

Fig. 2. Structures of short O-glycans. The structures of the Tn, STn and core 3 O-glycans are shown on the left. In some glycoproteins clusters of O-glycans shown here as the Tn antigen may occur on adjacent or closely spaced Ser/Thr residues, as shown on the right. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/CBM-130375>)

Fig. 3.

Cosmc is a chaperone for the T-synthase. Working model of the mechanism of Cosmc function in the expression of the T-synthase and postulated expression of Tn/STn antigens associated with dysfunctional Cosmc. Cosmc is an ER-localized chaperone, which may exist as an oligomeric complex. Cosmc directly interacts with newly synthesized non-native T-synthase, facilitating folding of the T-synthase (*left*). In the absence of functional Cosmc, T-synthase aggregates into non-productive aggregates (*right*) and associates with other chaperones, e.g. Grp78, and is subsequently ubiquitinated and degraded by the 26S proteasome system. The active dimeric form of the T-synthase exits to the Golgi apparatus. Normal expression of *Cosmc* and *T-synthase* leads to normal O-glycan expression and complex structures on plasma membrane glycoproteins, whereas lack of Cosmc and T-synthase leads to expression of Tn and Sialyl Tn antigen. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/CBM-130375>)

Fig. 4.

Altered O-glycosylation of proteins contributes to altered activities. Glycoproteins expressing the abnormal O-glycan the Tn antigen may have altered structure and stability, and may not oligomerize properly nor be expressed stably on the membrane. Abnormal expression of the O-glycan Tn antigen in glycoproteins, instead of normal, extended O-glycans, can lead to altered glycoprotein expression and function. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/CBM-130375>)

Table 1

Several factors that may contribute to expression of Tn and/or STn antigens

<ul style="list-style-type: none">• Altered expression of Cosmc through acquired mutations [66,136,137]• Altered expression of Cosmc/T-synthase through epigenetic silencing [81,138]• Altered expression of Cosmc/T-synthase through signaling pathways [139]• Altered expression or localization of the ppGalNAcTs [59,112,130–132,140–157]• Altered expression of glycoprotein substrates for ppGalNAcTs and/or T-synthase• Altered expression of nucleotide sugar transporters and/or nucleotide sugars [158]• GOLPH3-dependent alterations in vesicular and Golgi transport and glycosylation pathways [133]• Altered Golgi pH causing altered glycosylation and altered glycosyltransferase organization [159]• Altered oligomerization status of glycosyltransferases [160,161]• Altered expression of ST6GalNAc-I could lead to Sialyl Tn expression [108,112,162–166]

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