Resolving conflicting data on expression of the Tn antigen and implications for clinical trials with cancer vaccines

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Abstract
The tumor-associated Tn antigen has been investigated extensively as a biomarker and therapeutic target. Cancer vaccines containing the Tn antigen as a single tumor antigen or as a component of a polyvalent vaccine have progressed into phase I and II clinical trials. One major focus of Tn-based vaccines is the treatment of prostate cancer patients. Although expression of the antigen on prostate tumors is a critical prerequisite, previous reports investigating Tn expression in prostate tumors have produced conflicting results. Using a combination of immunohistochemistry and carbohydrate microarray profiling, we show that only 4% to 26% of prostate tumors express the Tn antigen. Based on our results, the majority of prostate cancer patients do not express the appropriate antigen. Therefore, efforts to preselect the subset of prostate cancer patients with Tn-positive tumors or apply Tn vaccines to other cancers with higher rates of antigen expression could significantly improve clinical response rates. Because conflicting information on carbohydrate expression is a general problem for the field, the approach described in this article of analyzing antigen expression with multiple antibodies and using carbohydrate microarray profiles to interpret the results will be useful for the development of other carbohydrate-based cancer vaccines and diagnostics. [Mol Cancer Ther 2009;8(4):971–9]

Introduction
Major changes in carbohydrate expression occur during the onset and progression of cancer. Alterations in glycosylation can result in both the loss of normal carbohydrate antigens and the high expression of inappropriate carbohydrate structures called tumor-associated carbohydrate antigens. Many of these changes occur on the surface of cells or on secreted glycoproteins, making them appealing targets for biomarker and therapeutic development. For example, Lewis Y, TF, Globo H, GM2, polysialic acid, sialyl Lewis A, and sialyl Tn have been evaluated in clinical trials as vaccine antigens (1). One of the most remarkable tumor-associated carbohydrate antigens is the Tn antigen, a carbohydrate containing a single GalNAc residue attached via an α-linkage to either a serine or a threonine residue of a polypeptide chain (see Fig. 1). Although this GalNAc residue is present in the majority of O-linked glycans, it is normally masked by the attachment of addition sugar residues. In the 1970s, Springer et al. discovered that this truncated form of O-glycosylation was present at high levels in ∼90% of breast carcinomas (2). After many years of study, the Tn antigen has also been reported to be expressed in 70% to 90% of colon, lung, bladder, cervix, ovary, stomach, and prostate tumors (3–5) as well as on a variety of pathogens such as parasitic worms (6, 7) and HIV (8); however, little or no expression has been observed in a broad range of normal adult tissues (3–5).

Due to its exceptional expression profile, the Tn antigen has been investigated extensively as a diagnostic marker, a prognostic marker, and a therapeutic target for cancer (3–5). In particular, several groups have developed Tn-based vaccines for the treatment and prevention of carcinomas (for some examples, see refs. 9–18). Tn vaccines have produced excellent results in animal models and several clinical trials in humans have been published (10, 13, 17). The Tn antigen is also one of the key structures present on polyvalent cancer vaccines (17, 19) and multitigantin glycoprotein cancer vaccines (20–22) that have been evaluated in clinical trials. One of the primary focuses of Tn-based vaccines has been the treatment of prostate cancer. For example, Slovin et al. published results from a phase I clinical trial in which a vaccine composed of a synthetic Tn antigen coupled to a carrier protein was administered to patients with biochemically relapsed prostate cancer (13). The vaccine was safe, showed no evidence of autoimmunity, and produced an immune response to the Tn antigen in all patients; however, only a subset (33%) of patients achieved a favorable clinical response as measured by a decrease in prostate-specific antigen slopes. Strategies to improve clinical response rates or preselect the subset of patients that will achieve the desired response could significantly aid the clinical application of these vaccines.

For a cancer vaccine to be effective, each patient’s tumor should express the appropriate antigen(s). Several groups have evaluated expression of the Tn antigen in prostate tumors, but the results are not consistent. Using antibody
1E3 to detect the Tn antigen, Zhang et al. found that 90% of primary tumors expressed the Tn antigen (23). Using antibody 83D4, however, Charpin et al. reported that only 20% of prostate tumors were Tn positive (24). In a third study, Huang et al. found weak staining of 50% of prostate tumors with antibody 91S8 and weak staining of 83% using antibody 10F4 (25). One likely explanation for these discrepancies is that the antibodies used to detect the Tn antigen have differing specificities. One or more of the antibodies could cross-react with other antigens in which case some or all of the positive staining could be due to expression of a completely different carbohydrate. The issue of cross-reactivity has certainly been appreciated; however, only limited information on the specificities of these antibodies has been available primarily due to the lack of high-throughput methods for studying carbohydrate-protein interactions and the limited access of structurally defined carbohydrate antigens.

Carbohydrate microarray technology has emerged over the last few years as a powerful tool for analyzing carbohydrate-protein interactions (26–30). Carbohydrate microarrays contain many different glycans immobilized on a solid support in a spatially defined manner. The miniaturized format allows high-throughput analysis of hundreds to thousands of potential interactions while using only tiny amounts of each precious carbohydrate sample. Our group has developed a carbohydrate microarray containing >100 different structurally defined oligosaccharides and glycoproteins; to date, we have analyzed the specificities of >60 different antibodies and lectins (31–35). Array profiles of antibodies 1E3, 83D4, 91S8, and 10F4 could provide insight into the reported discrepancies; however, not all of these antibodies were available, and variations in sample groups between the reported studies would make direct comparisons difficult.

To resolve the conflicting data and identify optimal patients for Tn-based vaccines, we carried out a systematic analysis of Tn expression in prostate tumors. Four different antibodies were used to measure Tn expression in 77 prostate tumors, and carbohydrate microarray profiles of each antibody were used to interpret the results. Importantly, we show that the Tn antigen is only expressed in a small subset of prostate tumors. The results have significant implications for the clinical development and implementation of Tn-based cancer vaccines.

Materials and Methods

General

Antibody 1E3 was kindly provided by Prof. Sen-itiroh Hakomori. Monoclonal antibody B1.1 was purchased from Biomeda and antibody HBTn-1 was purchased from DAKO. Prostate cancer tissue arrays, colon cancer control slides, and breast cancer control slides were purchased from U.S. Biomax. Tissues were classified using tumor-node-metastasis system, histotype, and stage grading according to the WHO criteria.

Immunohistochemical Staining

The immunohistochemical staining conditions for each antibody were optimized on colon cancer tissue and confirmed by staining breast cancer tissue. In general, the slide was deparaffinized in xylene and rehydrated in gradual ethanol and PBS. The tissues were then treated with 0.02% trypsin in PBS solution at 37°C for 20 min, rinsed, and then incubated in 0.6% hydrogen peroxide/methanol solution for 15 min. After rinsing in PBS, tissue slides were incubated for 30 min in blocking solution provided in the commercial kit [1E3 and HBTn-1, Vectastain Elite ABC kit for mouse antibody (Vector Laboratories); B1.1, CSA mouse link kit for mouse immunoglobulin antibody (DAKO); and PolyTn, Vectastain Elite ABC kit for rabbit IgG antibody (Vector Laboratories)] to eliminate the nonspecific binding. The primary antibodies were diluted to their working concentration (1E3, 1:250; HBTn-1, 1:50; B1.1, 1:20; and PolyTn, 1:30) in 0.1% BSA/PBS solution (250 μL) and applied to the tissue (1E3, room temperature for 30 min; HBTn-1, room temperature for 30 min; B1.1, room temperature for 30 min; and PolyTn, 4°C overnight). The tissue sections were rinsed with PBS followed by the standard protocol of secondary reagents and peroxidase system provided by the commercial staining kits. The slides were developed in diaminobenzidine (Sigma-Aldrich) solution, rinsed, counterstained by Gill’s hematoxylin (Fisher Scientific), and then blued by saturated lithium carbonate solution. The slides were then washed, dehydrated, and mounted. For negative controls, the primary antibody was replaced by 0.1% BSA/PBS solution. Images were taken by an Olympus PROVIS microscope custom assembled with a Zeiss AxioCam camera. AxioVision software was used.

Figure 1. Structures of the Tn antigen and related carbohydrates.
Scoring of Tissue Sections
Each tissue core was evaluated for intensity, location of staining, and percentage of tumor cells that stain positively. Intensity was scored from 0 to 4, with 4 being most intense. Intensities from 1 to 4 were considered positive. The percentage of tumor cells staining positively was scored from 0 to 4 (0, 0-10%; 1, 10-24%; 2, 25-49%; 3, 50-74%; and 4, 75-100%). For comparison with the Zhang et al. study, individual cores were considered positive if ≥50% of the tumor cells were stained positive. Cases were classified as positive if they had cytoplasmic staining, membrane staining, or both.

Preparation of Polyclonal Antibodies to the Tn Antigen (PolyTn)
Rabbit polyclonal antiserum was generated at Biocon. Briefly, the rabbit was inoculated with a 1:1 emulsion of 500 μg asialo-ovine submaxillary mucin [asialo-OSM, prepared as reported previously (32)] and complete Freund’s adjuvant. The rabbit was boosted on days 21 and 42 with a 1:1 emulsion of 250 μg asialo-OSM and Freund’s incomplete adjuvant and then on days 63, 133, and 154 with a 1:1 emulsion of 125 μg asialo-OSM and Freund’s incomplete adjuvant. The rabbit was boosted with a synthetic Tn hapten (Ac-Ser-Tn-Ser-Gly-hexanoic acid) coupled to KLH on days 179 and 217 with a 1:1 emulsion of 125 μg Tn-KLH and Freund’s incomplete adjuvant and then bled 12 days after the final boost.

Affinity Purification of PolyTn
Preparation of Ser-Tn-Ser resin. A mixture of Ac-Ser-Tn-Ser-Gly-hexanoic acid (ref. 33; 150 mmol/L in water, 30 μL) and N-hydroxysuccinimide in DMF (300 mmol/L, 15 μL) was added to a solution of EDC (300 mmol/L solution in 1:1 water/DMF, 15 μL). The mixture was kept at room temperature for 1 h to pre-form the N-hydroxysuccinimide ester. Aminoethyl agarose resin (2 mL; Sigma-Aldrich; 4 atom spacer) was washed with water and centrifuged (1,000 rpm, 5 min) in an ultrafree-MC PVDF filtration tube (0.22 μm; Millipore). To the dry resin were added water (800 μL), 10× bicine buffer (10 mmol/L, pH 8.5, 200 μL), and the pre-mixed carbohydrate antigen solution. The mixture was shaken at room temperature for 2 h and then treated with acetic anhydride (300 mmol/L in DMF, 200 μL) for another 2 h. The mixture was then centrifuged and washed with DMF/water, water, and PBS.

Affinity Purification. Crude PolyTn antibody (1.8 mL) was added to the dry resin (2 mL) in an ultrafree-MC PVDF filtration tube, shaken at room temperature for 2 h, and then centrifuged. The filtrate was removed and the resin was...
washed with PBS. The dry resin was treated with 1.4 mL elution buffer [solution of GalNAc (400 mmol/L), glycine (50 mmol/L, pH 2.0), and NaCl (500 mmol/L)] for 10 min. The mixture was centrifuged and immediately neutralized with PBS (20×, 1.5 mL). The filtrate was dialyzed using regenerated cellulose dialysis membranes tubes (Spectra/Por 7; SpectrumLabs) with PBS overnight. BCA protein assay (Pierce) kit was used to determine the concentration of antibody (184 μg/mL). The polyclonal antibody was then further incubated with 200 μL KLH-agarose resin (Sigma-Aldrich) for 2 h at room temperature.

Carbohydrate Microarray Profiling of Antibodies

The carbohydrate microarrays were prepared as described previously with the following modifications (31–33). Samples were printed on SuperEpoxy 2 protein glass slides (TeleChem International) using a Biorobotics MicroGrid II microarrayer (Genomic Solutions) fitted with Stealth pins (TeleChem International; SMP3, which produce ∼100 μm spots). The list of 112 components printed on the array can be found in Fig. 3 and in Supplementary Table S2.

Binding of 1E3, HBTn-1, B1.1, and PolyTn were evaluated using minor modifications of the previously reported protocol (31–33). After blocking with 3% BSA/PBS (200 μL/sub-array well), antibodies were incubated at a range of concentrations (diluted into 0.3% BSA/PBS, 100 μL/sub-array well) on the array. After washing, bound antibody was detected by incubating the array with Cy3-labeled AffiniPure goat anti-mouse IgG2a (100 μL at 2 μg/mL; Jackson ImmunoResearch Laboratories) for 1E3, Cy3-labeled AffiniPure goat anti-mouse IgM (100 μL at 2 μg/mL; Jackson ImmunoResearch Laboratories) for HBTn-1 and B1.1, or Cy3-labeled AffiniPure goat anti-rabbit IgG (100 μL at 2 μg/mL; Jackson ImmunoResearch Laboratories) for PolyTn. Slides were washed with PBS, dried by centrifugation at 500 × g in a 50 mL conical tube, and then scanned using a GenePix Scanner 4000B (Molecular Devices). Background-corrected median fluorescence intensities for each component were obtained using GenePix Pro 6.0 software. All median intensities <1 were set to 1 as the “floor value.”

Results

Staining of Prostate Tumor Samples with 1E3

Antibody 1E3 is a mouse IgG2a monoclonal antibody that selectively stains a variety of tumor tissues (23). Although the production and characterization of antibody 1E3 have not been published, it is reported by Hakomori's group to bind the Tn antigen and to be very similar to antibody CU-1 (36, 37). We began by verifying that our staining conditions were appropriate and the antibody was active. Colon and breast adenocarcinoma tissues were used as positive controls, because these tumor types have consistently been reported to express the Tn antigen by many groups using a variety of antibodies (5, 25, 38–43). Images of colon cancer and breast cancer tissues stained with 1E3 can be found in Fig. 2 and Supporting Information, respectively. Once the staining conditions have been verified, a prostate tumor tissue array containing 74 adenocarcinomas,
3 transitional cell carcinomas, 1 hyperplasia, and 2 normal prostate cores was stained with 1E3. A broad range of ages, cancer stages, and Gleason scores were present on the array (see Table 1). For comparison with the Zhang et al. study, we defined a positive case/tumor as a sample tissue core with staining of ≥50% of the cancer cells. Using this cutoff, 78% of the prostate tumor samples stained positively with 1E3 (see Table 1), whereas 0 of 2 normal samples were positive. This result was in good agreement with the results reported by Zhang et al. (90% of cancer samples were positive; 0 of 6 normal samples were positive).

To further verify that the staining was due to 1E3 and not a result of nonspecific staining or contamination with other antibodies, the antibody was preincubated with resin containing the clustered Tn antigen to inhibit active 1E3 (a >95% reduction in binding was observed by ELISA). Pre-treatment completely abolished staining of the prostate tumor samples in all but one sample, which retained very weak staining (see Supplementary Fig. S1 and Supplementary Table S1).

### Staining of Prostate Tumor Samples with HBTn-1 and B1.1

HBTn-1 is a mouse monoclonal IgM antibody that has been widely used to study the expression of the Tn antigen in human tissue (for some recent examples, see refs. 44–47). It is known to bind both the clustered form of the Tn antigen (epitopes composed of two or more Tn antigens linked consecutively on a polypeptide chain) and the single Tn units on peptide chains (48). In addition, it has been shown to have high specificity for the Tn antigen over related carbohydrate structures (31). B1.1 is a mouse IgM monoclonal antibody listed as a general Tn binder by the company that supplies it; however, the production and characterization have not been published. Using a carbohydrate microarray, we have previously found B1.1 to be highly specific for the clustered form of the antigen (33). For both antibodies, the suitability of the staining conditions was verified by positive staining of colon adenocarcinoma tissue (see Fig. 2) and breast carcinoma tissue (see Supporting Information).

Next, the antibodies were used to stain the prostate cancer tissue array. We found that only 4% of the tumor samples were stained positively with antibody HBTn-1 and 6% were stained positively with B1.1. For each antibody, some positive samples had strong staining on most tumor cells, indicating that the concentration of antibody was sufficient to produce an intense stain if the antigen was present. In addition, it did not appear that the cutoff was too high to detect all the positive cases. If the cutoff was lowered such that a sample was defined as positive if ≥25% of the cancer cells were positive, then the number of positive cases only increased to 6% for HBTn-1 and 12% for B1.1. Thus, even with a lower cutoff, only a small percentage of samples stained positively with either antibody.

### Carbohydrate Microarray Profiles of 1E3, HBTn-1, and B1.1

To shed light on the discrepancies between staining with 1E3 and the other Tn-binding reagents, we evaluated the specificity of each of these antibodies using a carbohydrate microarray containing 112 different carbohydrates and glycoproteins (see Fig. 3; for a detailed list of array components, see Supplementary Table S2; refs. 31–35). Antibodies were incubated on the array and binding was detected with Cy3-labeled anti-mouse antibody. As expected, B1.1 was found to selectively bind the clustered form (Tn3) of the Tn antigen. HBTn-1 bound selectively to the clustered Tn antigen and single Tn peptides. Antibody 1E3 was found to bind both clustered and single forms of the Tn antigen. Importantly, 1E3 also bound several other glycans in addition to various forms of the Tn

### Table 1. Correlations between clinicopathologic factors and staining results with different antibodies

<table>
<thead>
<tr>
<th></th>
<th>1E3</th>
<th>HBTn-1</th>
<th>B1.1</th>
<th>PolyTn</th>
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<tr>
<td><strong>Age (y)</strong></td>
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<td></td>
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<tr>
<td>&lt;65</td>
<td>19 (83)</td>
<td>0 (0)</td>
<td>3 (13)</td>
<td>5 (22)</td>
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<td>41 (76)</td>
<td>3 (5)</td>
<td>2 (4)</td>
<td>15 (28)</td>
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<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>58 (78)</td>
<td>2 (3)</td>
<td>4 (5)</td>
<td>18 (24)</td>
</tr>
<tr>
<td>Transitional cell carcinoma</td>
<td>2 (67)</td>
<td>1 (33)</td>
<td>1 (33)</td>
<td>2 (67)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Early (I)</td>
<td>10 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Advanced (II-IV)</td>
<td>50 (75)</td>
<td>3 (4)</td>
<td>5 (7)</td>
<td>18 (27)</td>
</tr>
<tr>
<td><strong>Gleason score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-4</td>
<td>14 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (29)</td>
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<tr>
<td>5-6</td>
<td>11 (92)</td>
<td>0 (0)</td>
<td>1 (8)</td>
<td>3 (25)</td>
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<td>7-10</td>
<td>33 (69)</td>
<td>2 (4)</td>
<td>3 (6)</td>
<td>11 (23)</td>
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<td><strong>Total</strong></td>
<td>60 (78)</td>
<td>3 (4)</td>
<td>5 (6)</td>
<td>20 (26)</td>
</tr>
</tbody>
</table>

*Positive case = ≥50% of tumor cells stain positively.

*Gleason scores are not available for transitional cell carcinomas.
antigen. In particular, cross-reaction with the blood group A trisaccharide, GalNAcα1-6Gal, tri-N-acetyllactosamine, and GalNAcα was observed. Binding to each of the cross-reactive epitopes was completely inhibited by preincubation of 1E3 with clustered Tn-BSA, indicating that the cross-reactive epitopes are recognized by the same binding site as the Tn antigen. In addition, the reactivity of antibody 1E3 with asialo-OSM and OSM was unusual. OSM contains ~90% sialyl-Tn antigen along with a small amount of the Tn antigen, the TF antigen, and other minor glycans (49, 50). While other Tn-binding reagents such as HBTn-1, B1.1, and Vicia villosa isolectin B4 bind 20 to 50 times better to asialo-OSM than OSM (31, 33), antibody 1E3 bound with only a 2-fold preference for asialo-OSM. The results suggest that the antibody may bind to both the Tn antigen and the sialyl-Tn antigen (and/or mixed clusters composed of adjacent Tn and sialyl-Tn antigens); however, we could not confirm this, because a structurally defined sialyl-Tn antigen was not on our array. It should be noted that 1E3 may also bind other glycans that are not on our array.

Preparation, Affinity Purification, and Carbohydrate Microarray Evaluation of PolyTn Antibody

The results with HBTn-1 and B1.1 indicated that the Tn antigen was only expressed in a small subset of prostate tumors. Because these results were significantly different from the results with 1E3, we decided to evaluate Tn expression with a purified polyclonal antiserum to the Tn antigen. Because the antiserum would contain a mixture of Tn-binding antibodies, this reagent would likely bind the antigen in a variety of contexts. The polyclonal antibody (PolyTn) was produced by immunizing a rabbit with asialo-OSM, a glycoprotein with >90% of its carbohydrate composed of the Tn antigen (49, 50). After immunization and several booster injections, the rabbit was given two booster injections with a short Tn peptide [Ser-Tn(Ser)-Ser] conjugated to KLH to further increase Tn-specific antibody production. Serum was collected and the polyclonal antibodies were affinity purified by (a) capturing using the Tn peptide conjugated to agarose resin, (b) elution, and (c) incubating with KLH-resin to remove any remaining cross-reactive antibodies.

Next, the specificity of PolyTn was evaluated on the carbohydrate microarray. The antibody was found to bind very well to the single and clustered forms of the Tn antigen. In addition, some minimal cross-reactivity with GalNAcα, the P1 antigen, and the nonglycosylated parent peptide, Ser-Ser-Ser-Gly (see Fig. 3), was also observed.

Staining of Prostate Tumor Samples with PolyTn

Next, we examined tissue staining with PolyTn. The staining conditions were optimized using colon adenocarcinoma tissue as a positive control (Fig. 2) and confirmed with breast carcinoma tissue (see Supporting Information). On the prostate cancer array, PolyTn was found to stain 26% of the cancer samples positively and 0 of 2 normal samples. Even if the cutoff was lowered such that only 25% of the cancer cells must stain positively to assign the tumor as Tn positive, the number of positive cases only increased to 30%. Although higher than HBTn-1 and B1.1, the percentage of positive cases with PolyTn was significantly lower than the 80% to 90% observed with 1E3 in our study and the Zhang et al. study.

Discussion

Over the last 30 years, there have been considerable efforts to understand the basic biological functions of tumor-associated carbohydrate antigens and to exploit their unique expression properties as biomarkers and therapeutic targets; however, both basic and applied research have proven to be extremely challenging. One of the primary problems comes from the lack of reliable methods to measure expression of carbohydrate antigens, especially in large numbers of tissue samples. Carbohydrate expression levels are extremely difficult to measure using direct detection methods, infer from gene expression levels, or gauge from protein expression patterns. As a result, expression levels have primarily been monitored indirectly by probing binding of anti-carbohydrate antibodies and/or lectins using techniques such as immunohistochemistry and Western blotting. The quality of the information obtained from these studies depends largely on the specificity of these proteins; however, most of the antibodies and lectins have not been well characterized. Moreover, different reagents used to monitor the same antigen frequently produce conflicting results. Therefore, one of the most basic and essential pieces of information (the presence or absence of the antigen) is frequently in dispute.

A prime example of inconsistent data involves expression of the Tn antigen. The antigen is reported to be expressed on a wide range of carcinomas including breast, prostate, lung, and colon, and the Tn antigen has been evaluated extensively as a biomarker and therapeutic target (3–5). In fact, Tn-based cancer vaccines have advanced into phase I and II clinical trials for the treatment of epithelial cancers (10, 13, 17, 19–22). Nevertheless, conflicting data on the expression of the Tn antigen in various tumors such as lung, larynx, cervix, and bladder are widespread. Most notably, reports of Tn expression in prostate tumors vary from 20% to 90% of cases (23–25). Given that recent and ongoing clinical trials with Tn vaccines are being evaluated in prostate cancer patients, resolution of conflicting data in this tumor type was imperative.

Our approach for resolving inconsistent expression data involved carrying out a systematic comparison of tissue staining with multiple Tn-binding antibodies and then using carbohydrate microarray profiles of the antibodies to interpret the results. To minimize differences arising from variations in tissue sample groups, preparation, and preservation, each antibody was used to stain the same set of 77 prostate tumors. In addition, equivalent criteria for assigning a tumor as positive or negative were used for each antibody. In total, four different Tn-binding antibodies were used in the study: 1E3, HBTn-1, B1.1, and PolyTn. We found significant differences in staining between the antibodies.
Antibody 1E3 was found to stain a high percentage (78%) of prostate tumors positively, whereas antibodies HBTrn-1, B1.1, and PolyTn stained 4%, 6%, and 26% of the prostate tumors, respectively.

One of the key challenges in interpreting previous studies is the lack of information on the specificities of the antibodies and lectins used to measure Tn expression. To address this, we profiled each of the antibodies with a carbohydrate microarray. HBTrn-1 and B1.1 were found to be highly specific for the Tn antigen and the clustered Tn antigen, respectively (31, 33). PolyTn bound well to the single and clustered forms of the Tn antigen, but it also showed some minimal reactivity to the nonglycosylated parent peptide (Ser-Ser-Ser-Gly) and GaINAc-α and the P1 antigen. In contrast, antibody 1E3 cross-reacted significantly with a variety of other glycans including the blood group A trisaccharide, GaINAcα1-6Gal, tri-N-acetyllactoaminosides, and GaINAC-α. In addition, binding to both asialo-OSM and OSM suggests that 1E3 may also bind the sialyl-Tn antigen. Thus, the remarkable tumor-binding properties of 1E3 are likely the result of recognition of multiple carbohydrate antigens. Although additional studies will be required to determine the exact nature of the other antigen(s), the most notable candidate is sialyl-Tn, a well-known tumor-associated carbohydrate antigen that is expressed in a wide range of cancers including prostate tumors (23). Of the other glycans, the blood group A antigen is frequently decreased or absent on tumors, and very little is known about the expression of GaINAcα1-6Gal and tri-N-acetyllactoaminosides antigens in tumor tissue. Binding to the simple monosaccharide GaINAc-α suggests that there may be other GaINAc terminal antigens that are recognized by 1E3 such as the core 5 O-glycan.

The results have significant implications for the development and implementation of Tn-based vaccines for prostate cancer patients. Taken together, the tissue staining and microarray results indicate that the Tn antigen is expressed on only a small subset of prostate tumors and that antibody 1E3 is likely binding to a different antigen in most prostate cancers. Therefore, only a minority of prostate cancer patients express the appropriate antigen for a Tn-based vaccine. Thus, certain patients included in the clinical trials may not have been expressing the Tn antigen. Interestingly, the results from one of the two published clinical trials are consistent with this possibility. These authors found that whereas all the patients generated an immune response to the Tn antigen, only ~30% achieved a clinical response as judged by a decrease in prostate-specific antigen slope (13). The results from the second clinical trial are harder to interpret due to the use of multiple antigens (17).

Although cross-reaction of 1E3 with other antigens is a likely explanation for the tissue staining data, it is possible that 1E3 binds a specialized form of the Tn antigen that is not recognized by any of the other Tn antibodies. For example, 1E3 may bind Tn within a particular peptide sequence, peptide secondary structure, or presentation that is not recognized by the other reagents. Given that HBTrn-1, B1.1, and PolyTn recognize a wide variety of forms of the Tn antigen, this explanation seems unlikely. Nevertheless, in this case, it would be vital to structurally characterize such a specialized form to design an appropriate vaccine antigen and determine if the immune response elicited by a vaccine was targeting the specialized form of the Tn antigen.

The results in this study suggest two simple strategies for improving clinical responses with Tn-based vaccines. The objective of both approaches is to identify a more optimal patient group. First, one could preselect the subset of prostate cancer patients that is expressing the Tn antigen (or the more general form of the Tn antigen). To identify this subset, one could stain biopsy samples with multiple Tn reagents such as HBTrn-1, B1.1, and/or PolyTn and select individuals with tumors that consistently stain positive. Second, one could apply Tn vaccines to a cancer type that consistently shows Tn expression with a wide variety of Tn-binding antibodies. For example, the Tn antigen has been reported to be expressed at high levels in the majority of colon cancer tumors by many groups using a wide range of antibodies, including HBTrn-1 (39), NCC-LU-35 (39), NCC-LU-81 (40), BaGS6 (40), TEC-02 (41), 12A8-C7-F5 (42), Cu-1 (42), 1.01 (42), V. villosa isoelecin B4 (43), MLS128 (25), 10F4 (25), and 91S8 (24). Only one study, using antibody 83D4, reported low expression (30% colon cancers stained positively). With either strategy, a more optimal patient group could significantly improve clinical response rates.

Finally, our results have broad implications for the field. Several other carbohydrate-based vaccines and immunotherapeutics that target antigens such as Lewis Y, TF, Globo H, GM2, polysialic acid, sialyl Lewis A, and sialyl-Tn have undergone clinical trials, and many others have been investigated in preclinical studies. Expression of the appropriate antigen is also an important prerequisite for these vaccines. Measurement of carbohydrate antigen expression is a general problem and conflicting reports permeate throughout the field. The lack of reliable, consistent expression information is a fundamental problem for basic research, biomarker development, and therapeutic development. The advent of carbohydrate microarray technology enables new approaches for addressing these problems. In particular, the approach described in this article of analyzing antigen expression with multiple antibodies and using carbohydrate microarray profiles to interpret the results provides a simple and convenient avenue for resolving conflicting data and selecting appropriate patients for clinical trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Tn Antigen Expression in Prostate Cancer

References


28. de Paz JI, Seebeger PH. Recent advances in carbohydrate microarrays. QSR Com Sci 2006;25:1027–32.


36. Inufusa H, Kojima N, Yasutomi M, Hakomori S. Human lung adenocarcinoma cell lines with different lung colonization potential (LCP), and a correlation between expression of sialosyl dimeric LeX (defined by MA6 FH6) and LCP. Clin Exp Metastasis 1991;9:245–57.


41. Cao Y, Schlag PM, Karsten U. Immunodetection of epithelial mucin (MUC1, MUC3) and mucin-associated glycoproteins (TF, Tn, and sialosyl-Tn) in benign and malignant lesions of colon epithelium: apolar localization corresponds to malignant transformation. Virchows Arch 1997;431:159–66.


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