Human Tumor Antigens Tn and Sialyl Tn Arise from Mutations in *Cosmc*

Tongzhong Ju,[1] Grainger S. Lanneau,[2] Tripti Gautam,[3] Yingchun Wang,[1] Baoyun Xia,[1] Sean R. Stowell,[1] Margaret T. Willard,[1] Wenyi Wang,[1] Jonathan Y. Xia,[7] Rosemary E. Zuna,[4] Zoltan Laszik,[4] Doris M. Benbrook,[2,3,6] Marie H. Hanigan,[5,6] and Richard D. Cummings[1]

[1]Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia; and Departments of [2]Obstetrics and Gynecology, [3]Biochemistry and Molecular Biology, [4]Pathology, and [5]Cell Biology, [6]Cancer Institute, University of Oklahoma Health Sciences Center, and [7]Oklahoma School of Sciences and Mathematics, Oklahoma City, Oklahoma

Abstract

Neoplastic lesions typically express specific carbohydrate antigens on glycolipids, mucins, and other glycoproteins. Such antigens are often under epigenetic control and are subject to reversion and loss upon therapeutic selective pressure. We report here that two of the most common tumor-associated carbohydrate antigens, Tn and sialyl Tn (STn), result from somatic mutations in the gene *Cosmc* that encodes a molecular chaperone required for formation of the active T-synthase. Diverse neoplastic lesions, including colon cancer and melanoma-derived cells lines, expressed both Tn and STn antigen due to loss-of-function mutations in *Cosmc*. In addition, two human cervical cancer specimens that showed expression of the Tn/STn antigens were also found to have mutations in *Cosmc* and loss of heterozygosity for the cross-linked *Cosmc* locus. This is the first example of somatic mutations in multiple types of cancers that cause global alterations in cell surface carbohydrate antigen expression. [Cancer Res 2008;68(6):1636–46]

Introduction

Neoplastic transformation of human cells is associated with multiple genetic and epigenetic changes that affect tumor growth, differentiation, and metastatic potential. These changes are commonly associated with the expression of tumor-associated carbohydrate antigens (TACA), which have been the targets of multiple studies in attempts to define the molecular basis for their expression (1–4). Many of these TACAs have also been the focus of potential antitumor vaccines (5). Two of the most common TACAs are the Tn and sialyl Tn (STn) antigens, which are expressed by >80% of human carcinomas and occur on multiple secreted and surface glycoproteins and mucins (5–7). However, little is known about the mechanisms controlling expression of Tn and STn. The expression of TACAs has been proposed to result from altered metabolism of tumor cells, changes in the tumor microenvironment, and consequent changes in expression of multiple genes within the glycosylation pathways (8–10). No specific genetic mutations have been found in glycosylation pathways in human tumor cells to explain the expression of these TACAs.

The Tn antigen (GalNAcα-Ser/Thr) is a biosynthetic intermediate in the formation of normal mucin-type O-glycans and is typically extended by the action of the enzyme core 1 β3 galactosyltransferase (core 1 β3Gal-T, T-synthase; ref. 11). Thus, Tn antigen expression results from lack of T-synthase activity. Expression of the Tn antigen is also often associated with expression of the STn antigen (NeuAcα6GalNAcα-Ser/Thr). We recently discovered that expression of the T-synthase is under the control of a unique molecular chaperone we termed Cosmc (12). Human *Cosmc* resides on Xq24 as a single exon gene. Acquired mutations of *Cosmc*, as seen in some human hematopoietic diseases (13), causes loss of the T-synthase activity and consequent expression of the Tn and STn antigens (14).

Such observations prompted us to examine whether human tumor cells expressing the Tn and STn antigens exhibit mutations in *Cosmc*. Here, we report that all human tumor cells examined, including human tumor cell lines and two human cervical cancer specimens, which express the Tn and STn antigens, harbor mutations in *Cosmc* that result in a loss of function of Cosmc and consequent loss of T-synthase activity. These results provide the first explanation at a genetic level for expression of TACAs in human cancers.

Materials and Methods

Cell culture. Human tumor cell lines, including T-lymphoid Jurkat (clone E6-1, ATCC TIB 152) colorectal carcinomas, LS174T (CL-188), were purchased from American Type Culture Collection. Human colorectal carcinoma LSC and LSB cells (15) were kindly provided by Dr. Steven Itzkowitz of Mount Sinai School of Medicine at New York. Human melanoma cell lines LOX and FEMX-I (16) were kindly provided by the group of Dr. Oystein Fodstad of Norwegian Radium Hospital Research Foundation. All cell lines were cultured either in DMEM [Life Technologies] plus 10% fetal bovine serum (FBS) for LSC, LSB, and LS174T cells or RPMI 1640 (Life Technologies) plus 10% FBS for Jurkat, LOX, and FEMX-I cells at 37°C and 5% CO_2.

Transfection, Southern blots, and hybridization. Tumor cells were transfected with wild-type Cosmc (wtCosmc) or T-synthase using Fugene 6 transfection reagent (Roche) according to the manufacturer's protocol. For Southern blotting, genomic DNA (gDNA; 5 μg) from LOX, FEMX-I, and normal control leukocytes was digested in a 50-μL reaction with 100 units of *Bam*HI and *Sac*I (New England BioLabs) at 37°C overnight. DNA was extracted by isopropanol precipitation and then resuspended in 20 μL of TE [10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0)] and analyzed on a 0.8% Agarose gel (11 × 14 cm) at 22 V for overnight. After denaturation in 0.5 N NaOH plus 1.5 mol/L NaCl and neutralization with 0.5 mol/L Tris-HCl (pH 7.0) containing 1.5 mol/L NaCl, the gel was equilibrated with 20× SSC [3.0 mol/L sodium chloride, 0.3 mol/L sodium citrate–HCl (pH 7.0)], and the DNA was transferred to a Nytran Supercharge TurboBlotter membrane (11 × 14 cm) using a TurboBlotter (Whatman Scheicher & Schuell) for 10 to 12 h. After washing with water for 5 min, the membrane

was heated at 80°C for 30 min and the DNA was cross-linked under UV. For hybridization, the DNA probe was the open reading frame (ORF) of human *Cosmc*. The DNA probe was prepared by PCR. Twenty nanograms of DNA were random primer–labeled with α-^{32}P-dCTP (>3,000 Ci/mmol; America Radiochemical Company) using Rediprime Random Prime Labeling System (GE HealthCare) according to manufacturer's protocol. After prehybridization with 12 mL of Hybrisol I (Chemicon International) containing 200 μg/mL of sheared salmon sperm DNA at 42.5°C for 4 to 6 h, the membrane was hybridized with denatured probe at 42.5°C overnight. The membrane was then washed twice with 2× SSC plus 0.1% SDS at room temperature, twice with 0.5× SSC plus 0.5% SDS at 65°C, and finally twice with 0.1× SSC plus 0.1% SDS at 65°C. The signal was detected by exposing the membrane to a BioMax film for 3 to 5 d at −80°C before the film was developed.

Reverse transcription–PCR, PCR, cloning, and sequencing. The total RNA and gDNA from cells were prepared using Absolutely RNA Nanoprep kit (Stratagene) and FlexiGene DNA kit (Qiagen, Inc.) following the manufacturer's protocols. The reverse transcription–PCR (RT-PCR) for human *Cosmc* and *T-synthase* were performed with 5 to 20 ng of total RNA as template with a two-step method (AccuScript High-Fidelity RT-PCR System from Stratagene) according to the manufacturer's protocol. The PCRs were carried out with Phusion High-Fidelity PCR kit (New England Biolabs) in a 50-μL reaction containing 100 ng of gDNA as template and 100 nmol/L of each primer. For cDNA of Cosmc, the forward primer was 5′-CGTGAGAGGAAACCCGTG-3′ and the reverse primer was 5′-TGTG-TGGTTATACCAGTGCC-3′. For PCR amplification of the coding region or Exon of *Cosmc* and Exons of *T-synthase,* the primers were the forward primer 5′-CTGTTTTAGCAGCAAATAGAGGGG-3′ and the reverse primer 5′-CAATTCCCTTCTCTTGAGGCAAAC-3′ for exon I, the forward primer 5′-CCTCCAGTGATGTCCTAGATAACAC-3′ and the reverse primer 5′-ACTACTGCTGATTGCTGATGTCCC-3′ for exon II, and the forward primer 5′-GTTCCTCTGCTTTACCTTGCCATC-3′ and the reverse primer 5′-TGGGATTCCAGTGTTCTATGTCAG-3′ for exon III. The CpG island was predicted by an online program.[8] For PCR amplification of the CpG island II of *Cosmc,* the forward primer was 5′-GCTGGCACTGTGGTTAAG-3′ and reverse primer was 5′-GGAAACAAAACTGCACACG-3′. The PCR products were analyzed on a 1% Tris-acetate EDTA agarose gel. The bands of the expected size were excised, and the DNAs were extracted from the gel with QIAquick Gel Extraction kit (Qiagen, Inc.). RT-PCR and PCR products for *Cosmc* and *T-synthase* were subjected directly to sequencing or subcloned into pCR4Topo blunt vector (Invitrogen).

Immunohistochemistry and fluorescence staining. Mock and wtCosmc stably transfected LOX and FEMX-I cells (5×10^4) were seeded in chambered slides and cultured overnight. After fixation with 4% paraformaldehyde, the cells in each chamber were treated with 5 milliunits of neuraminidase (*Arthrobacter ureafaciens*; Roche) in 300 μL of 50 mmol/L acetate buffer (pH 5.5) at 37°C for 1 h. For immunohistochemistry, the staining procedure was done according to the manufacturer's protocol (Zymed Laboratories). Briefly, the cells were incubated with 5 μg/mL anti-Tn monoclonal antibody (mAb) at room temperature for 1 h, then incubated in the peroxidase-labeled goat anti-mouse IgM (1:500) for 1 h at room temperature. The cells were washed after each incubation with PBS. The slides were developed with the chromogen AEC single solution for 3 to 5 min and finally counterstained with Mayer's hematoxylin. The cells were covered with crystal mount media and dried overnight. For fluorescence staining, the cells were blocked with 1% bovine serum albumin (BSA) and then incubated with anti-Tn mAb. After washing thrice with PBS, the cells were covered with Alexa568-labeled goat anti-mouse IgM (1:500) and 10 μg/mL FITC-labeled peanut agglutinin (PNA) in PBS in dark at room temperature for 45 min. The nuclei of cells were stained with 300 nmol/L 4′,6-diamidino-2-phenylindole (DAPI) dye in PBS. After washing, the cells were covered with mount media and visualized under a regular microscope for immunohistochemistry and under a fluorescence microscope. The images were recorded at 20× magnification.

---

[8] http://www.uscnorris.com/cpgislands2/cpg.aspx

Flow cytometry and cell sorting. Approximately 5×10^5 cells [wild type, stably mock-transfected with pcDNA3.1(+)] (Invitrogen) or transfected with expression vector expressing human wtCosmc were seeded in a T75 flask with complete media the day before the experiment and cultured overnight. Cells were washed with PBS, trypsinized, and harvested. After washing with Hank's balanced solution (Invitrogen), the cells were suspended in 600 μL of Hank's solution and divided into two fractions. Twenty milliunits of neuraminidase was added to one fraction and incubated at 37°C for 45 to 60 min, shaking the cells every 15 min to keep them in suspension. The cells were washed once with Hank's solution and suspended in 400 μL of Hank's solution. Both the neuraminidase-treated cells and the untreated cells were divided into four fluorescence-activated cell sorting (FACS) tubes (100 μL per tube). For anti-Tn antibody staining, 100 μL of mouse anti-Tn mAb (IgM, diluted at 1:100 with PBS) were added to one neuraminidase-treated and one untreated fraction. The controls were incubated with control mouse IgM. All fractions were incubated on ice for 60 min. Then cells were washed thrice with PBS and incubated with Alexa Fluor 488–labeled goat anti-mouse IgM (Molecular Probes) at 1:500 dilution in Hank's solution. The cells were incubated in the dark on ice for 45 min. The cells were then washed twice with PBS, resuspended in 500 μL of PBS, and analyzed on a flow cytometer (FACSCalibur, Becton Dickinson). Tn(+) cells were sorted on a cell sorter (FACSorter, Becton Dickinson).

For lectin staining, 100 μL of FITC-labeled PNA at 2.5 μg/mL in 50 mmol/L TBS [Tris-HCl (pH 7.4), 150 mmol/L NaCl] containing 1 mmol/L $CaCl_2$ and $MgCl_2$ were added to one of the neuraminidase-treated cells and one of the untreated cells, mixed, and incubated on ice for 1 h under the dark. The control was the same as above, except that 20 mmol/L of lactose were present in the FITC-PNA solution. After washing with 3 mL PBS thrice, the cells were resuspended in 500 μL of PBS, analyzed on a flow cytometer, and sorted.

Examination of Cosmc from human cervical tumor specimens. Two specimens of human cervical cancer samples (DH85 and DH86) were provided by the Obstetrics and Gynecology Department at University of Oklahoma Health Sciences Center under an institutional review board–approved protocol. These specimens were first stained immunohistochemically for expression of with Tn and STn antigens, using appropriate mAbs. The Tn/STn(+) tumor cells were then collected by laser capture microdissection (LCM). The gDNA was isolated from the cells using Qiagen micro-DNA isolation kit according to the manufacturer's protocol. The ORF of human *Cosmc* was analyzed by sequencing the PCR product, as described above. The gene and cDNA of *Cosmc* from cervical tissue samples were analyzed by PCR and RT-PCR using gDNA and total RNA from the tissue as templates, respectively, and DNA sequencing. To sequence two single-nucleotide polymorphisms (SNP; rs591040 and rs17327439) 1.2 to 1.4 kb downstream of the coding region of *Cosmc*, PCR was performed using 5′-AATGGCACAATCTCGGCTC-3′ as forward primer and 5′-TGCTCTAA-CACTCTATGCGGAC-3′as reverse primer covering those two SNPs, and PCR product was subjected to direct sequencing.

Preparation of membrane extracts. Approximately, 10^6 logarithmically growing cells were harvested. Membranes were solubilized with 150 μL of PBS containing protease inhibitors (Complete-mini, EDTA-free; Roche) and 0.5% Triton X-100 on ice for 30 min. A membrane extract was obtained by centrifugation at 3,000 rpm for 5 min and collection of the supernatant. Membrane extract (50 μL) was desialylated by incubation at 37°C overnight with *A. ureafaciens* neuraminidase (10 milliunits; Roche).

Additional assays. The activity of T-synthase was measured as previously described using the donor UDP-Gal and the acceptor GalNAc-α-phenyl (Sigma-Aldrich; ref. 11). The protein concentration in cell extracts was determined by the bicinchoninic acid method (Pierce) following the manufacturer's instructions with BSA as a standard. Cell extracts (20 μL) that were treated and untreated with neuraminidase were analyzed on SDS-PAGE (Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Western blots were analyzed by immunoblotting with mouse anti-STn mAb (IgG1, clone HB-STn1 from DakoCytomation), mouse anti-Tn antibody (IgM), and the horseradish peroxidase (HRP)–labeled lectins *Helix pomatia* agglutinin (HPA) and PNA,

as previously described (12, 17), using HighSignal West Pico Chemiluminescent Substrate (Pierce). Anti-Tn mAb (mouse IgM, CA3638, clone 12A8-C7-F5; ref. 18) was kindly provided by the late Dr. Georg F. Springer from University of Illinois. HRP-labeled lectins HPA and PNA and FITC-labeled PNA were purchased from E-Y Laboratories.

Results

LSC cells lacking T-synthase activity contain mutated Cosmc. To determine whether neoplastic cells previously shown to express the Tn antigen have mutations in *Cosmc*, we first examined colon carcinoma LSC cells. These cells were previously shown to lack T-synthase activity (15). LSC cells were originally established from the parental colorectal carcinoma cell line LS174T (15, 19). As a control, we examined LSB cells, a separate clone also isolated from LS174T that does not express the Tn antigen. Because O-glycan elongation requires the expression of both T-synthase and Cosmc (12), we examined T-synthase

expression in these two cell lines. Both cell populations expressed comparable levels of T-synthase transcript (data not shown), but LSC cells had very low T-synthase activity when compared with LSB cells (Fig. 1C). LSB cells expressed wtCosmc cDNA (Fig. 1A), whereas Cosmc cDNA from LSC cells had mutations, including a T-insertion, at position 53 (Fig. 1A). This mutation causes an ORF shift that generates a premature stop codon in Cosmc. The resulting transcript is predicted to encode a 28–amino acid peptide instead of the 318–amino acid wild-type protein (Fig. 1B). In addition, within the 28–amino acid peptide, only the 18 NH_2 terminal amino acids correspond to the normal NH_2 terminus of Cosmc, which represents the entire short cytoplasmic domain and a portion of the proximal transmembrane domain. We have shown previously that the function of Cosmc requires an intact COOH terminal domain (12, 13). We directly tested whether the mutation in LSC cells leads to loss of Cosmc and its function as a chaperone in the proper folding of the T-synthase, as described
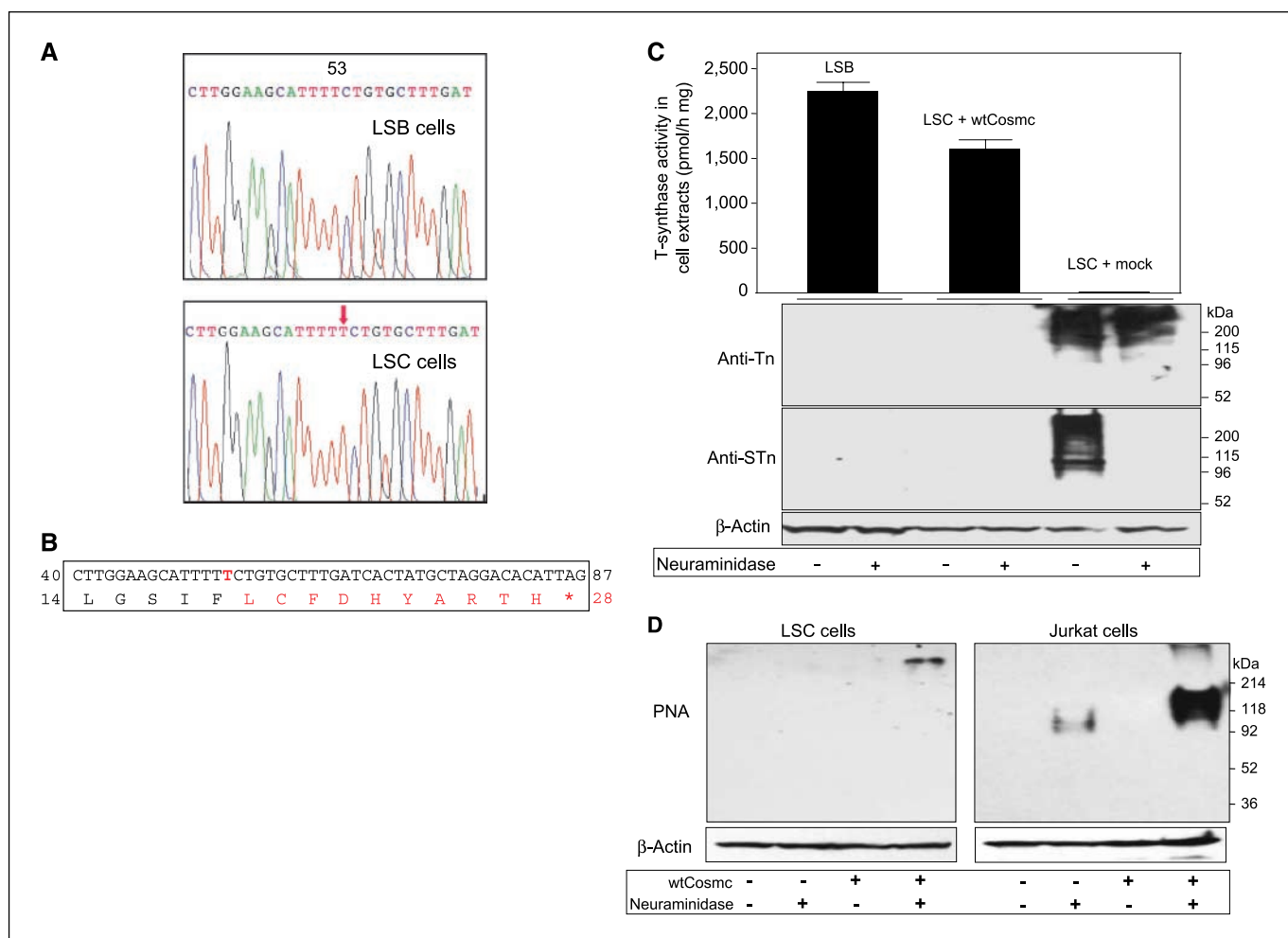
Figure 1. Mutation of *Cosmc* in human colon carcinoma LSC cells accounts for their lack of T-synthase activity leading to expression of Tn/STn antigens. LSB cells, a parallel cell line of LSC, have wtCosmc (*A*), whereas LSC cells have a mutated *Cosmc* with a T-insertion at position 53 (*arrow*; *A*) resulting in an ORF shift and a premature stop shown in *B*. The cDNA of *Cosmc* (a single exon gene) was obtained by RT-PCR using total RNA from LSB and LSC cells and sequenced. Portions of *Cosmc* cDNA sequences are shown. *C*, the T-synthase activity in LSC cells was restored, and Tn/STn antigens in LSC cells were abolished by transfection with a plasmid expressing human wtCosmc but not with a mock transfection (empty vector). T-synthase activity in the extracts of transfected LSC and LSB cells was measured using the acceptor GalNAc-α-phenyl. Cell extracts from mock-transfected or wtCosmc-transfected LSC and LSB cells were treated with neuraminidase and analyzed by Western blotting with mouse anti-Tn mAb (IgM) and anti-STn mAb (IgG). *D*, transfection of LSC cells with wtCosmc corrects the O-glycan structure on the cell surface. Cell extracts from mock-transfected or wtCosmc-transfected LSC cells were treated with neuraminidase and analyzed by Western blotting with HRP-labeled PNA that detects the normal T antigen. Cell extracts from Jurkat cells transfected with empty vector and wtCosmc were stained with PNA as a control. β-Actin was used as a loading control.
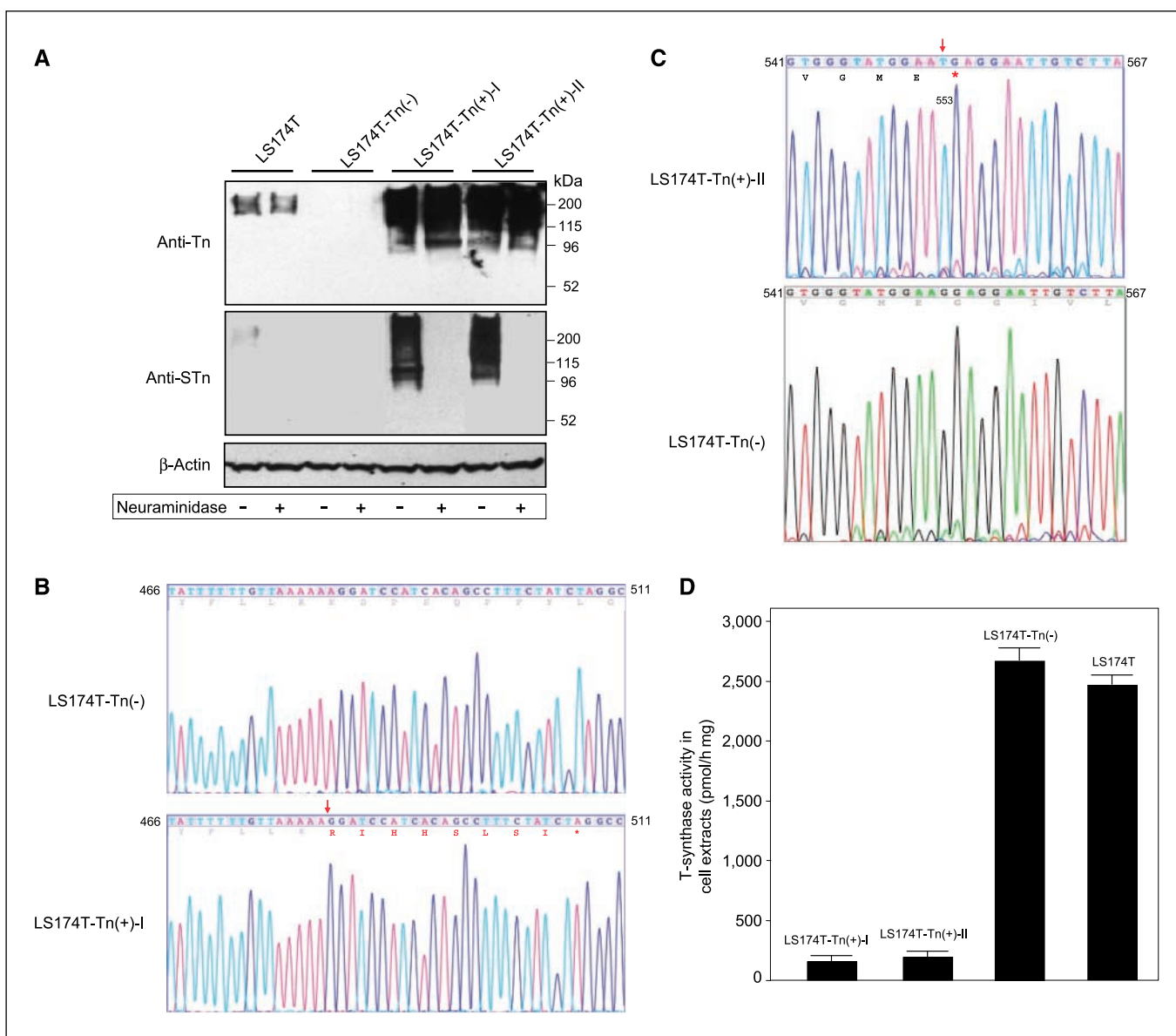
Figure 2. Mutations of *Cosmc* in LS174T-Tn(+) cells are associated with the Tn/STn phenotype of LS174T cells. Parental LS174T cells were stained with anti-Tn mAb and analyzed by FACS, and the Tn(+) cells were obtained by sorting. A portion of LS174T-Tn(+) cells was subcloned, and five single-cell clones were obtained. *A,* Tn/STn antigens were analyzed by Western blot of cell extracts with anti-Tn and anti-STn mAbs before and after neuraminidase treatment. *B* and *C,* the total RNA from those clones of LS174T-Tn(+) and LS174T-Tn(−) cells was prepared. RT-PCR for human *Cosmc* was performed, and the product was directly sequenced. The trace file shows the portion of *Cosmc* sequence corresponding to the mutation of 482A deletion in LS174T-Tn(+)-I (*B*) and G553T change in LS174T-Tn(+)-II (*C; red arrow*). 482A deletion resulted in an ORF shift of *Cosmc* in LS174T-Tn(+)-I, whereas the G553T mutation introduced a premature stop codon. *D,* the T-synthase activity from LS174T-Tn(+)-I, LS174T-Tn(+)-II, and Tn(−) cells, as well as parental cells, were measured.

below. Two other mutations of a silent nature were observed in *Cosmc* from LSC cells but not LSB cells, including a nucleotide change from G to A at position 226 and C to T at position 322 (data not shown).

Transfection of LSC cells with wtCosmc can restore the T-synthase activity and correct O-glycan expression. LSC cells were stably transfected with a plasmid encoding wtCosmc. As seen in Fig. 1*C*, transfection of LSC cells with wtCosmc restored T-synthase activity. Furthermore, restoration of T-synthase activity with wtCosmc eliminated expression of the Tn/STn antigens by Western blot analysis (Fig. 1*C*), demonstrating that the wtCosmc rescued normal surface O-glycan elongation. To confirm that loss

of Tn/STn expression resulted from renewed O-glycan elongation, we probed cells with PNA, a plant lectin that binds with high affinity to the T-synthase product, T-antigen (Galβ3GalNAcα-Ser/Thr; ref. 20). T-antigen is further elongated to form sialyl T-antigen, which is not recognized by PNA. Treatment with neuraminidase exposes the T-antigen and PNA recognition. Introduction of wtCosmc into LSC cells led to the expression of high molecular weight glycoproteins recognized by PNA following neuraminidase treatment. Similar staining followed expression of wtCosmc in Jurkat cells, which also contain mutations in *Cosmc* (Fig. 1*D*). It is noteworthy that PNA stained, to a lesser extent, Jurkat cells. This likely results from the minimal T-synthase activity present in Jurkat

cells, which express a truncated *Cosmc* capable of producing 2% to 5% of the activity of full protein of Cosmc (12). These results show that the mutation in *Cosmc* results in Tn/STn antigen expression in LSC cells and that wtCosmc can complement the defect and rescue the T-synthase activity and O-glycan extension.

Cosmc is mutated in subsets of LS174T cells with the Tn(+) phenotype, whereas Cosmc is normal in Tn(−) cells. LSC and LSB cells were originally derived from LS174T cells. We attempted to enrich for any Tn(+) cells within the LS174T population using the anti-Tn antibody and found that ∼5% of LS174T cells stained positive for Tn antigen, designated LS174T-Tn(+), which were subsequently subcloned into five separate subclones. Genetic analyses below showed that these subclones represented two types of populations that we designated as LS174T-Tn(+)-I and LS174T-Tn(+)-II.

We analyzed total expression of Tn and STn antigens by Western blotting before and after treatment with neuraminidase. As shown in Fig. 2A, the LS174T-Tn(−) cells, which had been sorted based on their lack of expression of Tn antigen, exhibited no staining with anti-Tn antibody. However, the parental LS174T cells, which is a mixture of Tn(+) and Tn(−) cell, exhibited some staining with the anti-Tn and anti-STn antibodies. By contrast, both LS174T-Tn(+)-I and LS174T-Tn(+)-II exhibited robust staining of high molecular weight glycoproteins with anti-Tn and with anti-STn antibodies (Fig. 2A). The staining with anti-STn was abolished by treatment with neuraminidase. These results show that LS174T-Tn(+)-I and LS174T-Tn(+)-II express many glycoproteins with Tn and STn antigens and that the LS174T cells selected based on their lack of Tn expression [LS174T-Tn(−) cells] did not express either Tn or STn antigens.

All *Cosmc* cDNAs isolated from either Tn(−) or Tn(+) clones isolated from LS174T cells exhibited two silent mutations, G266A and C322T, which were also seen in LSC cells. However, all five Tn(+) subclones exhibited mutations different from that observed in LSC cells. One Tn(+) clone designated LS174T-Tn(+)-I contained a unique mutation, which is an A-deletion at position 482 (482A). This 482A mutation resulted in an ORF shift with a predicted polypeptide of 168 amino acids, of which 160 amino acids belong to the NH_2 terminal portion of Cosmc (Fig. 2B). The other four Tn(+) clones designated LS174T-Tn(+)-II shared a mutation at position 553, a nucleotide change from G to T (G553T; Fig. 2C), which resulted in a 184–amino acid polypeptide. We then assayed the total T-synthase activity in different cell lines. LS174T cells, which our results show, are a mixed population of Tn(−) and Tn(+) cells, possessed slightly lower total activity than Tn(−) cells (Fig. 2D), whereas both LS174T-Tn(+)-I and LS174T-Tn(+)-II cell lines had very low T-synthase activity.

Thus, a total of three different mutations were found in subclones from LS174T cells that expressed the Tn antigen: a T-insertion at position 53 in LSC cells (Fig. 1A), an 482A in LS174T-Tn(+)-I cells (Fig. 2B), and G553T in LS174T-Tn(+)-II cells (Fig. 2C). These results show that Tn/STn expression in LS174T cells correlates with mutations in *Cosmc*.

Human melanoma LOX cells express Tn/STn antigens and do not express Cosmc transcripts. The above evidence shows that mutations in *Cosmc* occur in poly-T (Fig. 1A) and poly-A (Fig. 2B) islands, but these data are insufficient to conclude that mutations in Cosmc accumulate in high-frequency hotspots (Supplementary Fig. S1A and B). To further explore mutations in Cosmc associated with Tn antigen expression, we examined two melanoma cell lines, LOX and FEMX-I (21, 22). LOX cells, derived from a male patient (22), were previously shown to be stained by

HPA (23), a lectin that specifically recognizes α-GalNAc residues as in the Tn antigen (24). Interestingly, HPA binding correlated with experimental lung metastases in athymic nude mice (16).

Although transcripts for both *T-synthase* and *Cosmc* were expressed in FEMX-I cells, RT-PCR failed to detect the transcript for *Cosmc* in LOX cells (Fig. 3A). Loss of the *Cosmc* transcript could arise from several reasons, including gene deletion, alterations in the promoter, transcript instability, or possibly gene methylation. To explore these possibilities, we first investigated whether unbalanced genetic translocations caused deletion of *Cosmc* from the genome of these cells. However, PCR analysis of DNA isolated from LOX cells showed that *Cosmc* was present and that the genetic integrity of the encoding portion of *Cosmc* remained intact (data not shown).

We then questioned whether *cis*-acting regulatory elements might have mutations. According to the predicted promoter sequence obtained from the online promoter analysis software,[8] two CpG islands (CpG-I and CpG-II) reside upstream from the coding sequence. From the available human EST and the *Cosmc* transcript size analyzed using Northern blot analysis,[9] the Cosmc transcript likely begins within the CpG-II, which contains four Sp1 sites (specific transcription activator site), suggesting that the CpG-II may serve as a promoter for this gene. Interestingly, LOX cells exhibited a genomic deletion of this CpG-II sequence, although FEMX-I cells exhibited a normal CpG-II sequence. To insure that the coding region of T-synthase remained intact, we examined T-synthase and found that the coding region for *Cosmc* (Fig. 3B) and three exons of T-synthase did not have deletion mutations (data not shown). To confirm the regulatory element deletion, Southern blot analysis was performed. Restriction enzyme mapping revealed that there are three *Bam*HI sites spanning the *Cosmc*; between the regulatory sequence and coding region, inside the coding region, and at the 3′-end of the gene. There are also three *Sac*I cleavage sites in this gene; inside the regulatory element, inside the coding region, and downstream of the gene. Consistent with PCR, Southern blot showed that FEMX-I had an identical restriction pattern as the normal control, which generated 1.4 and 5.9 kb bands for *Bam*HI (B) and 4.3 and 7.0 kb bands for *Sac*I (S) digestion using a probe that targeted the ORF (Fig. 3C). By contrast, LOX cells lacked both 1.4 and 5.9 kb bands following *Bam*HI digestion. The 1.4-kb band spans the 5′ portion of coding and upstream noncoding region. Instead, restriction digestion of LOX cells revealed a single ∼9.0-kb band. *Sac*I digestion of LOX gDNA also failed to produce a 4.3-kb band, which covered a portion of promoter region and 5′ portion of coding region (Fig. 3C). These results show that LOX cells contain a genomic deletion upstream of the *Cosmc* coding region, corroborating PCR demonstration of a CpG-II deletion. Taken together, these results show that LOX cells have a genomic deletion upstream of the coding region that results in lack of expression of Cosmc transcripts.

To examine the correlation in LOX cells between loss of *Cosmc* transcripts and Tn expression, we examined whether LOX cells were stained by HPA, as previously reported (23). LOX cells bound well to HPA and had poor binding to PNA. FEMX-I stained well with PNA, yet failed to stain with HPA (Fig. 3D). Interestingly, Western blot analysis of LOX cells stained with HPA revealed four to five major bands, in contrast to LSC and LS174T Tn+ cells, both of which displayed a large molecular weight distribution of HPA-positive

---

[9] Unpublished result.

bands. LOX cells also expressed both Tn and STn antigens, similar to LSC cells. By contrast, FEMX-I did not express either the Tn or STn antigens. These results suggest that these cells likely exhibit differential expression of mucins compared with colorectal tumor cells.

LOX cells had no detectable T-synthase activity, whereas FEMX-I cells had significant activity, demonstrating that the inability of LOX cells to produce functional Cosmc correlates with loss of T-synthase activity (Fig. 4*A*). To define the role of Cosmc function in LOX cells, we transfected them with wtCosmc, which restored T-synthase activity (Fig. 4*A*). Furthermore, LOX cells transfected with wtCosmc also displayed decreased expression of Tn antigen expression and an increase in PNA binding as assessed by flow cytometry (Fig. 4*B*). Consistent with the activity of T-synthase in cells and flow cytometry results, the majority of LOX cells stably transfected with wtCosmc displayed Tn(−) phenotype, whereas mock LOX cells were uniformly Tn(+) by immunohistochemistry (Fig. 4*C*). By contrast, FEMX-I cells were Tn(−) (Fig. 4*C*). To further confirm the finding in Fig. 4*C*, LOX cells stably expressing wtCosmc were stained with both FITC-labeled PNA and Alexa568-labeled anti-Tn mAb. As shown in Fig. 4*D*, whereas mock-transfected LOX cells were stained uniformly by anti-Tn mAb in

red, and not stained by PNA, only a few LOX cells transfected with wtCosmc were stained by anti-Tn mAb (red) whereas the majority of the cells were bound by PNA (green). Importantly, there was no overlap between the Tn(+) cells and PNA(+) cells (Fig. 4*D*), which is consistent with the interpretation that, in those cells transfected to express wtCosmc, the Tn antigen expression is lost while T-antigen expression is gained. Taken together, these results show that expression of wtCosmc in LOX cells causes restoration of T-synthase activity and restoration of normal O-glycan extension.

Alteration of *Cosmc* in human cervical cancer. To investigate whether *Cosmc* is altered in human tumor cells that express Tn/STn antigens, we analyzed the sequence of *Cosmc* in two specimens of human cervical cancer samples (DH85 and DH86). As shown in Supplementary Fig. S2*A* and *B*, two sections were chosen that were intensively stained by both Tn and STn mAb with the same pattern, indicating that those tumor cells expressed both Tn and STn antigens. The Tn/STn(+) cells were selected by LCM, as described in Materials and Methods. In the Tn/STn(+) tumor cells from the DH85 sample, we identified two mutations (A152G and G385A; Fig. 5*A*). These two mutations resulted in D51G and A129T changes in its amino acid sequence, respectively. Because

Figure 3. Human melanoma cell line LOX lacks the transcript for *Cosmc* and expresses Tn and STn antigens because of the deletion of the promoter region for *Cosmc*. Total RNA and gDNA from human melanoma cell lines LOX and FEMX-I were prepared. RT-PCR for human *Cosmc*, *T-synthase*, and *glyceraldehyde-3-phosphate dehydrogenase* and PCR for the coding region, promoter region of *Cosmc*, were performed. The RT-PCR (*A*) and PCR (*B*) products were analyzed on an agarose gel. The size of expected products are on the right side of the gel. *C,* Southern blot analysis. gDNAs from normal control, LOX and FEMX-I, were digested with *Bam*HI (*B*) and *Sac*I (*S*), respectively, analyzed on 0.8% Agarose gel, and transferred to a membrane. The membrane was hybridized with a probe of ^{32}P-labeled *Cosmc* ORF and exposed to a BioMax film. The 1-kb marker is at the right side the figure. *D,* lectin blots of extracts from LOX and FEMX-I cells. Cell extracts with and without neuraminidase treatment were subjected to SDS-PAGE and transferred to a membrane. The membrane was then blotted with HRP-labeled HPA and PNA.
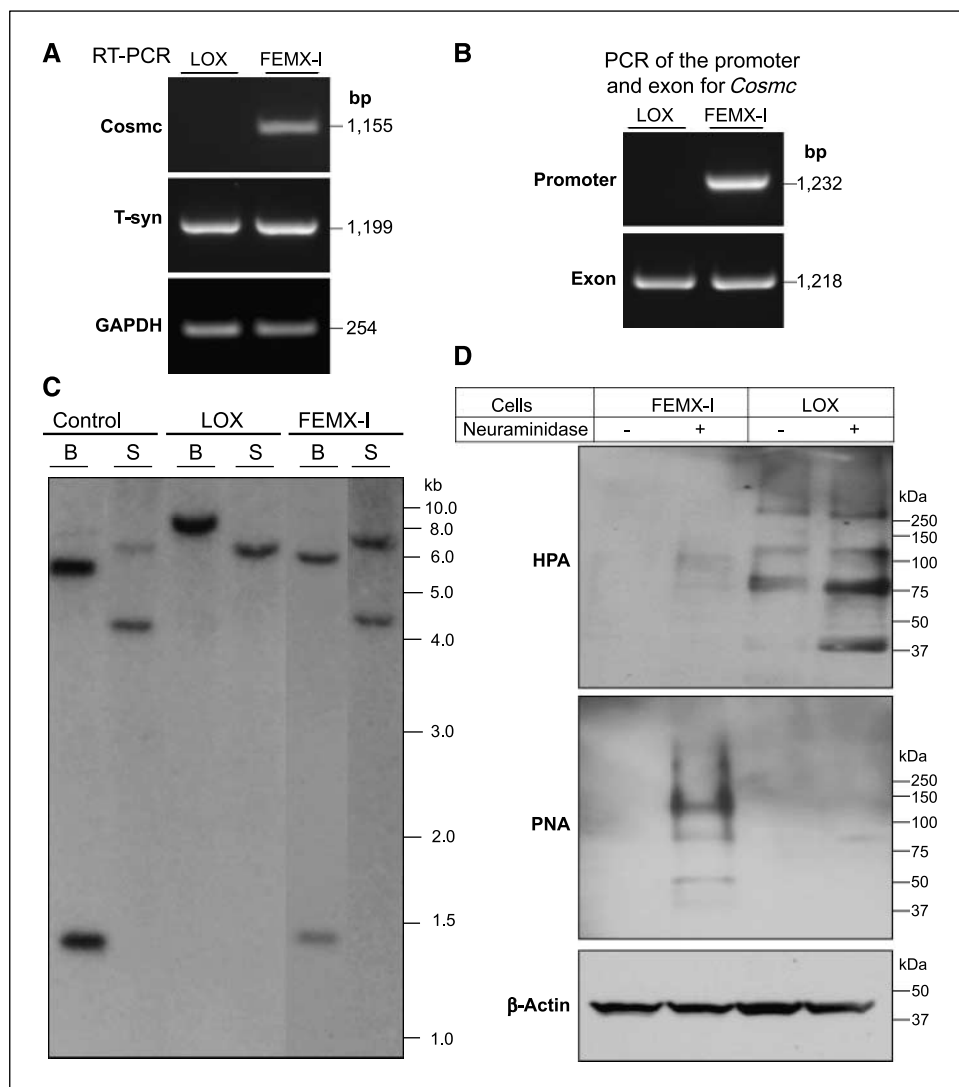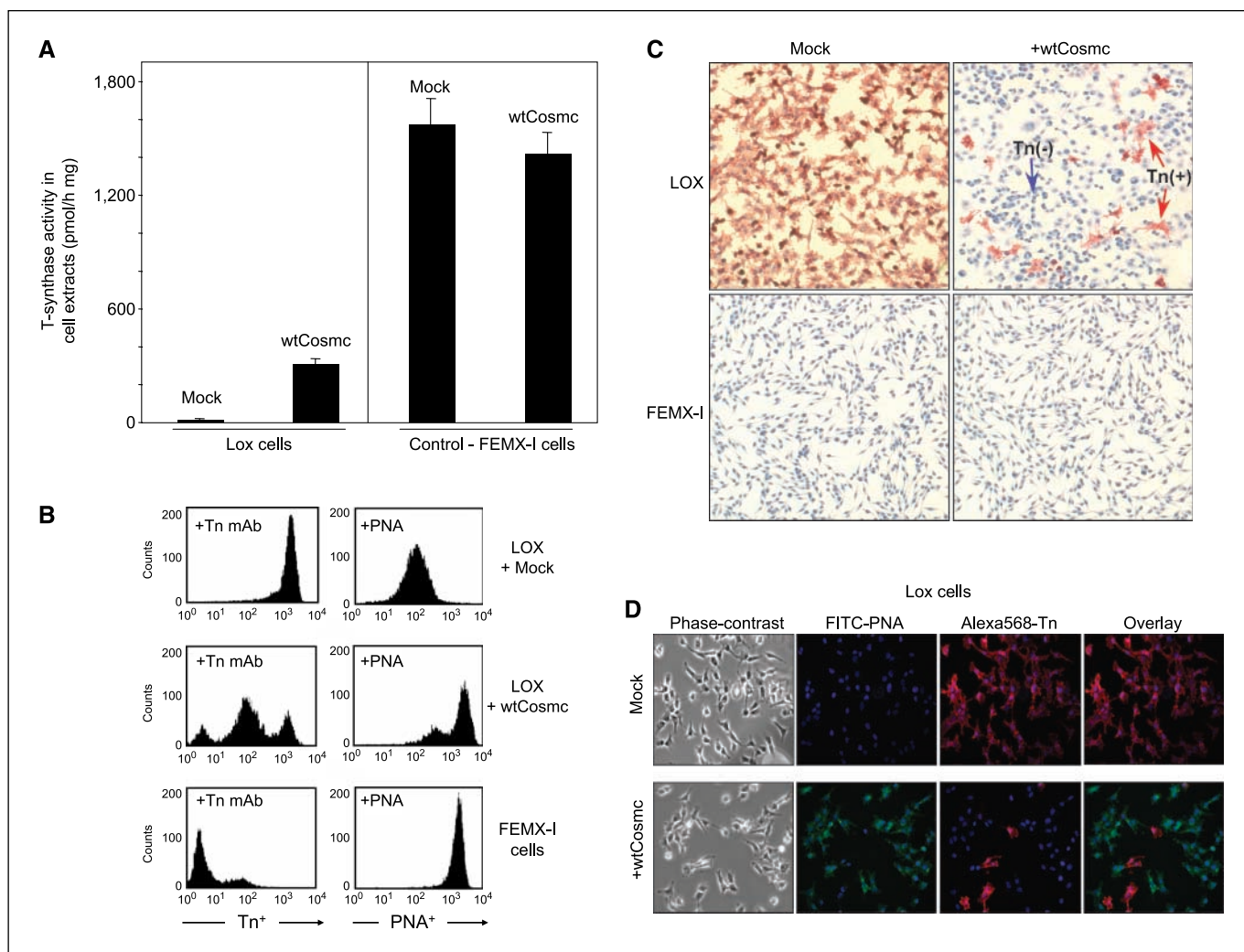
Figure 4. wtCosmc restores T-synthase activity in LOX cells, reduces Tn-antigen expression, and partially corrects the O-glycan structure on the cell surface. *A*, LOX and FEMX-I cells were mock transfected or stably transfected with wtCosmc, cell extracts were then prepared, and T-synthase activity in the cell extracts was measured using the acceptor GalNAc-α-Phenyl. *B*, transfected LOX and FEMX-I cells were stained with Tn mAb and FITC-labeled PNA after neuraminidase treatment and analyzed by flow cytometry. *C* and *D*, mock-transfected and wtCosmc–stably transfected LOX and FEMX-I cells were cultured on chambered slides overnight. The cells were fixed and desialylated by neuraminidase treatment. *C*, after blocking with goat serum, the cells were immunochemically stained with anti-Tn mAb. After developing with chromogen AEC substrate, the cells were counterstained with Mayer's hematoxylin and analyzed under a microscope. *D*, after blocking with BSA, the cells were first incubated with anti-Tn and then with Alexa568-labeled goat anti-mouse IgM and FITC-labeled PNA. Finally, the nuclei of cells were stained with DAPI. After washing, the cells were covered with mount media and analyzed by fluorescence microscopy. The image was collected at 20× magnification.

only a single DNA sequence for *Cosmc* was seen in this specimen, it is likely that the *Cosmc* on one allele from the tumor cells was deleted as a result of loss of heterozygosity (LOH); the chance for identical mutations simultaneously happening on both alleles is very low. To confirm this, we sequenced the 3′ region of *Cosmc* containing two SNPs (rs591040 and rs17327439), which are at +1,258 and +1,337 bp (downstream of the *Cosmc* coding region). As expected, whereas the whole tissue gDNA from DH85 sample contained those two SNPs and one more mosaic sequence (C/T) at +1,266 bp, the DNA from Tn/STn(+) tumor cells had only a single sequence (Fig. 5*B*). These results show that there was a loss of the active allele of *Cosmc* in this sample because no mutation was seen in the cDNA (Fig. 5*A*). Interestingly, *Cosmc* in the other sample DH86 contained a SNP (T393A, rs17261572) in its coding sequence and the A-allele is active, as showed by its cDNA sequence (Fig. 5*C*). There were two silent mutations (G226A and C322T)

found in *Cosmc* in both whole-tissue and tumor cells (data not shown); however, there was LOH in the tumor cells with the inactive allele remaining (Fig. 5*C*). These data indicate that there was a deletion (LOH) in the region on the active allele of the X-chromosome containing functional *Cosmc* in the tumor cells. Loss of *Cosmc* in these tumor cells accounts for the formation of an inactive T-synthase and the expression of Tn/STn antigens. Taken together, these preliminary studies show that the expression of Tn/STn antigens in human cervical cancer results from the mutations in *Cosmc*.

Discussion

The Tn antigen and its sialylated derivative STn are common tumor-associated carbohydrate antigens. Expression of the Tn antigen correlates with metastatic potential and poor prognosis

in many cancers (25, 26), including cervical (27, 28), lung adenocarcinomas (29), colorectal carcinomas (30), breast carcinomas (31), and gastric carcinomas (32). Many studies over the past 30 years have shown that the Tn antigen is expressed by >80% of human carcinomas (5–7). Our studies here show that expression of the Tn and STn antigens in human tumor cell lines and in two cervical cancer specimens results from mutations in *Cosmc*, leading to a loss of T-synthase activity and consequent inability to modify the Tn precursor. These results represent the first example of a single genetic locus regulating the global expression of a tumor-specific carbohydrate antigen in diverse types of human neoplastic disease.

Our study represents the first report of altered Cosmc expression in human cancers. We chose to examine cervical cancer because it has been well documented that Tn and STn antigens are expressed in at least 60% of human cervical carcinomas and their expression is associated with the poor prognosis of the disease (27, 28). However, until now, there has been no genetic or biochemical basis for the expression of the Tn/STn antigens in human cancers. Human cervical cancer is the second most common cancer among women worldwide (33). Most cervical cancers are caused by infection of human papillomavirus (HPV); HPV-16, HPV-18, and HPV-31 account for a vast majority of the cases (34). Understanding the genetic basis of the expression of Tn and STn should certainly shed light on developing novel diagnostic, prognostic, and, more importantly, therapeutic approaches against this disease. Whereas our studies should be viewed as very preliminary in nature, we have shown that, for those cervical tumor cells that are very positive for Tn/STn expression, mutations occur in *Cosmc*. One specimen contained mutations in *Cosmc* along with LOH, whereas the other specimen had LOH. Thus, our results show that LOH or deletion of *Cosmc* are among the molecular mechanisms accounting for Tn/STn expression in human

Figure 5. Alteration of *Cosmc* in human cervical cancer. Two specimens of human cervical cancer samples, DH85 (*A* and *B*) and DH86 (*C*), were first stained immunohistochemically with Tn and STn antigens, using appropriate mAbs. The gDNA and total RNA from the tissue and the gDNA from Tn/STn(+) tumor cells collected by LCM were isolated. The coding region and cDNA for *Cosmc* (*A* and *C*), as well as the downstream of the coding region (*B*), were amplified by PCR or RT-PCR and the products were directly sequenced.
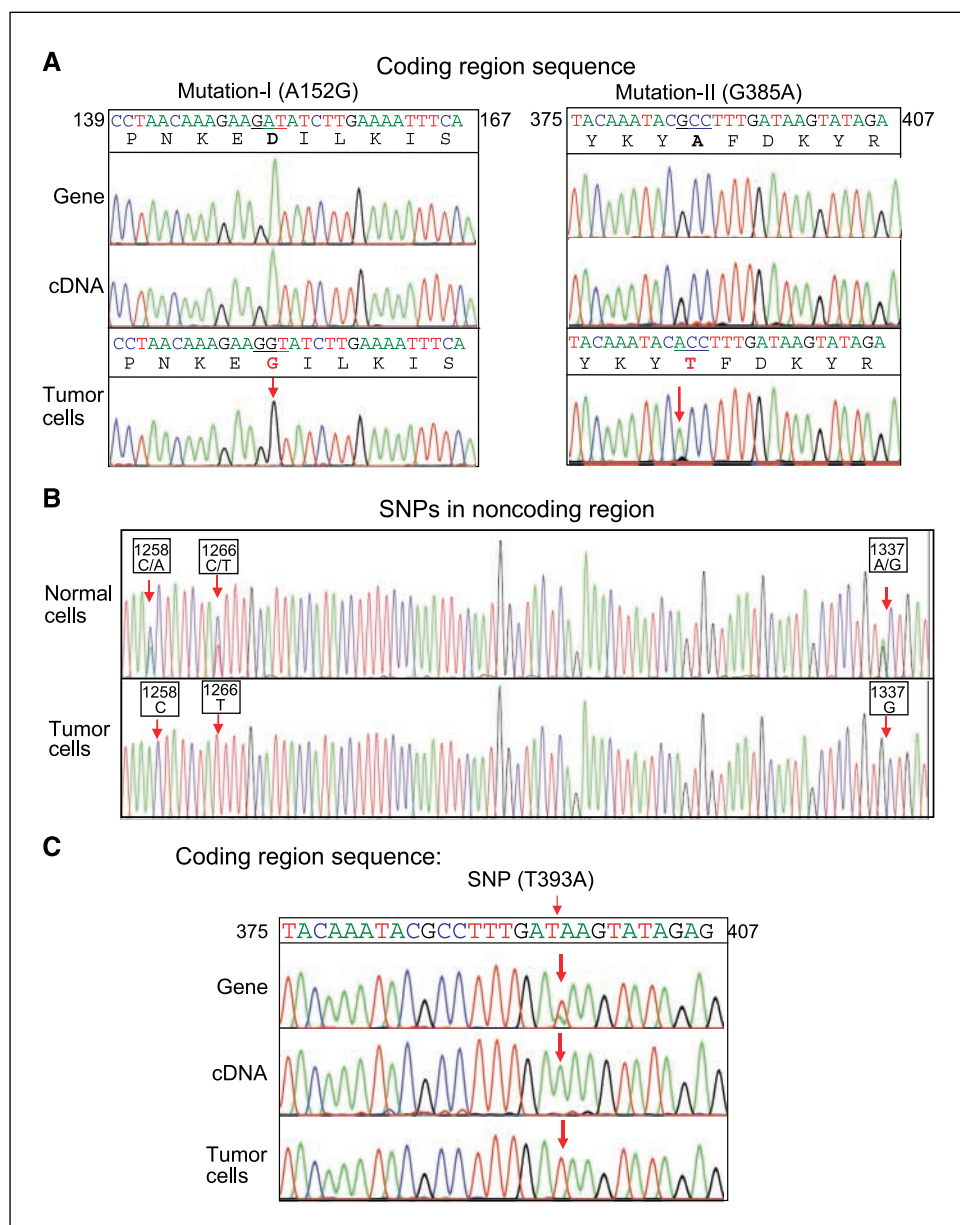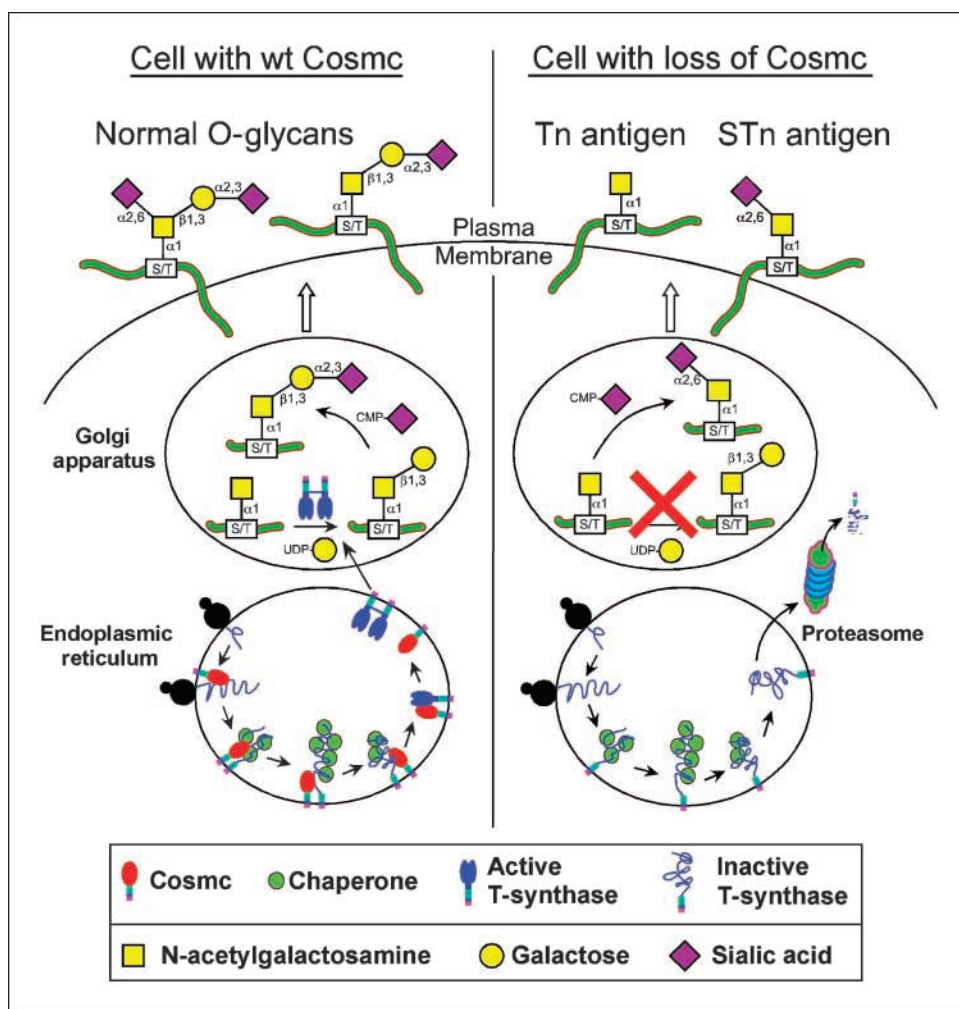
Figure 6. Mutations in *Cosmc* result in expression of Tn and STn antigens. The model illustrates the molecular mechanism for the expression of the tumor-associated carbohydrate antigens Tn and STn. On the left side, Cosmc (*red figure*), a core 1 β3GalT-specific molecular chaperone, resides in the endoplasmic reticulum and cooperates with other endoplasmic reticulum chaperones (*green*) to help the folding of newly synthesized T-synthase polypeptide (*blue line*) to a functional T-synthase (*blue figure*). The active dimeric enzyme then exits to the Golgi apparatus, where it converts Tn antigen to the core 1 structure (or T-antigen), which is a common precursor for most extended O-glycans. On the right side, when *Cosmc* is mutated, Cosmc is dysfunctional and unable to assist the folding of newly synthesized T-synthase. This results in a misfolded polypeptide leading to the ERAD pathway and degradation by the proteasome. Thus, loss of Cosmc leads to a loss of T-synthase in the Golgi apparatus and accumulation of Tn antigen, which can be converted to STn by the ST6GalNAc-I enzyme.

cervical cancers. Several chromosomal regions, including 3p, 4p, 4q, 6p, 6q, 11q, 13q, 17q, and 18q, have been observed to have LOH associated with cervical cancer (35). Our preliminary result is the first report of a LOH on X-chromosome (Xq24) in human cervical cancer. Our results indicate that new studies with much larger numbers of patients are warranted to examine LOH and additional mutations for the *Cosmc* locus in patients with cervical cancer. In cervical cancer, we did not observe the same mutations as found in the tumor cell lines. This may be because they are derived from different type of tissues. However, many more tumor specimens will need to be examined in the future to better map out the types of mutations that occur in *Cosmc* and how they relate to those found in cell lines derived from human tumors.

The localization of *Cosmc* on the X-chromosome increases the sensitivity of cells to loss of this gene due to the presence of only one X-chromosome in males and the inactivation of one X-chromosome in female cells during embryonic development through methylation and condensation (36). Thus, adult female tissues are cellular mosaics with approximately half of the cells expressing the paternal X-chromosome and the other half expressing the maternal X-chromosome. Women who have a significant deviation from this 1:1 ratio are considered to have skewed X-chromosome inactivation, a condition associated with

early development of lung and breast cancer (37). Thus, the key role of the Cosmc protein in T-synthase activity shown by this study, the frequent loss of T-synthase activity in cancer and metastases, and the localization of the *Cosmc* gene on the X-chromosome indicate that loss or mutation of the single *Cosmc* allele can contribute to tumorigenesis and metastases. This identifies *Cosmc* as a molecular target for diagnostic, prognostic, prevention, and treatment strategies.

The identification of novel neoplastic specific antigens or markers has been important in the diagnosis and treatment of neoplastic disease, and a wide variety of markers have been identified (38). However, whereas some of these markers have shown great promise and efficacy in treatment and diagnosis, such as Her2 and prostate-specific antigen; most have limited specificity and the genetic or epigenetic basis for the expression of most neoplastic antigens are unclear.

Altered expression of sialic acid on cell surface glycoproteins is associated with cancer cell invasiveness and metastasis (39). Previous studies have suggested that expression of STn antigen results from up-regulation of ST6GalNAc-I, the enzyme responsible for sialylating the Tn antigen (8, 40), although other studies failed to find a correlation between STn expression and expression of ST6GalNAc-I (41). More importantly, expression of the STn usually correlates with expression of the Tn antigen. These

results are consistent with the known biochemical pathway for formation of the Tn and STn antigens, which requires the inaction of the T-synthase, which normally adds a galactose to the Tn antigen to create the disaccharide Galβ3GalNAcα-Ser/Thr (Fig. 6). In this model, acquired or somatic mutations in the X-chromosome–encoded *Cosmc* in tumor cells or perhaps tumor stem cells would result in loss of Cosmc chaperone function and consequently the T-synthase protein is degraded in the proteasome (12, 13). The loss of the T-synthase results in Tn antigen expression and the capability of generating the STn antigen if the ST6GalNAc-I enzyme is also expressed. Complementation with wtCosmc causes loss of both Tn and STn expression, as we have shown. The lack of expression of the T-synthase in tumor cells is consistent with previous studies where the activity of the T-synthase was shown to be low in human breast tumors (42). In addition, it was recently reported that aging mouse fibrosarcoma cells that express the Tn antigen also contain a mutated form of *Cosmc* (43). Thus, in tumor cells expressing the Tn and STn antigens, there may be both loss of Cosmc function, leading to loss of T-synthase activity, and there may be up-regulation of expression for ST6GalNAc-I. The molecular basis for up-regulation of ST6GalNAc-I in tumor cells is unknown.

The expression of the Tn and STn antigens in tumor cells may have broad consequences. The Tn antigen is recognized by a C-type lectin, termed MGL, which is expressed by both dendritic cells and macrophages (44, 45). In many cases, Tn antigen is expressed on secreted and cell surface mucins, such as Muc-1, in colorectal tumor cells but not in normal colon (46). MGL-positive cells have been detected *in situ* in colorectal tumors (45). Such results suggest the provocative possibility that expression of the Tn antigen and its recognition by MGL may be involved in immune surveillance and tolerance (45). Clearly, future studies should now explore the relationship between *Cosmc* mutations, Tn antigen expression, and interactions with C-type lectins expressed by dendritic cells and macrophages.

Altered O-glycosylation, such as expression of the Tn and STn antigens, may have many other biological consequences in cancer. Mucin-associated STn antigen can inhibit natural killer cell–induced cytotoxicity of target tumor cells (47). In some tumors, mucin expression and altered glycosylation correlates with expression of galectins, such as galectin-3 (48), a member of the galectin superfamily shown to bind Tn antigen, contributing to metastatic extravasation and regulation of the adaptive immune response. Expression of truncated O-glycans also correlates with altered expression of cell surface mucins and integrins and can change the adhesive properties of cells (49). Reduced O-glycan content may also render mucins more susceptible to metalloproteinases, favoring detachment, migration, altered interactions with adhesion molecules, such as E-selectin and P-selectin, and subsequent extravasation of neoplastic cells, ultimately contributing to metastasis. For example, we recently studied the roles of O-glycans in intestinal mucins, which express both core 1 and core 3 O-glycans. Genetic ablation of the core 3 β1,3-*N*-acetylglucosaminyltransferase (C3GnT) leads to loss of core 3 structures and increased susceptibility to colitis and colorectal tumor formation, which is also correlated with a deficiency of colon surface expressed MUC2 (50). Clearly, much remains to be done to fully elucidate the consequences of Tn and STn antigen expression in tumor-derived glycoproteins, but mechanistic evidence is accumulating to support the hypothesis that abnormal expression of O-glycans is associated with altered cellular properties, immune functions, and metastatic potential. Our findings of mutations in *Cosmc* in human tumor cells, coupled with the strong evidence that the Tn and STn antigens are commonly expressed by human carcinomas, strengthens the potential for using these antigens and the *Cosmc* gene as targets for the diagnosis and treatment of human neoplastic diseases.

Acknowledgments

References

1. Hakomori S. Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv Exp Med Biol 2001; 491:369–402.
2. Feizi T. Carbohydrate antigens in human cancer. Cancer Surv 1985;4:245–69.
3. Xu Y, Sette A, Sidney J, Gendler SJ, Franco A. Tumor-associated carbohydrate antigens: a possible avenue for cancer prevention. Immunol Cell Biol 2005;83:440–8.
4. Dube DH, Bertozzi CR. Glycans in cancer and inflammation-potential for therapeutics and diagnostics. Nat Rev Drug Discov 2005;4:477–88.
5. Galonic DP, Gin DY. Chemical glycosylation in the synthesis of glycoconjugate antitumour vaccines. Nature 2007;446:1000–7.
6. Springer GF. T and Tn, general carcinoma autoantigens. Science 1984;224:1198–206.
7. Slovin SF, Keding SJ, Ragupathi G. Carbohydrate vaccines as immunotherapy for cancer. Immunol Cell Biol 2005;83:418–28.
8. Sewell R, Backstrom M, Dalziel M, et al. The ST6GalNAc-I sialyltransferase localizes throughout the Golgi and is responsible for the synthesis of the tumor-associated sialyl-Tn O-glycan in human breast cancer. J Biol Chem 2006;281:3586–94.
9. Escrevente C, Machado E, Brito C, et al. Different expression levels of α3/4 fucosyltransferases and Lewis determinants in ovarian carcinoma tissues and cell lines. Int J Oncol 2006;29:557–66.
10. Serpa J, Mesquita P, Mendes N, et al. Expression of Lea in gastric cancer cell lines depends on FUT3 expression regulated by promoter methylation. Cancer Lett 2006;242:191–7.
11. Ju T, Brewer K, D'Souza A, Cummings RD, Canfield WM. Cloning and expression of human core 1 β1,3-galactosyltransferase. J Biol Chem 2002;277:178–86.
12. Ju T, Cummings RD. A unique molecular chaperone Cosmc required for activity of the mammalian core 1 β3-galactosyltransferase. Proc Natl Acad Sci U S A 2002; 99:16613–8.
13. Ju T, Cummings RD. Protein glycosylation: chaperone mutation in Tn syndrome. Nature 2005;437:1252.
14. Piller V, Piller F, Fukuda M. Biosynthesis of truncated O-glycans in the T cell line Jurkat. Localization of O-glycan initiation. J Biol Chem 1990;265:9264–71.
15. Brockhausen I, Yang J, Dickinson N, Ogata S, Itzkowitz SH. Enzymatic basis for sialyl-Tn expression in human colon cancer cells. Glycoconj J 1998; 15:595–603.
16. Rye PD, Fodstad O, Emilsen E, Bryne M. Invasion potential and N-acetylgalactosamine expression in a human melanoma model. Int J Cancer 1998;75:609–14.
17. Xia L, Ju T, Westmuckett A, et al. Defective angiogenesis and fatal embryonic hemorrhage in mice lacking core 1-derived O-glycans. J Cell Biol 2004;164: 451–9.
18. Avichezer D, Springer GF, Schechter B, Arnon R. Immunoreactivities of polyclonal and monoclonal anti-T and anti-Tn antibodies with human carcinoma cells, grown *in vitro* and in a xenograft model. Int J Cancer 1997;72:119–27.
19. Ogata S, Chen A, Itzkowitz SH. Use of model cell lines to study the biosynthesis and biological role of cancer-associated sialosyl-Tn antigen. Cancer Res 1994;54: 4036–44.
20. Novogrodsky A, Lotan R, Ravid A, Sharon N. Peanut agglutinin, a new mitogen that binds to galactosyl sites exposed after neuraminidase treatment. J Immunol 1975;115:1243–8.
21. Fodstad O, Kjonniksen I, Aamdal S, Nesland JM, Boyd MR, Pihl A. Extrapulmonary, tissue-specific metastasis formation in nude mice injected with FEMX-I human melanoma cells. Cancer Res 1988;48:4382–8.
22. Fodstad O, Aamdal S, McMenamin M, Nesland JM, Pihl A. A new experimental metastasis model in athymic

nude mice, the human malignant melanoma LOX. Int J Cancer 1988;41:442–9.

23. Kjonniksen I, Rye PD, Fodstad O. *Helix pomatia* agglutinin binding in human tumour cell lines: correlation with pulmonary metastases in nude mice. Br J Cancer 1994;69:1021–4.

24. Baker DA, Sugii S, Kabat EA, Ratcliffe RM, Hermentin P, Lemieux RU. Immunochemical studies on the combining sites of Forssman hapten reactive hemagglutinins from *Dolichos biflorus, Helix pomatia*, and *Wistaria floribunda*. Biochemistry 1983;22:2741–50.

25. Mitchell BS, Schumacher U. The use of the lectin *Helix pomatia* agglutinin (HPA) as a prognostic indicator and as a tool in cancer research. Histol Histopathol 1999;14:217–26.

26. Kawaguchi T. Cancer metastasis: characterization and identification of the behavior of metastatic tumor cells and the cell adhesion molecules, including carbohydrates. Curr Drug Targets Cardiovasc Haematol Disord 2005;5:39–64.

27. Numa F, Tsunaga N, Michioka T, Nawata S, Ogata H, Kato H. Tissue expression of Sialyl Tn antigen in gynecologic tumors. J Obstet Gynaecol 1995;21:385–9.

28. Inoue M, Ogawa H, Nakanishi K, Tanizawa O, Karino K, Endo J. Clinical value of sialyl Tn antigen in patients with gynecologic tumors. Obstet Gynecol 1990;75:1032–6.

29. Laack E, Nikbakht H, Peters A, et al. Lectin histochemistry of resected adenocarcinoma of the lung: *Helix pomatia* agglutinin binding is an independent prognostic factor. Am J Pathol 2002;160:1001–8.

30. Konno A, Hoshino Y, Terashima S, Motoki R, Kawaguchi T. Carbohydrate expression profile of colorectal cancer cells is relevant to metastatic pattern and prognosis. Clin Exp Metastasis 2002;19:61–70.

31. Fernandez Madrid F, Tang N, Alansari H, Karvonen RL, Tomkiel JE. Improved approach to identify cancer-associated autoantigens. Autoimmun Rev 2005;4:230–5.

32. Kakeji Y, Tsujitani S, Mori M, Maehara Y, Sugimachi K. *Helix pomatia* agglutinin binding activity is a predictor of survival time for patients with gastric carcinoma. Cancer 1991;68:2438–42.

33. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.

34. Chan JK, Berek JS. Impact of the human papilloma vaccine on cervical cancer. J Clin Oncol 2007;25:2975–82.

35. Miyai K, Furugen Y, Matsumoto T, et al. Loss of heterozygosity analysis in uterine cervical adenocarcinoma. Gynecol Oncol 2004;94:115–20.

36. Chow JC, Yen Z, Ziesche SM, Brown CJ. Silencing of the mammalian X chromosome. Annu Rev Genomics Hum Genet 2005;6:69–92.

37. Struewing JP, Pineda MA, Sherman ME, et al. Skewed X chromosome inactivation and early-onset breast cancer. J Med Genet 2006;43:48–53.

38. Faried A, Kimura H, Faried LS, et al. Expression of carbohydrate antigens in human esophageal squamous cell carcinoma: prognostic application and its diagnostic implications. Ann Surg Oncol 2007;14:960–7.

39. Wang PH, Lee WL, Juang CM, et al. Altered mRNA expressions of sialyltransferases in ovarian cancers. Gynecol Oncol 2005;99:631–9.

40. Julien S, Adriaenssens E, Ottenberg K, et al. ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. Glycobiology 2006;16:54–64.

41. Vazquez-Martin C, Cuevas E, Gil-Martin E, Fernandez-Briera A. Correlation analysis between tumor-associated antigen sialyl-Tn expression and ST6GalNAc I activity in human colon adenocarcinoma. Oncology 2004;67:159–65.

42. Zhuang D, Yousefi S, Dennis JW. Tn antigen and UDP-Gal:GalNAc α-R β 1–3Galactosyltransferase expression in human breast carcinoma. Cancer Biochem Biophys 1991;12:185–98.

43. Schietinger A, Philip M, Yoshida BA, et al. A mutant chaperone converts a wild-type protein into a tumor-specific antigen. Science 2006;314:304–8.

44. Higashi N, Fujioka K, Denda-Nagai K, et al. The macrophage C-type lectin specific for galactose/N-acetylgalactosamine is an endocytic receptor expressed on monocyte-derived immature dendritic cells. J Biol Chem 2002;277:20686–93.

45. Saeland E, van Vliet SJ, Backstrom M, et al. The C-type lectin MGL expressed by dendritic cells detects glycan changes on MUC1 in colon carcinoma. Cancer Immunol Immunother 2007;56:1225–36.

46. Itzkowitz SH, Yuan M, Montgomery CK, et al. Expression of Tn, sialosyl-Tn, and T antigens in human colon cancer. Cancer Res 1989;49:197–204.

47. Ogata S, Maimonis PJ, Itzkowitz SH. Mucins bearing the cancer-associated sialosyl-Tn antigen mediate inhibition of natural killer cell cytotoxicity. Cancer Res 1992;52:4741–6.

48. Byrd JC, Bresalier RS. Mucins and mucin binding proteins in colorectal cancer. Cancer Metastasis Rev 2004;23:77–99.

49. Paszkiewicz-Gadek A, Porowska H, Lemancewicz D, Wolczynski S, Gindzienski A. The influence of N- and O-glycosylation inhibitors on the glycosylation profile of cellular membrane proteins and adhesive properties of carcinoma cell lines. Int J Mol Med 2006;17:669–74.

50. An G, Wei B, Xia B, et al. Increased susceptibility to colitis and colorectal tumors in mice lacking core 3-derived O-glycans. J Exp Med 2007;204:1417–29.