

Hypersialylation of β_1 Integrins, Observed in Colon Adenocarcinoma, May Contribute to Cancer Progression by Up-regulating Cell Motility

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Abstract

Colon adenocarcinomas are known to express elevated levels of $\alpha 2$ -6 sialylation and increased activity of ST6Gal-I, the Golgi glycosyltransferase that creates $\alpha 2$ -6 linkages. Elevated ST6Gal-I positively correlates with metastasis and poor survival, and therefore ST6Gal-I-mediated hypersialylation likely plays a role in colorectal tumor invasion. Previously we found that oncogenic *ras* (present in roughly 50% of colon adenocarcinomas) up-regulates ST6Gal-I and, in turn, increases sialylation of β_1 integrin adhesion receptors in colon epithelial cells. However, we wanted to know if this pattern held true *in vivo* and, if so, how β_1 hypersialylation might contribute to colon tumor progression. In the present study, we find that β_1 integrins from colon adenocarcinomas consistently carry higher levels of $\alpha 2$ -6 sialic acid. To explore the effects of increased $\alpha 2$ -6 sialylation on β_1 -integrin function, we stably expressed ST6Gal-I in a colon epithelial cell line lacking endogenous ST6Gal-I. ST6Gal-I expressors (with $\alpha 2$ -6 sialylated β_1 integrins) exhibited up-regulated attachment to collagen I and laminin and increased haptotactic migration toward collagen I, relative to parental cells (with completely unsialylated β_1 integrins). Blockade of ST6Gal-I expression with short interfering RNA reversed collagen binding back to the level of ST6Gal-I nonexpressors, confirming that $\alpha 2$ -6 sialylation regulates β_1 integrin function. Finally, we show that β_1 integrins from ST6Gal-I expressors have increased association with talin, a marker for integrin activation. Collectively, these findings suggest that β_1 hypersialylation may augment colon tumor progression by altering cell preference for certain extracellular matrix milieus, as well as by stimulating cell migration. (Cancer Res 2005; 65(11): 4645-52)

Introduction

Cell surface proteins are typically elaborated with a complex array of asparagine-linked (*N*-linked) glycans, which, given their localization to the extracellular protein domain, are well positioned to modulate numerous cell/cell and cell/matrix interactions. For example, altered *N*-linked glycosylation has been shown to control diverse processes such as B-cell regulation, leukocyte rolling and adhesion to vascular endothelium, and embryonic development (1). Not surprisingly, altered *N*-linked glycosylation is also thought to

play a role in tumor invasion and metastasis, processes involving profound derangements in the interaction of cancerous cells with their extracellular matrix environment.

Supporting the idea that variant *N*-glycosylation has functional relevance to cancer, there are consistent trends in the types of sugar modifications that appear on tumor cells, relative to normal epithelial cells. For example, sialyl Lewis A and sialyl Lewis X structures, which are commonly overexpressed in tumors, are strongly correlated with metastasis and a poor prognosis (2). Because these sialylated/fucosylated Lewis structures can help form tumor ligands for selectins, they may facilitate tumor cell dissemination by promoting tumor-endothelial cell interactions (2). Other well-documented changes in glycan composition include increased $\beta 1$ -6 branching of polylactosamine chain structures (mediated by the GnT-V glycosyltransferase) and alterations in the abundance and/or linkage pattern of sialic acid, a negatively charged sugar that caps the terminal galactose residue of polylactosamine chains. Numerous studies have shown that human tumors display elevated levels of GnT-V as well as the $\alpha 2$ -6 sialyltransferase, ST6Gal-I (reviewed in ref. 3). Furthermore, *in vitro* cell culture studies indicate that GnT-V and ST6Gal-I are up-regulated by oncogenes such as *ras* (3) and that increased enzyme expression is highly correlated with altered cell adhesion and motility on selected extracellular matrix ligands. ST6Gal-I is not the only enzyme capable of creating $\alpha 2$ -6 linkages; however, it specifically targets terminal Gal $\beta 1$ -4GlcNAc structures (*N*-acetyl-lactosamine), as opposed to ST6GalNAc-I, the enzyme that generates the sialyl Tn antigen on O-linked glycans (4). In addition to ST6Gal-I and ST6GalNAc-I, another $\alpha 2$ -6-specific sialyltransferase has been identified, ST6Gal-II (5). This enzyme seems to prefer oligosaccharides to glycoproteins as a substrate, and unlike ST6Gal-I, ST6Gal-II has not been detected in human tumors (5).

It has long been known that the *N*-glycans of the β_1 subunit of the integrin family of cell adhesion receptors have a different carbohydrate composition after cell transformation (3, 6), although the specific changes in glycan structure have not been well defined nor have the physiologic consequences of such changes been established. Accumulating evidence suggests that the β_1 integrin is a substrate for GnT-V (3, 6). Overexpression of GnT-V in several different cell types causes β_1 integrins to acquire increased levels of $\beta 1$ -6 branched polylactosamine structures, and, importantly, the acquisition of these structures is associated with a profound alteration in the activity of β_1 -containing integrin heterodimers. For example, forced expression of GnT-V in both human hepatocellular carcinoma cells (7) and fibrosarcoma cells (8) leads to reduced cell adhesion and spreading on the $\alpha_5\beta_1$ ligand, fibronectin, but stimulates increased $\alpha_5\beta_1$ -mediated invasion through Matrigel. Interestingly, glycosylation of the $\alpha 5$ integrin

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subunit does not seem to be modified in cells with overexpressed GnT-V (8, 9), suggesting that certain glycosyltransferases may target a selected number of integrin subunits.

Recent studies from our laboratory suggest that, as with GnT-V, β_1 integrins are a substrate for ST6Gal-I (10, 11). Previously, we showed that stable expression of oncogenic *ras* in colon epithelial cells induced up-regulated ST6Gal-I expression and, in turn, led to dramatically increased α 2-6 sialylation of β_1 integrins (10). In light of these results, we hypothesized that β_1 integrins would likely be a functional target for elevated α 2-6 sialylation *in vivo*. In the present study, we find that β_1 integrins from colon adenocarcinomas carry increased α 2-6 sialylation relative to integrins from pair-matched normal epithelial tissues. To determine how ST6Gal-I-mediated β_1 hypersialylation might affect the adhesive and migratory capacity of tumor cells, we expressed ST6Gal-I in SW48 cells, a colon epithelial cell line that lacks endogenous sialyltransferase activity (12). ST6Gal-I expressors show enhanced attachment to the β_1 ligands, collagen I and laminin, elevated haptotactic migration toward collagen I, and increased association with talin, a cytoskeletal-associated protein with known involvement in integrin activation. These results strongly suggest a functional role for ST6Gal-I-mediated sialylation of β_1 integrins in colon cancer progression.

Materials and Methods

Frozen tissues. Primary site colon adenocarcinomas and pair-matched normal colon tissues were procured from the Cooperative Human Tissue Network with prior approval from the University of Alabama Institutional Review Board. As necessary, adenocarcinoma samples were microdissected to remove inflammatory or necrotic tissues.

Western blotting. Frozen tissues (0.1-0.4 g) were homogenized using a polytron in 1 mL of 50 mmol/L Tris-HCl buffer (pH 7.4) containing 1% Triton X-100, 0.5 mmol/L phenylmethylsulfonylfluoride, 20 μ g/mL leupeptin, 4 mmol/L NaF, and 200 μ mol/L sodium pervanadate ("lysis buffer"). For SW48 cells, lysis buffer was added directly to cells adherent to tissue culture dishes. After centrifugation of cell/tissue homogenates, supernatant protein concentrations were determined using a modified Bradford Assay (Sigma, St. Louis, MO). Lysate protein was boiled in SDS-PAGE sample buffer under reducing conditions, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride. Membranes were blocked with nonfat dry milk in TBS containing 0.05% Tween 20 (TBST) and then incubated with a primary antibody against the β_1 integrin (Transduction Laboratories, Lexington, KY). For ST6Gal-I-expressing SW48 cell lines, membranes were incubated with an antibody against the V5 tag (Invitrogen, Carlsbad, CA). After washing with TBST, blots were incubated with horseradish peroxidase-coupled secondary antibody (Amersham, Piscataway, NJ), and labeled proteins were visualized by enhanced chemiluminescence. Images were scanned with a Hewlett-Packard Scanjet 5470c (Wilmington, DE), and densitometric analysis was done with the Scion Image program (Frederick, MD).

Lectin affinity assays. One milligram of either tissue homogenate or cell culture lysate was incubated for 3 hours at 4°C with 10 to 50 μ g/mL of one of the following biotinylated lectins: SNA [specific for Sia α 2-6Gal(NAC)], MAA (specific for Sia α 2-3Gal β 1-4GlcNAc), UEA (specific for terminal Fuc α 1-2Gal β 1-4GlcNAc), LTL (specific for Gal β 1-4[Fuc α 1-3]GlcNAc), or ECL (specific for terminal, uncapped Gal β 1-4GlcNAc; Vector Laboratories, Burlingame, CA). Streptavidin-agarose beads (Sigma) were then added, and samples were incubated for an additional 2 hours at 4°C with rotation. Lectin-glycoprotein complexes were collected by brief centrifugation, washed thrice with lysis buffer, and then washed once with PBS. Precipitated proteins were released from the bead complexes by boiling in SDS-PAGE sample buffer, resolved by reducing SDS-PAGE, then immunoblotted to detect β_1 integrins as described above.

Immunofluorescent double-labeling. Frozen tissue sections were fixed in 3.7% formaldehyde and blocked in 3% goat serum. Sections were incubated with biotinylated SNA followed by a streptavidin-coupled green fluorescent dye, AlexaFluor 488 (Molecular Probes, Eugene, OR). Sections were simultaneously incubated with the glycosylation-insensitive MAB2000 monoclonal antibody to β_1 (Chemicon International, Temecula, CA), followed by a secondary antibody coupled to the red fluorescent marker, AlexaFluor 594 (Molecular Probes). Nuclei were counterstained with Hoechst dye.

SW48 cell lines. Human SW48 colon epithelial cells were purchased from the American Type Culture Collection (Bethesda, MD) and grown as suggested by the supplier in Leibovitz's L-15 medium with 2 mmol/L L-glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and gentamicin. Cells were maintained at 37°C in a CO₂-free incubator and passaged two to three times per week.

Stable ST6Gal-I-expressing SW48 cells. Rat liver ST6Gal-I cDNA (generous gift from Dr. Karen Colley, University of Illinois, Chicago, IL) was stably introduced into SW48 cells using TranzVector (Tranzyme, Inc., Research Triangle Park, NC). The TranzVector system represents an HIV-based lentiviral vector with unique safety features as described (13). To generate vector stock, ST6Gal-I cDNA tagged with a V5 epitope at its COOH terminus was first cloned into the gene transfer component placing it under the control of the human cytomegalovirus (hCMV) promoter. Expression of ST6Gal-I was also coupled to that of the puromycin-N-acetyltransferase gene (*puro*) gene via the internal ribosomal entry site (IRES) of encephalomyocarditis virus. Linking expression of ST6Gal-I and *puro* (bicistronic vector), allowed for relatively rapid selection of SW48 cells expressing ST6Gal-I by growth in media containing puromycin. TranzVector stock expressing CMV-ST6Gal-I-IRES-*puro* was generated as previously described (13). As an empty vector control, TranzVector stock expressing CMV-null-IRES-*puro* was also generated. SW48 cells were transduced with either ST6Gal-I or control empty vector particles. Puromycin-resistant cells were expanded to form a pool of stable ST6Gal-I expressors, and ST6Gal-I expression was confirmed by Western blot detection of the attached V5 epitope tag.

Collagen and laminin attachment assays. SW48 cells were disengaged from culture flasks with nonenzymatic CellStripper solution (Cellgro, Herndon, VA), and then resuspended in serum-free medium. Cells were plated onto tissue culture-treated dishes precoated with either 20 μ g/mL of basement membrane laminin isolated from Engelbreth-Holm-Swarm murine sarcoma cells (Sigma) or 30 μ g/mL of bovine collagen I (Cohesion, Vancouver, BC, Canada) and blocked with 2% denatured bovine serum albumin (BSA). Cells were also plated onto dishes coated with 2% denatured BSA alone to control for nonspecific binding. After 30 minutes of incubation at 37°C, nonadherent cells were removed by PBS washing. Attachment was quantified by crystal violet staining as previously described (10).

Short interfering RNA transfection. Four synthetic short interfering RNA (siRNA) duplexes were generated against the following ST6Gal-I (SIAT1) gene sequences: Duplex 1, 5'-ACTCAGATATCCCAAAGTG-3'; Duplex 2, 5'-CATCCAAGCGCAAGACTGA-3'; Duplex 3, 5'-AGAAGAATTT-GGTGAAGCA-3'; and Duplex 4, 5'-GGACATCTACCTGCTTGA-3'. The four duplexes, as well as *siCONTROL* nontargeting siRNA, were purchased from Dharmacon (Lafayette, CO). The 21-nucleotide duplexed siRNAs were provided in desalted/deprotected form, with symmetrical 2-nucleotide 3' overhangs and a 5' phosphorylated antisense strand. Transfection of siRNA duplexes into ST6Gal-I-expressing SW48 cell lines was done with the TransIT-TKO reagent (Mirus, Madison, WI) according to the manufacturer's recommendations. One day before transfection, SW48 cells were trypsinized, resuspended in L-15 media (no antibiotics) plus 10% FBS, and then seeded at 2.5×10^5 cells per well in 24-well tissue culture-treated dishes. The following day, cells (roughly 50% confluent) were transfected either with individual siRNA duplexes at a concentration of 40 nmol/L or with transfection reagent alone. Cells were incubated at 37°C in a CO₂-free incubator for 48 hours, lysed, and then assayed for ST6Gal-I protein expression by Western blotting for the V5 tag. To verify even protein loading, blots were stripped and reblotted with an antibody against β -actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Collagen attachment assays: short interfering RNA–transfected cells. ST6Gal-I–expressing and empty vector SW48 cells were transfected as described above with either the siRNA duplex 2 (which induced the greatest ST6Gal-I down-regulation), nontargeting siRNA, or with transfection reagent alone. Cells were detached at 24 to 72 hours posttransfection and used for collagen I attachment assays as described above.

Haptotactic migration assays. Collagen I haptotaxis was evaluated using the QCM Collagen I Quantitative Cell Migration Assay kit (Chemicon International). Cells were serum-starved for 24 hours, detached with CellStripper solution, and then resuspended in serum-free medium. Cells were then seeded 2.5×10^5 cells per well into the upper wells of Boyden chambers lined with 8.0- μm polyethylene terephthalate (PET) membranes coated on the underside with a collagen I concentration gradient. The lower chambers were filled with 300 μL per well of media plus 2% FBS (as a chemoattractant). After 20 hours incubation at 37°C, cell migration was quantitated using the vendor's staining protocol. For laminin haptotaxis assays, 6.5-mm-diameter Transwell migration chamber inserts with 8.0- μm pore size PET membranes (Corning, Acton, MA) were coated on the underside with 20 $\mu\text{g}/\text{mL}$ laminin overnight at 4°C in PBS. The undersides of the chamber inserts were then overcoated with 1% denatured BSA. After a wash with PBS, laminin haptotaxis was analyzed using the conditions described above for collagen I.

β_1 Integrin/talin coimmunoprecipitation. SW48 cells were seeded onto tissue culture–treated dishes that had been precoated with 30 $\mu\text{g}/\text{mL}$ collagen and then overcoated with 2% denatured BSA. When cells reached 80% to 90% confluence, they were lysed in immunoprecipitation buffer [50 mmol/L Tris-HCl buffer (pH 7.4) containing 1% Triton X-100, 0.5% NP40, 150 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 20 $\mu\text{g}/\text{mL}$ leupeptin, 4 mmol/L NaF, and 200 $\mu\text{mol}/\text{L}$ sodium pervanadate]. Lysate proteins (2.0 mg/sample) were immunoprecipitated with glycosylation-insensitive anti- β_1 antibody MAB2000 (Chemicon International) and the Seize Primary Mammalian Immunoprecipitation kit from Pierce (Rockford, IL). After 2.5 hours of immunoprecipitation at 25°C, beads were washed and then boiled in SDS-PAGE sample buffer. Precipitated proteins were resolved by SDS-PAGE and then Western blotted with an antibody against talin (Chemicon International). Membranes were stripped and reblotted for β_1 to verify equal loading.

Results

β_1 Integrins are hypersialylated in colon tumors. Since our *in vitro* studies identified the β_1 integrin as an ST6Gal-I substrate (10, 11), we speculated that β_1 integrins were targets for ST6Gal-I–mediated hypersialylation in colon adenocarcinomas. To test this hypothesis, we used SNA reactivity as a relative measure of β_1 integrin α 2-6 sialylation in both colon adenocarcinomas and pair-matched normal tissues. Briefly, tissue homogenates were incubated with biotinylated SNA followed by streptavidin-coated beads to precipitate SNA-bound glycoproteins. Precipitated proteins were resolved by SDS-PAGE, and β_1 integrins were detected by immunoblotting. Total tissue homogenates were also Western blotted for β_1 to quantify total β_1 integrin expression in normal and tumor tissues. Relative β_1 α 2-6 sialylation was then expressed as a ratio of the densitometric value of SNA-precipitated β_1 to total β_1 in the tissue homogenate. As shown in Fig. 1, tumor β_1 integrins from all eight patients examined carried elevated α 2-6 sialylation compared with β_1 integrins from pair-matched normal colon tissues. Western blots of total β_1 and SNA-precipitated β_1 for patient 1 are shown in Fig. 1 as a representative example. On average, a 4-fold increase in β_1 integrin α 2-6 sialylation was observed in tumor samples ($P < 0.02$ by a paired Student's *t* test), although there was patient-to-patient variability in the degree of β_1 hypersialylation. In contrast to the consistently elevated levels of β_1 sialylation, we did not observe any consistent trend in β_1 expression

levels, an interesting finding given that some studies have reported down-regulated β_1 expression in colon carcinoma (14–17).

α 2-6 Sialic acids colocalize with β_1 integrins in normal and tumor tissues. Using an immunofluorescent double-labeling protocol, we evaluated localization of β_1 integrins (using a β_1 -specific antibody) and α 2-6 sialic acids (using SNA lectin) in pair-matched colon tumor and normal epithelial tissue sections (note that β_1 from normal tissues does carry some sialylation; see Fig. 1). As shown in Fig. 2A to D, both α 2-6 sialic acids and β_1 integrins were enriched in the basolateral domain of normal colon epithelium, which is the expected site for β_1 integrins. In contrast, basolateral enrichment was lost in tumor tissues (Fig. 2E–H), which is consistent with the absence of a polarized monolayer in transformed epithelium. Importantly, α 2-6 sialic acids and β_1 integrins clearly colocalize in both normal and tumor tissues. Of note, it has been reported that in addition to ST6Gal-I–mediated sialylation, SNA can recognize the O-linked sialyl-Tn antigen (18). Because β_1 integrins potentially express this epitope (19), we cannot currently exclude the possibility that sialyl-Tn moieties might have contributed to the SNA staining in our study. However, in a more recent investigation, it was concluded that the sialyl-Tn antigen did not contribute significantly to SNA labeling of either

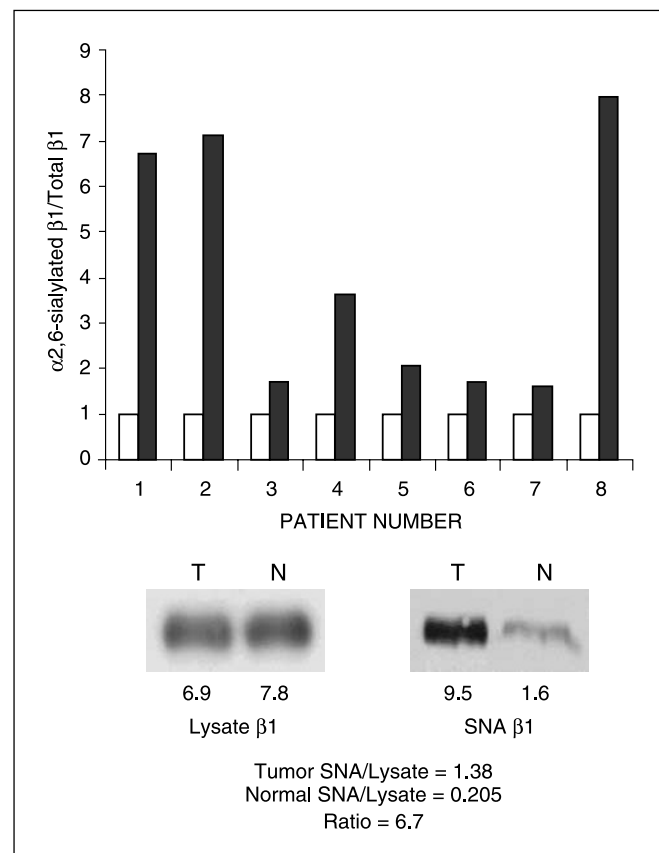


Figure 1. α 2-6 Sialylation of β_1 integrins in pair-matched normal (\square) and adenocarcinoma (\blacksquare) colon tissues. Tissue homogenates (N, normal; T, tumor) from eight patients were precipitated with SNA (a lectin specific for α 2-6–linked sialic acids), resolved by SDS-PAGE, and then immunoblotted for β_1 . Homogenates were also Western blotted for total β_1 integrin expression. Densitometry was used to quantitate both SNA-precipitated β_1 and total β_1 levels, and the relative level of β_1 α 2-6 sialylation was defined as a ratio of SNA-precipitated β_1 over total β_1 . The value for normal tissues was normalized to one. Immunoblots of total β_1 and SNA-precipitated β_1 from patient 1 were included as a representative example.

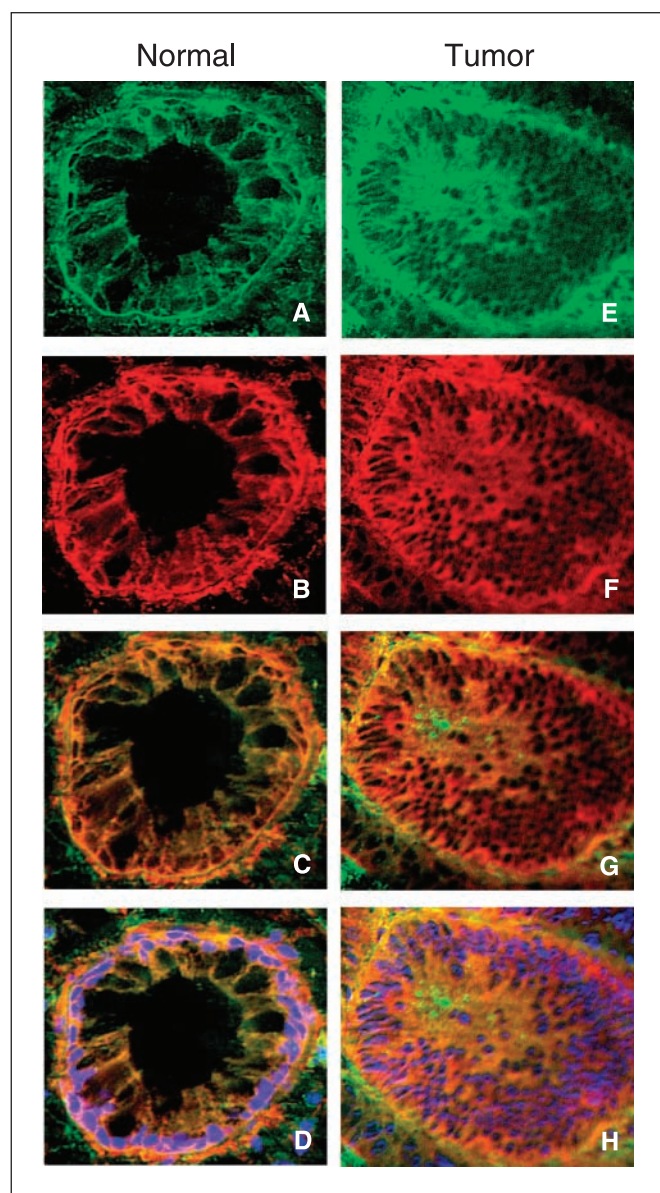


Figure 2. β_1 Integrins and $\alpha 2-6$ sialic acid colocalize in normal and adenocarcinoma colon tissues. Using an immunofluorescent double-labeling protocol, we evaluated the localization of $\alpha 2-6$ sialic acids (green) and β_1 integrins (red) in both normal and tumor tissues from the same patient. A and E, $\alpha 2-6$ sialic acids; B and F, β_1 integrins; C and G, merged image; D and H, merged image plus nuclei.

colon tumors or selected colon carcinoma cell lines (20). There are two other lectins, TJA and PSL, that seem to be more selective for $\alpha 2-6$ Gal $\beta 1-4$ GlcNAc structures (21, 22); however, to our knowledge, these are not commercially available.

Forced expression of ST6Gal-I in a colon epithelial cell line increases β_1 integrin sialylation. To explore the mechanistic role of β_1 hypersialylation in colon cancer progression, we generated stable ST6Gal-I expression in human SW48 colon epithelial cells. The SW48 cell line reportedly lacks any detectable $\alpha 2-6$ or $\alpha 2-3$ sialyltransferase activity against *N*-acetylglucosamine structures (12). SW48 cells were transduced with a lentiviral vector encoding a full-length ST6Gal-I cDNA fused to a 5-amino acid V5 tag, or with the lentiviral vector alone as a control. Expression of the ST6Gal-I

construct was verified by Western blotting for the V5 tag. As shown in Fig. 3A, cells carrying the ST6Gal-I construct (ST6) expressed the V5 tag, whereas no V5 signal was detected in parental (P) or empty vector-transduced cells (EV).

To determine if β_1 integrins were targets for ST6Gal-I-mediated $\alpha 2-6$ sialylation in these cells, lysates were Western blotted for β_1 . As shown in Fig. 3B, two bands were present in the β_1 integrin blot from total cell lysates. The top band ("mature form") represents the fully glycosylated, functional receptor, whereas the bottom band represents a partially glycosylated precursor form that resides in the endoplasmic reticulum. Electrophoretic mobility of mature, but not precursor, β_1 integrins from ST6 cells was decreased relative to β_1 integrins from both P and EV cells, suggesting an increased molecular mass. Increased mass of the Golgi-processed mature form, but not the ER-resident precursor form, is consistent with the addition of $\alpha 2-6$ sialic acids by ST6Gal-I. Interestingly, we observed only minimal, and sometimes undetectable, levels of precursor β_1 isoforms in intact tissues (Fig. 1).

To confirm that $\alpha 2-6$ sialylation was responsible for the increased mass of ST6 cell β_1 integrins, we precipitated $\alpha 2-6$

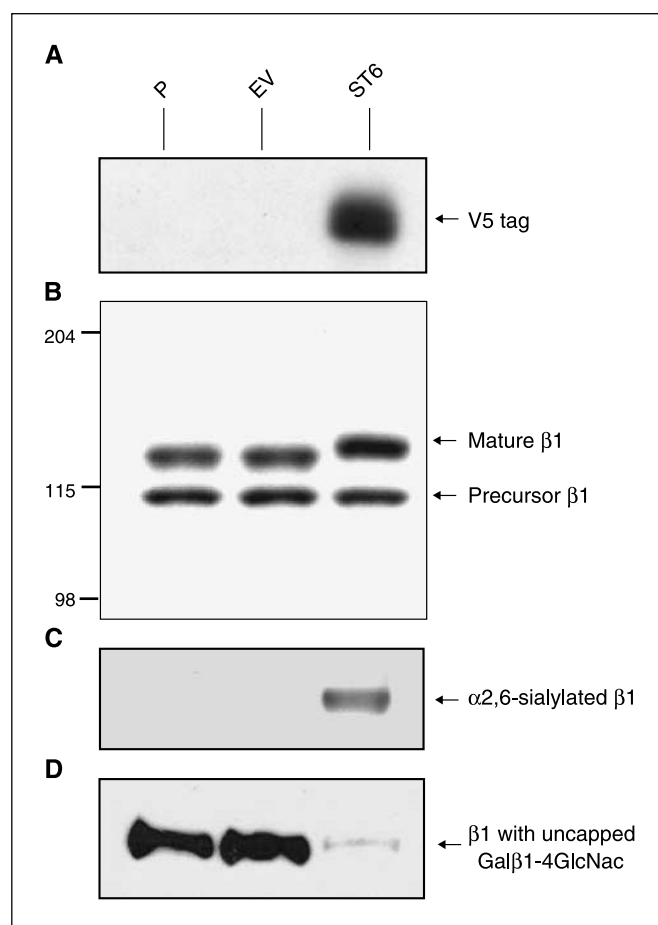


Figure 3. $\alpha 2-6$ Sialylation of β_1 integrins in ST6Gal-I-expressing SW48 cells. SW48 colon epithelial cells were transduced with a lentiviral vector encoding a full-length rat liver ST6Gal-I cDNA fused to a V5 tag to facilitate detection by immunoblotting (labeled ST6). Both nontransduced parental cells (P) and cells transduced with the empty lentiviral vector (EV) were used as controls. A, lysates were immunoblotted for the V5 tag to verify expression of ST6Gal-I. B, lysates were immunoblotted for the β_1 integrin. Top band, mature, Golgi-processed form of β_1 . Bottom band, the precursor, ER-resident form of β_1 . C, lysates were SNA-precipitated and immunoblotted for β_1 . D, lysates were ECL-precipitated and immunoblotted for β_1 .

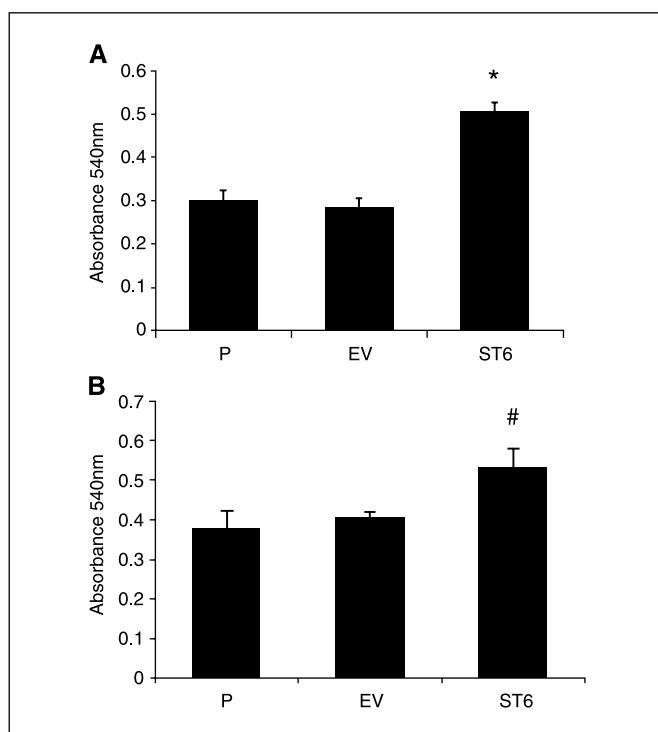


Figure 4. Collagen and laminin attachment of ST6Gal-I-expressing cell lines. SW48 cells (P, EV, and ST6) were detached from tissue culture flasks using a nonenzymatic chelating solution. Cells were plated onto tissue culture dishes coated with either (A) collagen I or (B) laminin, which had been overcoated with denatured BSA, or onto dishes coated with denatured BSA alone (to control for nonspecific binding). Cells were allowed to adhere for 30 minutes at 37°C, and then stained with crystal violet as previously described (10). Where indicated, ST6 binding was significantly greater than P or EV binding. *, $P = 0.015$; #, $P = 0.0135$, evaluated by a paired Student's *t* test. Columns, mean of four independent experiments done in duplicate; bars, SD.

sialylated glycoproteins from cell lysates using SNA, and then Western blotted for β_1 integrins as before. As shown in Fig. 3C, β_1 integrins from ST6, but not P or EV, cells were precipitated by SNA. We then did the same type of assay using ECL, a lectin specific for uncapped terminal Gal β 1-4GlcNAc residues (Fig. 3D). We suspected that *N*-glycans on the parental cells would have uncapped galactoses, given that SW48 cells reportedly have no endogenous sialyltransferase activity (12). As shown in Fig. 3D, ECL labeling, which was very strong in P and EV cells, was nearly lost in ST6 cells, suggesting that β_1 integrin *N*-glycans in these cells switch primarily from galactose-terminated to α 2-6 sialic acid-terminated structures. To further verify that *N*-glycans were uncapped in parental cells, we incubated lysates with MAA (specific for Sia α 2-3Gal β 1-4GlcNAc), UEA (specific for Fuc α 1-2Gal β 1-4GlcNAc), and LTL (specific for Gal β 1-4[Fuc α 1-3]GlcNAc) and found that there was no detectable amount of β_1 in any of these lectin precipitates (not shown).

Cells expressing ST6Gal-I show increased attachment to collagen I and laminin. Having shown that stable expression of ST6Gal-I induces α 2-6 sialylation of β_1 integrins, we proceeded to evaluate the role of α 2-6 sialylation in integrin-mediated attachment to β_1 ligands such as collagen I and laminin. As shown in Fig. 4, ST6 cells exhibited significantly enhanced attachment to collagen I (Fig. 4A) and laminin (Fig. 4B) relative to P and EV cells, although the greatest increase was in collagen binding.

To confirm that ST6Gal-I caused the up-regulated collagen I binding in ST6 cells, we used siRNAs to inhibit ST6Gal-I expression in these cells. To find a siRNA duplex that effectively down-regulates ST6Gal-I, ST6 cells were initially transfected with several candidate siRNA duplexes targeted against ST6Gal-I mRNA, a nontargeting siRNA, or transfection reagent alone. At 48 hours posttransfection, ST6Gal-I expression was measured by Western blotting for the V5 tag. As shown in Fig. 5A, ST6Gal-I protein expression was markedly reduced in cells transfected with siRNA duplex 2 (D2), whereas nontargeting siRNA or transfection reagent alone had no effect. ST6 and EV cells were subsequently transfected with D2, nontargeting siRNA, or transfection reagent alone. After 24 to 72 hours of transfection, collagen I attachment was measured, and lysates were immunoblotted for V5. As shown in Fig. 5B, D2 siRNA transfection reduced collagen binding in ST6 cells back to the level of EV cells by 48 to 72 hours posttransfection. This reversal was temporally correlated to D2 siRNA-mediated blockade of ST6Gal-I expression at 48 to 72 hours, demonstrating that ST6Gal-I was responsible for the elevated collagen binding observed in ST6 cells.

ST6Gal-I expression increases haptotactic migration on collagen I but not on laminin. To determine if α 2-6 sialylation of β_1 might lead to acquisition of a more migratory and/or metastatic cell phenotype, we evaluated cell migration on collagen I and laminin. Using migration chamber assays, we measured the migratory capacity of our SW48 cell lines on either a collagen I or laminin concentration gradient (haptotaxis) using 2% FBS as a chemoattractant. As shown in Fig. 6A, ST6 cells were more migratory on a collagen I concentration gradient than either P or EV cells. In contrast, laminin did not support cell migration in any of the cell lines tested (not shown).

β_1 Integrins from ST6Gal-I expressors show increased association with talin. The interaction of talin with the β_1 cytoplasmic tail is a well-accepted marker for integrin activation (23). Given that ST6Gal-I expressors exhibited up-regulated binding and migration on collagen I, we anticipated that more talin would be associated with β_1 integrins from these cells. To test this hypothesis, we immunoprecipitated β_1 integrins from cells seeded onto collagen I, and then Western blotted the immunoprecipitates for coprecipitated talin. As shown in Fig. 6B, substantially more talin coimmunoprecipitated with β_1 integrins from ST6 cells, as compared with EV cells. Blots were stripped and reblotted for the β_1 integrin to confirm that equivalent amounts of β_1 were precipitated (not shown).

Discussion

Neoplastic acquisition of terminal N-linked carbohydrate modifications is consistently linked with tumor progression in a number of different cancers (2). Intriguingly, an inhibitor of terminal glycosylation, swainsonine, has shown promise as an anticancer agent in two phase I clinical trials (24), and blocking expression of cell surface sialyl Lewis-type glycans inhibits the metastatic potential of colon tumor cell lines (25). Although these findings suggest that cancer-induced alterations in glycosylation contribute to tumor progression, neither the signaling cascades that direct differential glycosylation nor the cell surface glycoproteins targeted by Golgi glycosyltransferases have been well characterized.

Elevated levels of ST6Gal-I and α 2-6 sialic acid have been observed in several types of tumors, including colon adenocarcinomas (reviewed in refs. 3, 26). ST6Gal-I mRNA and enzyme activity are particularly high in metastasizing tumors (27, 28), and

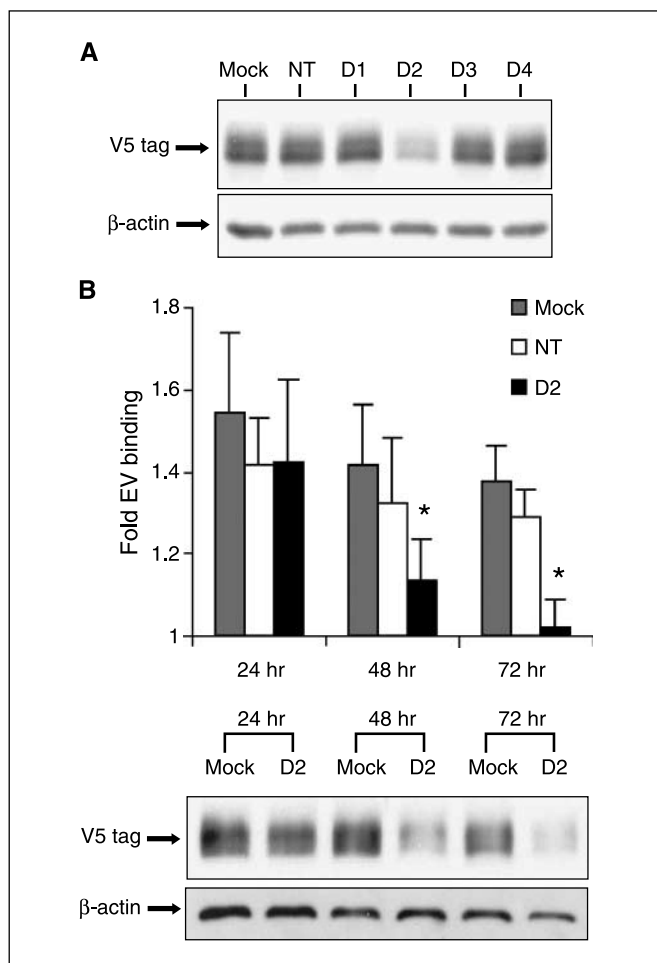


Figure 5. Collagen attachment of siRNA-transfected ST6Gal-I-expressing cell lines. *A*, ST6Gal-I expressors were transfected 48 hours with one of four siRNA duplexes designed to target ST6Gal-I (*D1-D4*), with a nontargeting siRNA (*NT*) to control for nonspecific effects of siRNA transfection, or with transfection reagent alone (*Mock*). Cell lysates were then Western blotted with antibodies against the V5-tagged ST6Gal-I or β -actin. *B*, ST6Gal-I expressors were treated with siRNA duplex *D2* (which was shown to effectively down-regulate ST6Gal-I), nontargeting siRNA, or transfection reagent alone. Empty vector cells were transfected with nontargeting siRNA as a control. At 24 to 72 hours posttransfection, cells were assayed for collagen I attachment as previously described. The attachment values for ST6Gal-I expressors transfected with siRNA duplex *D2* (*D2*), nontargeting siRNA (*NT*), or transfection reagent alone (*Mock*) were graphed as fold increases over the binding of NT-siRNA-transfected empty vector cells. *, binding of *D2* cells was significantly less than binding of *Mock* and *NT* cells ($P \leq 0.04$, evaluated by a paired Student's *t* test). *Columns*, mean of four independent experiments done in duplicate; *bars*, SE. To confirm siRNA-mediated down-regulation of ST6Gal-I, cell lysates were Western blotted for both the V5-tagged ST6Gal-I and β -actin.

elevated ST6Gal-I expression correlates with a poor prognosis in patients with colorectal and breast cancer (29, 30). *In vitro* cell culture studies suggest that ST6Gal-I up-regulation may contribute to metastasis by regulating invasiveness and/or cell motility (31, 32). For example, transfection of ST6Gal-I increases invasiveness of mammary carcinoma cells (33), and down-regulation of ST6Gal-I with antisense RNA blocks Matrigel invasion of HT29 colon epithelial cells (32). Although these findings implicate ST6Gal-I in tumor progression, the cell surface proteins modified by ST6Gal-I in these cancers have, up until this time, not been identified. Our present work establishes the β_1 integrin adhesion receptor as a target for up-regulated α 2-6 sialylation in colon adenocarcinomas.

Furthermore, we show that ST6Gal-I-mediated hypersialylation of β_1 in colon epithelial cells stimulates both cell attachment and migration on collagen I, behaviors that may contribute to the more migratory/invasive phenotype of colon tumor cells. Recently, Amano et al. found that ST6Gal-I-mediated sialylation of CD45 (a T-cell galectin-1 receptor) negatively regulates galectin-1-induced clustering and T-cell death (34), providing a similar example where ST6Gal-I mediates a phenotypic effect through a specific cell surface protein target.

Several investigators (including our group) have shown that ST6Gal-I expression is up-regulated in response to oncogenic ras (10, 35, 36). Because only 50% of colon tumors are reported to carry oncogenic *ras* mutations (37), it was somewhat surprising that all of the colon tumors we tested expressed hypersialylated β_1 integrins (i.e., it is statistically unlikely that all of these tumors carry oncogenic *ras*). We speculate that the tumor samples not carrying oncogenic *ras* likely harbor other activating mutations in the *ras* signaling pathway. Indeed, studies have shown that a substantial subset of colon tumors carry activated B-RAF (a downstream *ras* effector; refs. 38, 39) and increased activity of growth factor receptors upstream of *ras*, such as transforming growth factor- β and epidermal growth factor receptor (40, 41). Recently, it has been reported that oncogenic H-*ras* induces ST6Gal-I expression in murine fibroblasts via activation of the RalGEF effector pathway (42), but the role of this pathway in human colon adenocarcinoma

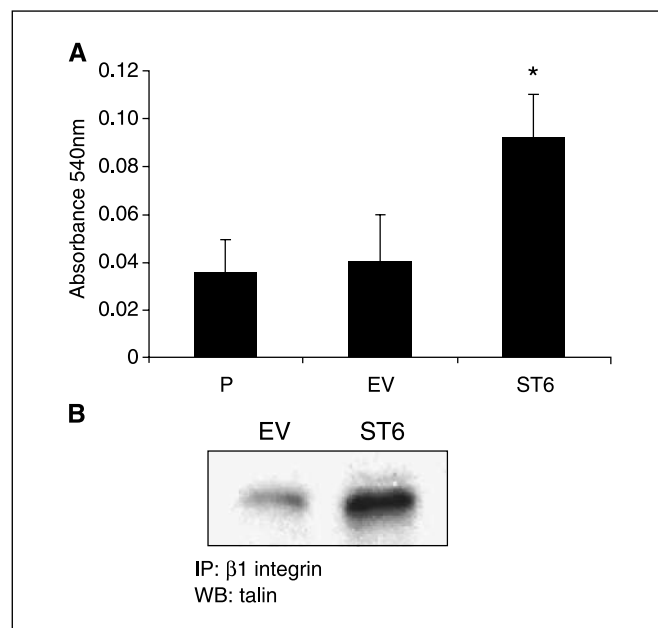


Figure 6. *A*, haptotactic collagen migration of ST6Gal-I-expressing cell lines. SW48 cell lines (*P*, *EV*, and *ST6*), which had been serum-starved for 24 hours, were detached from tissue culture flasks using a nonenzymatic chelating solution. Cells were resuspended in serum-free medium and seeded into the upper wells of Boyden chambers lined with 8.0- μ m PET membranes coated on the underside with a collagen I concentration gradient. The lower chambers contained medium plus 2% FBS as a chemoattractant. After 20 hours, migration along a collagen I concentration gradient (haptotaxis) was measured using the vendor's protocol. *, haptotactic migration of *ST6* cells was significantly greater than *P* or *EV* cell migration ($P \leq 0.011$, evaluated by a Student's paired *t* test). *Columns*, mean of five independent experiments done in duplicate; *bars*, SE. *B*, empty vector (*EV*) and ST6Gal-I-expressing (*ST6*) SW48 cells grown on the β_1 integrin ligand collagen I were lysed and immunoprecipitated using an antibody against β_1 . Immunoprecipitates were resolved by SDS-PAGE and Western blotted for talin. To confirm equal immunoprecipitation of β_1 from *EV* and *ST6* cell lines, talin blots were stripped and reblotted for β_1 (not shown). *IP*, immunoprecipitation; *WB*, Western blotting.

has not been elucidated. Alternately, ST6Gal-I may be up-regulated by unidentified *ras*-independent signaling mechanisms. For instance, Li et al. found that treatment of colon cancer cells by the secondary bile acid deoxycholate down-regulated ST6Gal-I in a Ca^{2+} -dependent manner (43). As colon adenocarcinomas grow, the authors speculate, their reduced exposure to fecal bile salts, which normally repress ST6Gal-I expression, might favor invasion or metastasis through increased $\alpha 2$ -6 sialylation.

The β_1 integrin heterodimerizes with one of 12 possible α subunits and mediates adhesion, spreading, and migration on multiple ligands including collagen, laminin, and fibronectin (44, 45). Accordingly, this integrin is ideally suited to influence tumor cell behavior in diverse extracellular matrix milieus. As evidence of the central role of β_1 in the colon adenocarcinoma phenotype, blocking antibodies against β_1 integrins were shown to reduce metastasis of human colon carcinoma cells in an *in vivo* nude mouse model (46). The elevated $\alpha 2$ -6 sialylation of β_1 we have observed in colon adenocarcinoma tissues likely alters interactions of colon tumor cells with their local matrix environment. As verification of the role of sialylation in β_1 function, we found that forced ST6Gal-I expression in SW48 cells led to increased β_1 -mediated attachment and migration on collagen I and increased coupling of the β_1 subunit to the cytoskeletal-associated protein, talin. Additional studies will be needed to fully elucidate the mechanism by which altered sialylation regulates β_1 function; however, other data from our laboratory indicate that sialylation directly affects the ligand-binding activity of collagen receptors. Using purified $\alpha_1\beta_1$ collagen-specific integrins, we previously showed that enzymatic desialylation inhibited the binding of these receptors to collagen I (10).

Integrin activity is known to be conformationally regulated (47), and recent protein modeling work shows that carbohydrate structures occupying N-linked glycosylation sites are often found in low-accessibility regions of glycoproteins or folded into surface grooves, suggesting they can intimately regulate conformation and ligand-binding site access (48). Given the terminal location and negative charge of sialic acids, they are well suited for this role. Using site-directed mutagenesis to introduce a consensus sequence for N-linked glycosylation into both β_1 and β_3 integrins, Luo et al. have shown that these artificial "glycan wedges" alter both the conformation and ligand-binding activity of the integrin heterodimers that carry them (49). Although not addressing the role of

terminal sialylation directly, these studies do provide "proof of principle" that N-linked carbohydrate structures can dramatically regulate the conformation, and hence, activity level, of the integrins that carry them. Although the specific sites of N-glycosylation have not yet been mapped, there are three sites that carry the appropriate consensus sequence (NxS/T) within a region of the β_1 integrin known as the "I-like" domain (50). The β_1 I-like domain is hypothesized to allosterically regulate ligand binding when β_1 is paired with I-domain-containing α subunits such as $\alpha 1$ and $\alpha 2$, or to bind ligands directly when the β_1 is paired to α subunits without I-domains, such as $\alpha 3$ and $\alpha 5$ (51).

In addition to directly modulating β_1 integrin-ligand interactions, ST6Gal-I-mediated sialylation could influence other, more indirect mechanisms of integrin activation. For example, up-regulated $\alpha 2$ -6 sialylation might alter the lateral association of β_1 -containing integrins with other membrane-associated proteins, such as tetraspanins (52, 53) or the urokinase-type plasminogen activation receptor (54), to coordinately regulate integrin-dependent processes. In particular, the interaction of $\alpha_3\beta_1$ and $\alpha_5\beta_1$ heterodimers with tetraspanin CD82 seems to be dependent on the glycosylation state of both the respective integrin and CD82 (55).

Regardless of the mechanisms by which hypersialylation might alter the β_1 -mediated behavior of colon tumor cells, phenotypic changes due to altered glycosylation are likely to be long-lasting. In contrast to rapid and transient forms of integrin affinity/avidity regulation, altered glycosylation patterns are present from integrin maturation and cell surface localization all the way until turnover and degradation. As such, the ST6Gal-I-mediated hypersialylation of integrins by colon cancer cells is a mechanism well suited for effecting the long-term changes in β_1 -mediated cell adhesiveness and motility so characteristic of neoplastic cells.

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