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Sialyltransferase-Based Chemoenzymatic Histology for the Detection of *N*- and *O*-Glycans

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Abstract

Profiling specific glycans in histopathological samples is hampered by the lack of selective and sensitive tools for their detection. Here, we report on the development of chemoenzymatic histology of membrane polysaccharide (CHoMP)-based methods for the detection of *O*- and *N*-linked glycans on tissue sections via the use of sialyltransferases ST3Gal1 and ST6Gal1, respectively. Combining these two methods, we developed tandem labeling and double labeling strategies that permit the detection of unsialylated and sialylated glycans or the detection of *O*- and *N*-linked glycans on the same tissue section, respectively. We applied these methods to screen murine tissue specimens, human multiple-organ cancer arrays, and lymphoma and prostate cancer arrays. Using tandem labeling with ST6Gal1 to analyze *N*-glycans in a prostate cancer array, we found striking differences in expression patterns of both sialylated and unsialylated *N*-glycans between cancerous and healthy samples. Such differences were also observed between normal tissue from healthy donors and healthy tissue adjacent to tumors. Our double labeling technique identified significant differences in unsialylated *O*-glycans between B-cell and T-cell lymphomas and between B-cell lymphomas and normal adjacent lymph nodes. Remarkable differences were also detected between adjacent lymph nodes and spleen tissue samples. These new chemoenzymatic histology methods therefore provide valuable tools for the analysis of glycans in clinically relevant tissue samples.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

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Chemoenzymatic Histology

INTRODUCTION

It has been known for decades that the structures of cell-surface glycans change with the onset of cancer.^{1–3} The most widely occurring cancer-associated changes in protein glycosylation are upregulated sialylation and branched glycan structures as well as overexpression of “core” fucosylation.⁴ In addition, aberrant expression of truncated *O*-glycans is a hallmark of virtually all epithelial cancers.⁵ Because of this well-documented evidence, glycans are attractive candidates to improve early cancer diagnosis, determine prognosis and risk stratification, and serve as markers of specific therapeutic targets.

Traditionally, glycan detection in histological samples relies on antibodies or glycan-binding proteins known as lectins.⁶ Although there are a few highly specific antibodies against distinct glycan epitopes, it is difficult to generate high-affinity IgG antibodies for glycans in general because glycans by themselves are weakly immunogenic and can hardly activate helper T lymphocytes to produce strong immune responses. Likewise, lectins generally have low affinities for their glycan targets and often lack stringent specificity.^{7,8}

Recently, we developed a highly sensitive and specific histological technique capable of probing glycans in tissue sections. This technique, termed chemoenzymatic histology of membrane polysaccharides (CHoMP),⁹ is based on chemo-enzymatic glycan labeling, a method in which a glycosyltransferase is used to transfer a monosaccharide equipped with a reactive handle that can be further derivatized to incorporate a detection probe. This method takes advantage of the inherent substrate specificity of glycosyltransferases for their acceptors as well as their tolerance for unnatural modifications on the donor substrates.

In our previous work, we demonstrated the utility of the α -1,3-fucosyltransferase-based CHoMP for the analysis of LacNAc expression patterns on select cell types in tissue samples when combined with antibody-based staining (immunohistochemistry; IHC) of specific cell markers. Using this method to screen a lung adenocarcinoma microarray, we observed a 13-fold decrease in LacNAc expression from normal lung tissues to grade one adenocarcinoma specimens.⁹ This observation has recently been validated by Miramoto, Lebrilla, and co-workers in MS-based glycomics analysis.¹⁰

Here, we report on the expansion of CHoMP for the detection of *O*- and *N*-linked glycans leveraging sialyltransferase ST3Gal1- and ST6Gal1-mediated chemoenzymatic glycan labeling (Figure 1), respectively, and showcase the application of these methods to the

detection and analysis of glycosylation patterns in murine tissue specimens and human clinical samples.

RESULTS AND DISCUSSION

ST3Gal1 for O-Glycan Detection

The recombinant human sialyltransferase ST3Gal1 is expressed as a secretory protein fused to GFP, which can be cleaved before use. As demonstrated by Steet, Boons, and co-workers, this enzyme has been employed in the chemoenzymatic labeling of *O*-linked glycans in cell lines.¹¹ ST3Gal1 transfers sialic acid or its C-5 modified analogues to Gal β 1-3GalNAc α Ser/Thr (TF antigen/core 1) and GlcNAc β 1-6(Gal β 1-3)GalNAc α Ser/Thr (core 2) in mucin-type *O*-linked glycans.¹²⁻¹⁴ In healthy tissues, core 1 and 2 structures are often elongated or capped by sialic acid. However, the TF antigen is known to be exposed in a variety of carcinomas.^{5,15} The workflow of the ST3Gal1-based CHoMP for the detection of *O*-glycans is illustrated in Figure 2a. The procedure starts with the enzymatic transfer of *N*-(4-pentynoyl)neuraminic acid (SiaNAI) from the donor CMP-SiaNAI by ST3Gal1 onto the free Gal in core 1 and 2 *O*-glycan acceptors. The alkyne is then reacted with a chelating azide-biotin probe through the 3-[4-({bis[(1-*tert*-butyl]-1*H*-1,2,3-triazol-4-yl)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]-propanol (BTTP)-assisted, copper-catalyzed azide-alkyne cycloaddition (CuAAC). The use of this “accelerated” azide and the BTTP ligand have been shown to improve reaction efficiency by ~38-fold in cell labeling experiments,^{16,17} and these reagents proved equally effective in histological applications.⁹ Biotin tags attached to *O*-glycans are then probed with Neutravidin linked to horseradish peroxidase (HRP). Subsequently, HRP-mediated coupling of a dye-labeled tyramide is used for signal amplification (TSA). As described in the first report of CHoMP, tissues are treated with the conventional histology blocking reagents, including avidin/biotin block, H₂O₂, and 1% FCS in TBST to minimize the interference induced by endogenous biotin, peroxidases, and nonspecific binding, respectively.

In healthy tissues, core 1 and 2 epitopes are often elongated or capped by sialic acid.¹⁸ Pretreatment of these samples with neuraminidase, an enzyme that catalyzes the hydrolysis of α 2-3, α 2-6, and α 2-8 linked sialic acids from glycoconjugates, exposed previously sialylated epitopes to increase the sample pool of *O*-glycans that could be detected by the ST3Gal1-based chemoenzymatic labeling.¹¹ As expected, normal mouse tissue specimens without neuraminidase treatment showed very little staining. After pretreating the samples with neuraminidase, detectable signals increased dramatically (Figure 2b). Consistent with our previous reports, the combination of an alkyne-functionalized donor coupled to the “accelerated” biotin produced higher labeling efficiency compared to what was achieved by using an azide-bearing donor and an alkyne-biotin probe (Figure S1a).⁹ Likewise, the use of a fluorescently labeled avidin did not produce signals above background, and detectable signals were only observed when TSA was used (Figure S1b). Titrations for the enzyme (Figure S1c) and donor concentrations (Figure S1d) revealed that 80 μ g/mL of ST3Gal1 and 500 μ M CMP-SiaNAI were the optimal reagent concentrations for tissue labeling. Recently, Boons and coworkers reported that the one-step labeling of glycans is more efficient than the two-step process if a glycosyltransferase can accept the biotin-conjugated nucleotide sugar

as the substrate.¹⁹ We found that ST3Gal1 was able to transfer a C-5 biotinylated sialic acid from CMP-Sia-Biotin; however, the background signal produced was much higher than that afforded by the 2-step procedure when applied to tissue specimens, and the effective signal after background subtraction was consequently much lower (Figure S1e).

To confirm that ST3Gal1 sialylates primarily *O*-glycans, we pretreated the tissue samples with *O*-glycosidase, PNGaseF, or base-induced β -elimination (Figure 2c). *O*-Glycanase cleaves core 1 and 3 (GlcNAc β 1–3GalNAc α Ser/Thr) glycans from glycoproteins. As expected, pretreatment with this endoglycosidase significantly reduced the labeling intensity. According to the known substrate specificity of ST3Gal1, the residual fluorescent signal may be attributed to the core 2 glycans that remained on the tissue sample.^{12,14} β -Elimination effectively removed *O*-glycans, and as the result, the treated tissues produced no detectable signal. Pretreatment with PNGaseF, an amidase that cleaves between the innermost GlcNAc and asparagine residues from *N*-linked glycoproteins, did not affect the labeling intensity, confirming that ST3Gal1 labeling is restricted to *O*-glycans.

Using ST3Gal1 to label tissue specimens obtained from healthy murine organs with and without neuraminidase pretreatment confirmed that most tissues exhibited little staining without neuraminidase treatment with the exception of the lung tissue, which showed detectable signals under both conditions (Figure S2). Strong labeling was observed in mouse heart, liver, spleen, stomach, preputial gland, and lung tissues after neuraminidase treatment. In heart, liver, and stomach specimens, the labeling was mostly restricted to the cell membranes. The sebaceous secretory cells in the preputial gland tissue stained strongly compared to that of the acini. The spleen and lung showed very homogeneous labeling in both cytoplasm and membrane throughout the tissue samples with a slight higher signal intensity in the T-cell area of the white pulp of the spleen, whereas the central areas of the lung artery showed remarkably little glycosylation (Figure S2).

ST6Gal1 for *N*-Glycan Labeling in Tissue Specimens

Similar to ST3Gal1, ST6Gal1 has been used to label a broad acceptor subset of hybrid and complex *N*-glycans in cell lines.²⁰ ST6Gal1 adds α 2–6-linked *N*-acetylneuraminic acid (Neu5Ac; Sialic acid; Sia) and its analogues mainly to the terminal galactose in *N*-linked glycans.²¹ The recombinant rat ST6Gal-1 is expressed as a secretory protein fused to a cleavable GFP.²²

The chemoenzymatic histology method exploiting ST6Gal1 shared similar labeling steps with the procedure for *O*-glycan labeling (Figure 3a) with the only differences being the addition of Mg²⁺ and alkaline phosphatase to the enzymatic reaction mixture. Because *N*-glycans are abundant in most cells, no pretreatment with neuraminidase was necessary to detect significant signals; nevertheless, such a treatment could be applied to include naturally sialylated *N*-glycans in the acceptor pool for ST6Gal1. It was found via titrations for the concentrations of the enzyme (Figure S3a) and CMP-SiaNAI (Figure S3b) that 80 μ g/mL of ST6Gal1 and 500 μ M CMP-SiaNAI were the optimal concentrations to obtain the highest signal intensity. Similar to our observations with ST3Gal1, onestep treatment with ST6Gal1 was successful but generated significant background. After background subtraction, the labeling intensity was comparable to those obtained with a two-step protocol

(Figure S3c); however, because CMP-SiaNAI is more accessible than CMP-Sia-Biotin, we chose to maintain the two-step protocol for subsequent experiments. The selective labeling of *N*-glycans by ST6Gal1 was previously confirmed in cell-based assays by treatment with PNGaseF.²⁰ For our histological application, pretreatment of tissues with PNGaseF prevented chemoenzymatic labeling by ST6Gal1 in a similar fashion (Figure 3b).

A comparison of murine tissue specimens chemoenzymatically labeled with ST3Gal1 (with neuraminidase pretreatment) or ST6Gal1 (without neuraminidase pretreatment) highlighted some interesting differences in glycan distribution (Figure S4). Although the intestine, pancreas, and heart specimens were found to express both *O*- and *N*-glycans, the brain and spleen specimens showed abundant expression of *O*-glycans (ST3Gal1) but little expression of *N*-glycans (ST6Gal1). The lung specimen was found to express both glycans with the exception of the arteries, which show little glycosylation. However, the inner brush border of the epithelial cells lining the artery showed signals of *O*-glycans but not *N*-glycans. (Figure S4).

The chemoenzymatic histological methods described above can be combined among themselves or with neuraminidase treatment to enable the following applications: (1) *Tandem labeling*, in which the same enzyme is used before and after neuraminidase treatment to identify in the same specimen naturally unsialylated and sialylated acceptors, respectively. (2) *Double labeling*, in which sequential application of ST3Gal1 and ST6Gal1 allows the detection of both *N*- and *O*-glycans in the same tissue section. As an option, double labeling can be combined with pretreatment with neuraminidase.

These two applications, based on the sequential use of the same or different sialyltransferases, were enabled by the covalent nature of the TSA procedure through which no signal from the first labeling was lost during the second labeling. Because different tissue structures might have different glycan levels and therefore produce different background signals, we ensured that each enzymatic step had labeled all accessible acceptor glycan substrates. This was confirmed by omitting the neuraminidase treatment after the first labeling step and applying the same enzyme directly to a second run of labeling, which produced no detectable fluorescence, strongly suggesting that all acceptor structures are capped during the first labeling (Figure S5). Importantly, the blocking protocols developed for the original CHoMP could be seamlessly applied to a sequential labeling process. During a second labeling round, these protocols were essential to block any nonspecific signals arising from residual components of the first step labeling. For instance, the avidin/biotin block that was designed to eliminate any signal from endogenous biotin could also block any biotin not bound by Neutravidin during the first protocol application. Similarly, the H₂O₂ treatment that was intended for deactivating endogenous peroxidases during the initial labeling could deactivate the residual Neutravidin-HRP left from the initial labeling step, which in turn permitted the introduction of a secondary fluorophore by TSA in the second labeling process.

Tandem Labeling

In the tandem labeling strategy, the same sialyltransferase, either ST3Gal1 or ST6Gal1, is used twice in sequential labeling before and after neuraminidase treatment. This procedure

enables the detection of naturally unsialylated and sialylated acceptors in the same specimen (Figure 4a). The premise for successfully conducting tandem labeling is that the unnatural sialic acid introduced in the first step will not be removed during the neuraminidase treatment. To verify this, we pretreated a tissue specimen with neuraminidase to remove all naturally sialylated glycans before conducting the first run of labeling followed by neuraminidase treatment and the second run of labeling. After the second neuraminidase treatment, we did not observe any new signals arising from the second labeling (Figure S6A), suggesting neuraminidase treatment does not remove the unnatural sialic acids introduced in the first step of labeling. Furthermore, analysis of tissues with tandem labeling revealed areas where only fluorescence corresponding to the initial labeling was observed (Figure S6B), confirming that the sialic acid analogues introduced during the first labeling are not cleaved and reintroduced during a subsequent labeling.

After we demonstrated the feasibility of applying tandem labeling to tissue samples, we used this strategy to analyze sialylation patterns in two small arrays of different human organs including one core each of healthy and cancerous tissues. One array was labeled with ST3Gal1 (Figure 4b) and the other with ST6Gal1 (Figure 4c). Figure 4b summarizes the observations of the array labeled using ST3Gal1, showing the MFI of the cancerous tissues normalized to the MFI of the matching normal tissues, in which the MFI of the normal tissues was set to one. Testis, larynx, lymph node, thyroid, breast, lung, and prostate cancerous tissue specimens exhibited increased levels of both sialylated and unsialylated *O*-glycans, suggesting an increase in overall glycosylation upon malignant transformation. In the breast cancer tissue specimen, we detected an increase in the labeling intensity after neuraminidase treatment, indicating that there is a larger portion of sialylated glycans in the cancerous tissue compared with the healthy tissue. This observation is consistent with the published data showing that an increase in sialic acids and sialyltransferases is often observed in invasive ductal carcinoma.^{23–26} Pancreas and muscle cancerous tissue specimens were found to express lower levels of both sialylated and unsialylated glycans than normal tissue samples, suggesting a decrease in overall glycosylation. Finally, colon cancerous tissue was detected to have more unsialylated glycans and less sialylated glycans. Overall, changes in *N*-glycans between healthy and cancerous tissues were less pronounced than those observed for *O*-glycans, except for the thyroid tissue. Regarding the array labeled with ST6Gal1, most cancerous tissues seem to express more unsialylated than sialylated *N*-glycans (Figure 4c).

On the basis of the results obtained from the small arrays, we decided to pursue the analysis of *N*-glycans in a large prostate cancer array to obtain clinically relevant conclusions with a larger sample pool (Figure 5). This array includes 80 cores with 60 being prostatic adenocarcinoma cases, 10 adjacent normal tissues, and 10 normal tissues. Representative images of a sample pair after tandem labeling using ST6Gal1 are shown in Figure 5a. Consistent with what was observed for the small arrays, cancerous tissues exhibited significantly less staining before and after neuraminidase treatment, suggesting complex and hybrid-type *N*-linked glycans are downregulated (Figure 5b). Interestingly, we observed a striking difference in the expression of both sialylated and unsialylated *N*-glycans between tissues from healthy patients and healthy tissues adjacent to tumors (also known as adjacent normal tissues) with dramatically reduced staining in the latter. These findings suggest that

aberrant expression of glycans in adjacent normal tissues may reflect a diseased state either forestalling a cancerous transition or signaling a neighboring diseased area that can be exploited to develop biomarkers for malignant risk.^{27,28} By comparing the average MFI of tissues known to have undergone metastasis to those without reported metastasis, we discovered that there was no significant difference between metastatic and nonmetastatic tumors among sialylated *N*-glycans, however, metastatic tumors expressed less unsialylated acceptors as revealed by the first ST6Gal1 labeling (Figure 5c), suggesting that the ratio of sialylated to unsialylated glycans may serve as a marker for metastasis.

Double Labeling of *N*- and *O*-Linked Glycan

The double labeling strategy aims to simultaneously detect both *N*- and *O*-glycans on the same tissue section. This strategy exploits the sequential application of the ST3Gal1- and ST6Gal1-based chemoenzymatic labeling each coupled to a different fluorophore (Figure 6a).

Although acceptors for ST3Gal1 and ST6Gal1 are mutually exclusive, we found that the order of the enzymatic reactions used was a major factor for achieving reliable labeling of both glycan epitopes. The ST3Gal1 treatment followed by the ST6Gal1-mediated labeling was the ideal choice (Figure S7). The initial labeling of *N*-glycans by ST6Gal1 followed by the *O*-glycan labeling by ST3Gal1 was unsuccessful. *N*-Glycans usually present more branches than *O*-glycans, consequently the initial *N*-glycan labeling might have induced significant steric hindrance to block the second step *O*-glycan labeling, which was presumably the cause of this observation. As mentioned above, most healthy murine tissues required pretreatment with neuraminidase to enable a strong signal to be detected for *O*-glycans; consequently, the pool of *N*-glycans detected in the double labeling included both originally sialylated and unsialylated acceptors. However, this optional pretreatment could be omitted to detect solely unsialylated *N*- and *O*-glycans. The use of this double labeling strategy to examine several murine organs (Figure S8) revealed subtle variations among the distribution of *N*- and *O*-glycans. Among these, the most striking differential distribution was observed in mouse kidney tissue sections (Figure 6b) with abundant expression of *O*-glycans found in the brush borders of distal tubules and a strong signal of *N*-glycans found in distal tubules and collecting ducts.

As the first discovery-driven application of the double labeling strategy, we used this approach to analyze changes in glycosylation patterns in murine spleen tissues collected 7 days postinfection with *Listeria monocytogenes* (LM) and the spleen tissue from naive uninfected mice. It is known that lymphocyte glycosylation changes dramatically upon infection.²⁹ At the peak of T-cell activation (~7 dpi), we detected an increase in both unsialylated *N*- and *O*-glycan levels (Figure 7a), particularly in the T-cell region of the white pulp. Performing double labeling following neuraminidase treatment, we observed significantly higher signals for both *N*- and *O*-linked glycans in the infected spleen tissues versus those of the uninfected, suggesting that T-cell activation leads to an increase in overall glycosylation (Figure S9). Previous glycomics studies reported a decrease in sialylated biantennary *N*-glycans in activated T cells with no such changes detectable in activated B cells.²⁹ Because of its nature, our chemoenzymatic histology method could not

differentiate between T and B cells without including a cell marker by IHC; nevertheless, we detected an overall increase in glycan acceptors in activated splenocytes.

Finally, we decided to evaluate naturally unsialylated *N*- and *O*-glycan expression in a large lymph node and lymphoma array (Figure 7b, c). This array contains a total of 80 cores with 60 cases of lymphoma (47 B-cell lymphoma, 9 T-cell lymphoma, and 4 other types of lymphoma), 10 cores of adjacent normal lymph node tissues, and 10 cores of spleen tissues. We observed significantly lower unsialylated *O*-glycans using ST3Gal1-based labeling in B-cell lymphoma compared to that of adjacent lymph node tissues. It is known that sialylation and sialyltransferase expression increase in certain forms of cancer.^{30,31} The lower *O*-glycan labeling observed for B-cell lymphoma compared with healthy lymph node tissue could be a consequence of higher sialic acid presence masking the natural acceptors for ST3Gal1. Interestingly, no significant difference in *O*-glycans was observed between healthy lymph nodes and T-cell lymphoma (Figure 7b). Neither were any significant differences observed for unsialylated *N*-glycans among healthy lymph nodes, T-cell, or B-cell lymphomas (Figure 7c). In addition, the spleen showed significantly lower levels of unsialylated *O*- and *N*-glycans (Figure 7b, c) compared to those found in normal lymph nodes despite having similar structures and consisting mainly of lymphocytes. Labeling was also variable among the different normal lymph nodes based on their provenance, although the number of cores for each type of normal lymph node was too small, and consequently, variations observed could just be attributed to sample-to-sample variations (Figure S10).

CONCLUSIONS

In 1979, Paulson et al. demonstrated for the first time that sialic acid residues could be directly introduced onto the cell surface of erythrocytes using recombinant sialyltransferases.³² Nine years later, using the same enzymes supplied by Paulson, Brossmer and Gross demonstrated that it was possible to modify glycoproteins with a fluorescently tagged sialic acid transferred from the CMP-9-fluoresceinyl-NeuAc donor.³³ Likewise, a radioactive isotope-labeled CMP-*N*-acetyl-[³H]-neuraminic acid and a rat liver β -galactoside α -2,6-sialyltransferase were used in conjugation with *Vibrio cholerae* sialidase by Whiteheart and Hart to probe the sialylation state and the level of penultimate Gal β 1-4GlcNAc residues on the surfaces of murine T lymphocytes.³⁴ On the basis of these pioneering studies, we have developed new CHoMP methods for the detection of *O*- and *N*-glycans in tissue specimens by exploiting sialyltransferases ST3Gal1 and ST6Gal1, respectively. Labeling with ST3Gal1 enables the detection of nonelongated core 1 and 2 *O*-glycans. Further elongation of these glycan epitopes by core 1 GlcNAc transferase or by terminal sialylation prevents their detection by ST3Gal1. Nevertheless, the glycan epitopes modified by sialylation can be included in the detectable acceptor pool by pretreatment with neuraminidase. Labeling with ST6Gal1 permits the detection of complex and hybrid *N*-glycans with unsialylated terminal galactose residues. By nature, high mannose and terminally sialylated *N*-glycans are not accessible by this strategy, but the latter can be transformed into detectable acceptor types by neuraminidase pretreatment. The tandem use of either ST3Gal1 or ST6Gal1 before and after neuraminidase treatment enables the survey of unsialylated and sialylated *O*- or *N*-linked glycans, respectively, in the same specimen. Applying the tandem labeling to a small array of multiple human cancers, we identified

various differences in glycan expression between normal and cancerous tissues. A follow up study for *N*-glycans on a large prostate cancer array detected significant differences between unsialylated glycans in cancerous, adjacent, and normal tissues as well as between metastatic and nonmetastatic tumors. A double labeling strategy was also designed to analyze both *N*- and *O*-glycans on the same tissue section. Analysis of murine tissue sections using this double labeling strategy uncovered patterns of differential glycan expression in various organs, particularly in kidney sections, and differential glycan expressions in the spleens of naïve and bacteria-infected mice. This strategy also revealed that in a large human lymphoma array *O*-glycans could differentiate between B- and T-cell lymphoma as well as between B-cell lymphoma and normal tissues.

It is worth mentioning that although the main application for CHoMP described here is imaging, the incorporation of a bioorthogonal probe on specific glycan acceptors can be used for other downstream applications such as glycoproteomics, as has been recently reported by using metabolic oligosaccharide labeling.³⁵ Currently, we are applying these methods to analyze samples from patients who have undergone therapeutic treatment to determine if our methods can be used for tumor prognosis.

EXPERIMENTAL PROCEDURES

General Methods and Materials

All reagents and solvents were acquired from Fisher Scientific unless otherwise stated. Photomicrographs were acquired on a BZ-X700 Keyence Microscope, and all images were processed using ImageJ software. Statistical analysis and graph generation was completed using GraphPad Prism software. Human tissue arrays MC245a, PR806, and LM801a were purchased from US Biomax. Enzymes ST3Gal1 and ST6Gal1 were expressed in the Moremen lab (University of Georgia, Complex Carbohydrate Research Centre). Tyramide Signal amplification kits were obtained from PerkinElmer. The structures for BTTP and the accelerated azide-biotin probe can be found in Figure S11, detailed methods for their synthesis have been previously published.^{16,36}

Nucleotide Sugar Donor Synthesis

CMP-sialic acid-alkyne was synthesized according to a published protocol.³⁷ Briefly, a reaction mixture (10 mM mannose-alkyne, 50 mM sodium pyruvate, 100 mM Tris-HCl pH 8.8, 20 U of *E. coli* K12 aldolase, 20 mM MgSO₄, *N. meningitidis* CMP-sialic acid synthetase, and 20 mM CTP) was incubated for 2 h at 37 °C in a shaker (225 rpm). The reactions were monitored by TLC analysis (10 mM tetrabutylammonium hydroxide in 80% aqueous acetonitrile). When the reaction had gone to completion, 5 mL of cold ethanol was added, and the mixture were incubated for 30 min on ice to quench the reaction and precipitate the enzymes. The precipitate was removed by centrifugation (5,000 × *g* for 30 min), and the supernatant was concentrated using rotary evaporation to remove ethanol. The reaction products were purified by Bio-Gel P2 gel filtration chromatography (1.5 × 75 cm) and eluted with 50 mM ammonium bicarbonate buffer. Fractions containing the nucleotide sugar donors were lyophilized and used without further purification.

Mice

All animal studies were carried out under a protocol approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute (TSRI). C57BL/6 mice were bred and housed in the vivarium of The Scripps Research Institute, and they were euthanized at 6–8 weeks and immediately perfused with 4% formalin. Organs were harvested and immediately immersed in zinc formalin fixative for 24 h. Tissues were paraffin embedded by the TSRI's Histology Core Facility and then sectioned on a microtome at 5 μm . For effector-stage spleen tissue, the mouse was infected with 10,000 *Listeria monocytogenes* (LM; grown in brain heart infusion broth from a stock inoculum on the day of infection, cultured at 37 °C until OD600 = 0.08–0.2, and then diluted in PBS) via intraperitoneal injection. The infected mouse was euthanized, and the spleen was harvested as described above 7 days post infection.

Chemoenzymatic Histology

Tissue sections were deparaffinized and rehydrated according to standard protocols. In summary, the slides were sequentially immersed in coplin jars for 5 min in 40 mL: 2 $\times$ Histo-Clear II (National Diagnostics), 2 $\times$ ethanol, 2 $\times$ 70% aq. ethanol, 2 $\times$ 50% aq. ethanol and dH₂O. A hydrophobic barrier was drawn around the tissues on the slides using a PAP pen. Tissues were immersed in 50 mL of TBS + 0.1% Tween-20 (TBST) for 10 min. If necessary, slides were then placed in a humidified chamber and 100 μL of α 2–3,6,8 neuraminidase (NEB) reaction solution was added (2 μL hydrolase in GB1 buffer). Slides were incubated overnight at 37 °C followed by TBST washes (3 $\times$ 5 min). Slides were then again placed in a humidified chamber, and the enzyme labeling solution was added. The enzyme solution contained 500 μM CMP-SiaNAI in TBST and 80 ng/ μL of enzyme (ST3Gal1 or ST6Gal1). For ST6Gal1, the reaction mixture also contained 5 mM MgCl₂ and 1 μL of alkaline phosphatase (NEB) per 100 μL reaction solution. The slides were incubated for 1 h at 37 °C and then washed with TBST (3 $\times$ 5 min). The slides were again placed in a humidified chamber and blocked with avidin/biotin at RT according to standard protocols. In summary, 1 drop of avidin solution (Biolegend kit) was added to the tissue, and the slides were incubated for 10 min followed by TBST wash (3 $\times$ 3 min). One drop of biotin solution was then added, followed by a 10 min incubation and TBST washes (3 $\times$ 3 min) afterwards. The slides were then again placed in a humidified chamber and “clicked” to a biotin probe with a solution containing 50 μM accelerated azide-biotin probe,¹⁶ 75 μM CuSO₄, 150 μM BTTP ligand, and 2.5 mM sodium ascorbate in TBST for 1 h at RT. Following three washes in TBST (5 min each), the tissues were blocked for 10 min in 0.3% hydrogen peroxide diluted in TBS at RT and then washed with TBST (3 $\times$ 5 min) to remove the H₂O₂. The slides were placed in a humidified chamber and incubated with neutravidin-HRP (Biolegend, 1:100 in TBST) for 1 h at RT and then subsequently washed with TBST (3 $\times$ 5 min). The slides were then placed in a humidified chamber and incubated with TSA-Plus-Fluorophore reagent according to the manufacturer's protocol (1:50 dilution for 5 min at RT, protected from light), then washed with TBST (3 $\times$ 5 min) and mounted with Prolong antifade gold with DAPI (Invitrogen).

Chemoenzymatic Histology Variations for Method Development

For comparison between azide and alkyne donors, CMP-SiaNAz was used instead of CMP-SiaNAI, and it was clicked to an alkyne-PEG₄-biotin (Click Chemistry Tools) with the same conditions and concentrations as described above. For comparison with direct detection with streptavidin, tissue sections were incubated with Streptavidin-AlexaFluor 488 (Thermo Fisher, 2 $\mu\text{g}/\text{mL}$) in TBST for 1 h at RT, washed with TBST (3×5 min), and mounted directly with Prolong antifade gold with DAPI. For comparison with one-step labeling, slides were blocked with avidin/biotin prior to the enzymatic step. The reaction conditions were maintained with the exception of the substitution of CMP-Sia-Biotin (prepared as published¹⁹) instead of CMP-SiaNAI, and the click reaction was skipped; the rest of the procedure was conducted as described above. For PNGaseF treatment, slides were treated after deparaffinization with 100 μL of PNGaseF NEB standard reaction solution ($1 \times$ G2 buffer, 1% NP-40, and 2 μL of PNGaseF) overnight at 37 °C followed by TBST washes (3×5 min) and subsequently stained using the normal protocol. β -Elimination was conducted by incubating deparaffinized tissues with 0.5 M NaOH in 70% aqueous EtOH overnight at 4 °C, followed by washes and rehydration as done after the deparaffinization step (5 min each in $2 \times 70\%$ EtOH, $2 \times 50\%$ EtOH, dH₂O) and subsequently stained using the normal protocol. For *O*-glycosidase treatment, slides were treated after deparaffinization with 100 μL of deglycosylation solution ($1 \times$ G7 buffer, 1% NP-40, and 5 μL of *O*-glycosidase) overnight at 37 °C followed by TBST washes (3×5 min) and subsequently stained using the normal protocol.

Tandem Labeling

Tissues were labeled using either ST3Gal1 or ST6Gal1 with the normal chemoenzymatic histology protocol described above. After the last wash, instead of mounting with Prolong Gold, tissues were placed in a humidified chamber, and 100 μL of $\alpha 2$ –3,6,8 neuraminidase (NEB) reaction solution was added (2 μL of hydrolase in GB1 buffer). Slides were incubated overnight at 37 °C followed by TBST washes (3×5 min). Tissues were then subjected to a second round of normal chemoenzymatic histology protocol using the same enzyme used during the first labeling and substituting TSA-FITC with TSA-Cy5 during signal amplification. After the last wash, slides were mounted with Prolong antifade gold with DAPI.

Double Labeling

Tissues were labeled with ST3Gal1 with the normal chemoenzymatic histology protocol described above. After the last wash, instead of mounting with Prolong Gold, tissues were subjected to a second round of normal chemoenzymatic histology protocol using ST6Gal1 substituting TSA-FITC with TSA-Cy5 during signal amplification. After the last wash, slides were mounted with Prolong antifade gold with DAPI. During method development for this strategy, the fluorophores and enzyme orders were inverted for comparison. If total glycan detection rather than only naturally unsialylated glycan detection was intended, before any chemoenzymatic labeling, slides were placed in a humidified chamber, and 100 μL of $\alpha 2$ –3,6,8 neuraminidase (NEB) reaction solution was added (2 μL of hydrolase in

GB1 buffer). Slides were incubated overnight at 37 °C followed by TBST wash (3 × 5 min). Normal double chemoenzymatic labeling was then conducted on the desialylated tissues.

Image Processing

All tissues were prepared with a negative control (same treatment without sialyltransferase) in a serial section. Images were acquired at the maximum microscope gain to achieve 80% signal saturation with a monochrome camera, and negative controls were acquired with the same acquisition parameters. Using ImageJ, the minimum intensity was increased until the signal was undetectable in negative controls, and this value was established as background and subtracted from each positive image. Pseudo RGB color was assigned to each channel, usually green for the first glycan detected, red for the second glycan detected, and blue for nuclear DAPI stain. Each sample pair in the small multiple organ array (healthy and cancerous sample from the same organ) was processed with the same parameters. All cores in the large arrays were processed with the same parameters. MFI quantification was performed on selected areas for the full core at 10× acquisition after background subtraction. Selected areas included epithelial cells for adenocarcinomas and their healthy tissue counterparts, or total tissue avoiding any obvious fat or connective tissue for all other samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Glycan acceptors for ST3Gal1 and ST6Gal1 activity. ST3Gal1 catalyzes the transfer of sialic acids to *O*-glycans (cores 1 and 2; gray squares). *N*-Glycans with uncapped terminal LacNAc (orange squares) act as acceptors for sialyltransferase ST6Gal1. New disaccharides are shown in red squares.

Figure 2.

Development of a chemoenzymatic histology method for *O*-glycan detection using ST3Gal1. (A) General scheme for *O*-glycan labeling on histological samples. Histology images were acquired from 5 μ m FFPE murine liver tissue serial sections; green = *O*-glycans, blue = DAPI nuclear stain. (B) Effect of neuraminidase pretreatment to expose *O*-glycan acceptors for ST3Gal1 labeling. (C) *O*-Glycan specificity analysis for ST3Gal1 chemoenzymatic labeling. *O*-Glycosidase removes a large portion of acceptors for ST3Gal1, significantly decreasing *O*-glycan labeling. Base-induced β -elimination removes all *O*-glycan acceptors leading to complete signal absence after ST3Gal1 chemoenzymatic labeling. PNGaseF treatment removes *N*-glycans and does not have an effect on ST3Gal1 labeling.

Figure 3. Development of a chemoenzymatic histology method for *N*-glycan detection using ST6Gal1. (A) General scheme for *N*-glycan labeling on histological samples. (B) *N*-Glycan specificity analysis for chemoenzymatic labeling with ST6Gal1. PNGaseF treatment to remove *N*-glycans eliminates all signal for ST6Gal1; the negative control lacks ST6Gal1. Histology images were acquired from 5 μm FFPE murine kidney tissue serial sections; green = *N*-glycans, blue = DAPI nuclear stain.

Figure 4.

Tandem labeling strategy for identifying unsialylated and sialylated glycans. (A) Workflow for applying tandem labeling on tissue samples. (B, C) Summary of selected tissues from a human multiple organ cancer array analyzed by the tandem labeling strategy. MFI was quantified for each organ pair (cancerous and healthy cores of the same organ) and normalized to the healthy MFI = 1. Bar graphs represent normalized MFI from unsialylated glycans (green) and sialylated glycans (red) for *O*-glycans analyzed with ST3Gal1 (B) or *N*-glycans labeled with ST6Gal1 (C); error bars depict MFI standard deviation.

Figure 5.

Analysis of a large prostate cancer array via tandem labeling with ST6Gal1 for unsialylated and sialylated *N*-glycans. (A) Representative examples of cancerous and normal cores from a prostate array stained by tandem labeling. Green = unsialylated *N*-glycans, red = sialylated *N*-glycans, blue = DAPI nuclear stain. (B) MFI of unsialylated *N*-glycans (left) and sialylated *N*-glycans (right) for healthy, normal adjacent, and cancerous tissues. (C) Comparison of MFI of the cancerous cores with distant metastasis versus the cores without observed metastasis. Error bars depict MFI standard deviation; unpaired *t* tests (*p*-value: ns = nonsignificant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001).

Figure 6.

Double labeling of *N*- and *O*-glycans via the sequential use of ST3Gal1 and ST6Gal1. (A) The work flow of double labeling on tissue samples. (B) Double labeling applied to the detection of *O*-glycans (green) with ST3Gal1 and *N*-glycans (red) with ST6Gal1 after neuraminidase pretreatment of a mouse kidney tissue section (5 μ m FFPE). The overlay highlights regions with both glycan types, e.g. the distal tubules (arrows), or with a higher proportion of one type of glycan, e.g. higher *N*-glycan levels in collecting ducts (arrowheads). Blue = DAPI nuclear stain.

Figure 7.

Application of double labeling strategy. (A) Double labeling of 5 μm FFPE murine spleen tissue sections from either a naive mouse or a mouse 7 days after infection with LM. Green = O-glycans, red = N-glycans, blue = DAPI nuclear stain. Negative controls shown in the overlay inlets were prepared without enzymes during the chemoenzymatic step. (B, C) Results from large lymphoma array analyzed by double labeling strategy for (B) O-glycans with ST3Gal1 labeling and (C) N-glycans with ST6Gal1 labeling. Bar graphs compare MFI from B-cell lymphoma, T-cell lymphoma, normal adjacent lymph node, and spleen tissue samples. Dots represent single measurements; error bars depict MFI standard deviation with unpaired *t* tests (*p*-value: ns = nonsignificant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001).