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Cancer Biomarkers Defined by Autoantibody Signatures to Aberrant O-Glycopeptide Epitopes

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Abstract

Autoantibodies to cancer antigens hold promise as biomarkers for early detection of cancer. Proteins that are aberrantly processed in cancer cells are likely to present autoantibody targets. The extracellular mucin MUC1 is overexpressed and aberrantly glycosylated in many cancers; thus, we evaluated whether autoantibodies generated to aberrant O-glycoforms of MUC1 might serve as sensitive diagnostic biomarkers for cancer. Using an antibody-based glycoprofiling ELISA assay, we documented that aberrant truncated glycoforms were not detected in sera of cancer patients. An O-glycopeptide microarray was developed that detected IgG antibodies to aberrant O-glycopeptide epitopes in patients vaccinated with a keyhole limpet hemocyanin-conjugated truncated MUC1 peptide. We detected cancer-associated IgG autoantibodies in sera from breast, ovarian, and prostate cancer patients against different aberrant O-glycopeptide epitopes derived from MUC1. These autoantibodies represent a previously unaddressed source of sensitive biomarkers for early detection of cancer. The methods we have developed for chemoenzymatic synthesis of O-glycopeptides on microarrays may allow for broader mining of the entire cancer O-glycopeptidome. *Cancer Res*; 70(4); 1306–13. ©2010 AACR.

Introduction

Cancer-associated autoantibodies represent appealing biomarkers. Autoantibodies may develop early in carcinogenesis when tumor-associated antigens appear on premalignant or malignant lesions. Antibody responses can produce relatively high concentrations in circulation with a long circulation time, and they can be detected with sensitive and specific methods (1, 2). In contrast, antigens produced by small premalignant or malignant lesions are generally produced in vanishingly small levels, which, due to dilution and clearance from blood, may not be detectable. The discovery of specific autoantibodies to cancer antigens has been undertaken through different approaches.

Classic studies identified autoantibodies reactive with tumor cells, tissues, or isolated proteins. Analysis of human monoclonal antibodies (mAb) with cancer-selective reactivity has generally identified IgM antibodies reactive with carbohydrate epitopes (3) or glycosylation-dependent epitopes (4). Proteome-wide screening techniques include expressed cDNA libraries (SEREX; ref. 5), protein and peptide arrays (6, 7), random or designed phage displays (8), and more recently self-assembling protein arrays (9, 10). Cancer-associated autoantibodies characterized to date have been found to bind intracellular proteins with functions important in cell cycle regulation, such as GPR78 (8), p53 (11), NY-ESO-1 (12), and CDC25 (13), but also some cell membrane glycoproteins such as MUC1 (14), HER2 (15), and mesothelin (16). In the case of p53, the induction of autoantibodies is thought to be induced by increased expression and presentation caused by somatic mutations, although these mutations are often not included in the epitopes bound by antibodies (17). Autoantibodies to cell membrane and secreted proteins appear to be underrepresented, which may be due to the low frequency of somatic mutations in these genes (18) combined with the need for immunologic tolerance to exposed self-proteins. Another contributing factor may be related to technical and experimental limitations, as most broad proteomic screens conducted to date were designed to interrogate the proteome in the absence of posttranslational modifications, and in particular without unique posttranslational modifications associated with cancer. Although the latter problem is widely recognized by investigators in the field, there have been few solutions brought forward.

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Malignant transformation of cells is always accompanied by alterations in posttranslational modifications of proteins, and a well-documented example hereof is the abundant mucin-type O-glycosylation found on mucins and other O-glycoproteins (19). Tumor-associated changes in the types and levels of mucins expressed as well as their aberrant glycosylation create a diverse set of unusual molecular structures found on the surface of cancer cells and in secretions. These molecular structures generally represent glycoproteins with truncated immature O-glycans, which may only exist as brief biosynthetic intermediates in the early Golgi apparatus of normal cells. The immune system may thus not be exposed to these structures, and lack of immunologic tolerance may provoke both autoantibodies and cell-mediated immunity when expressed by cancer cells. We therefore hypothesized that autoimmunity to cancer antigens would be directed to cancer-specific epitopes generated by the combination of the normal protein backbone and aberrant O-glycosylation. The MUC1 mucin is heavily O-glycosylated in a large 20-amino-acid tandem repeated region, which is aberrantly glycosylated in cancer (20) and can be detected in serum of late-stage cancer patients (21, 22). Man is in general immunologically tolerant to the MUC1 tandem repeat protein core and its normal glycosylated forms (20, 23), although several studies have shown that up to 10% to 15% of healthy controls and cancer patients have antibody reactivity to MUC1 peptides as detected by ELISA assays; however, the detected levels are very low and have led to contradictory results by different investigators (24–27). We recently identified an immunodominant O-glycopeptide epitope (Tn/STn-MUC1) that results from aberrant glycosylation and for which there does not appear to be immunologic tolerance (refs. 28–30; see Supplementary Fig. S1A for structures of O-glycans). We have also shown that the cancer-associated Tn-glycoform of MUC1 is selectively taken up by dendritic cells and delivered to the MHC class I and II pathways (31), and the Tn-MUC1 glycoform induces potent IgG responses in MUC1 transgenic mice (28) and cancer patients (30). Importantly, the elicited immune response is specifically directed to the combined glycopeptide epitope with little or no antibody specificity for the Tn carbohydrate hapten. The Tn/STn-MUC1 glycopeptide epitope is broadly expressed in essentially all breast cancers as well as many other adenocarcinomas (29). Cancer-associated O-glycopeptide epitopes from MUC1 therefore represent likely candidates for natural autoantibodies induced in cancer patients.

In this report, we present a versatile chemoenzymatic approach to produce libraries of cancer-associated O-glycopeptides that, combined with a microarray platform, allows high-throughput detection of autoantibodies to the O-glycopeptidome. Analysis of such an array with a glycopeptide library derived from MUC1 showed sensitive detection of cancer-associated autoantibodies to distinct O-glycopeptide epitopes.

Materials and Methods

Human sera. Human sera were obtained from the following sources after written informed consent was acquired (the

protocol used was approved by Memorial Sloan-Kettering Institutional Review Board and Food and Drug Administration): preimmune and postvaccination (five s.c. injections biweekly of 2 to 4 μ g 25Tn-106mer-MUC1-KLH conjugate) sera from $n = 20$ breast cancer patients (stage III/IV after treatment and disease-free) enrolled in a phase I study. Sera from breast cancer ($n = 28$), ovarian cancer ($n = 20$), and prostate cancer ($n = 10$) patients collected close to time of first diagnosis of cancer and before treatment, including surgery, were obtained from Asterand, Inc. (six ovarian sera were from the Cooperative Human Tissue Network; Supplementary Table S1). Age and sex matched healthy control sera ($n = 39$) were obtained from Asterand, Inc. All sera from Asterand were collected and stored following the same standard operating procedure that involved clotting for 30 min and freezing within 60 min of collection. A second set of healthy control sera ($n = 33$) was obtained from blood donors.

Chemoenzymatic synthesis of O-glycopeptides. Peptides were synthesized and O-glycosylated *in vitro* using recombinant glycosyltransferases as previously described (28). Briefly, different polypeptide GalNAc-transferase isoforms were used to direct GalNAc O-glycan occupancies on peptides and core 1 β 3GalT, core 3 β 3GlcNAc-T, and ST6GalNAc-I were used to produce T, core 3, and STn glycoforms (see Supplementary Fig. S1 for the structures of glycopeptides). All glycopeptides were purified by high performance liquid chromatography and characterized by matrix-assisted laser desorption/ionization—time-of-flight. The MUC1 glycopeptides were essentially homogenous compounds with 6, 9, or 15 O-glycans attached, except that the 15 O-glycan MUC1 glycopeptide contained a mixture of 14 to 15 O-glycans as reported previously (28).

O-Glycopeptide array print and analysis. (Glyco)peptides and control structures were printed on Schott Nexterion Slide H or Schott Nexterion Slide H MPX 16 (Schott AG). Quadruplicates of all compounds were printed at 20, 5, and 1 μ mol/L in 150 mmol/L sodium phosphate (pH 8.5) with 0.005% CHAPS and printed on a BioRobotics MicroGrid II spotter (Genomics Solution) with a 0.21-mm pitch using Stealth 3B Micro Spotting Pins (Telechem International ArrayIt Division). Ovine submaxillary mucin (OSM; Isosep) and its desialylated counterpart asialo-OSM (AOSM) were printed as controls at 10, 2.5, and 0.5 μ mol/L. After printing, slides were incubated for 1 h in a humidified hybridization chamber with 70% to 100% relative humidity and stored until use at 4°C. Before use, unspotted slide areas were blocked for 1 h with 25 mmol/L ethanolamine in 100 mmol/L sodium borate (pH 8.5). Human sera serially diluted from 1:25 to 1:400 or mAbs (1 μ g/mL or hybridoma supernatants) were incubated in a closed container with gentle agitation for 1 h, washed three times in PBS with 0.05% Tween 20 (PBS-T), and followed by 1-h incubation with appropriate secondary antibodies. Human IgM and IgG antibodies were detected with Cy3-conjugated goat anti-human IgG (Fc-specific) and goat anti-human IgM (Sigma) diluted 1:5,000 in PBS-T. Murine mAbs were detected with Cy3-conjugated goat anti-mouse IgM (μ chain-specific) and

goat anti-mouse IgG (H+L) (The Jackson Laboratory) diluted 1:5,000. After washing, slides were rinsed shortly in H₂O, dried by centrifugation (200 × g), and scanned in a ProScanArray HT Microarray Scanner (Perkin-Elmer) followed by image analysis with ProScanArray Express 4.0 software (Perkin-Elmer). For comparison, slides were scanned with identical scanning parameters calculated by automatic sensitivity calibration. Data were analyzed and plotted using Microsoft Excel or GraphPad Prism software.

Results

A prerequisite for the existence of circulating autoantibodies would presumably be that the corresponding circulating antigen would be found in immune complexes and/or removed from circulation. We therefore developed and tested highly sensitive ELISA glycoprofiling assays for the two well-known cancer-associated mucins, MUC1 detected in the CA15-3 serum assay and MUC16 detected in the CA125 assay (Supplementary Data). We were essentially unable to detect the short truncated cancer glycoforms of either of these mucins in circulation, and the detectable glycoforms included only sialylated core 1 and complex-type O-glycans (Supplementary Figs. S1 and S2). This is in agreement with recent studies showing that cancer mucins without sialic acids are removed by scavenger lectin receptors on liver macrophages (32). The glycoprofiling results using mAbs with well-defined specificities (33) showed that circulating MUC1 does not include the aberrant Tn, STn, and T glycoforms and opens for the possibility that circulating autoantibodies directed to such glycoforms participate in their removal in addition to lectin receptors.

The primary aim of this study was therefore to develop a discovery platform for selective detection of cancer-associated autoantibodies directed to aberrant O-glycopeptide epitopes without interference by anticarbohydrate hapten antibodies. The size of the potential O-glycopeptidome is vast, with thousands of distinct O-glycoproteins combined with numerous distinct aberrant O-glycan structures. We have, for that reason, developed a toolbox of recombinant glycosyltransferase enzymes that allow enzymatic synthesis of diverse O-glycan structures with some degree of control of sites of attachment of O-glycans at serine and threonine residues in peptides (28). The first step involves site-directed synthesis of GalNAc-glycopeptides using a panel of human polypeptide GalNAc-transferases with different peptide substrate specificities by which a high degree of control of sites of O-glycan attachment to peptides can be achieved. Subsequent steps use a panel of galactosyltransferases, GlcNAc-transferases, and sialyltransferases to produce truncated O-glycan structures associated with cancer. These reagents were used to synthesize a library of MUC1 cancer-associated glycopeptides in solution using a synthetic 60-mer tandem repeat peptide as substrate (Fig. 1; ref. 28). To accommodate large glycopeptide libraries, we chose to use a slide microarray format on NHS-activated hydrogel slides, as Blixt and colleagues (34, 35) have shown that these provide a remarkably low background for detection of

human serum antibodies. We based our initial microarray on MUC1 and control peptides given the evidence that aberrant glycosylation of MUC1 is associated with the progression of a number of different adenocarcinomas; however, the available toolbox could be applied to peptides from any O-glycoprotein. The conditions for the array assay were standardized by using a panel of mAbs with peptide, carbohydrate hapten, and O-glycopeptide specificities, which confirmed quality and specificity of the array assay (Fig. 1A).

Subsequently, the utility of the assay was tested by analysis of human sera derived from a pilot clinical trial in which a MUC1 Tn-glycopeptide vaccine was administered to 20 breast cancer patients who had undergone radical treatment and were classified as disease-free. Patients received up to five injections of a 106-mer MUC1 tandem repeat peptide with 25Tn O-glycans conjugated to keyhole limpet hemocyanin (KLH) with trial design and protocol essentially as previously reported (30). Results of ELISA assays showed that patients had no preexisting IgG antibodies and all developed detectable IgG Tn-MUC1 glycopeptide antibodies after vaccination (30). Analysis of these sera with the O-glycopeptide array confirmed these results and showed that there was induction of IgG antibodies reactive with only the Tn-MUC1 glycopeptides with at least two O-glycans in the immunodominant -GSTAP- epitope, i.e., the 9Tn and 15Tn-MUC1 and not the 6Tn-MUC1 glycoforms (Fig. 1C). In five subjects, antibodies to STn-MUC1 were also detected. The induced antibodies did not cross-react with Tn or STn haptens, as evidenced by the lack of reactivity with other Tn and STn glycopeptides. This reactivity pattern is similar to that of the cancer-specific 5E5 antibody, except that these antibodies also react with a single O-glycan in the -GSTAP- epitope (28). Some reactivity to the truncated core 3 MUC1 glycoform (GlcNAc β 1-3GalNAc α 1-O-Ser/Thr) was also observed, and these may have resulted from "epitope spreading." The analysis of IgM antibodies revealed, as expected, broad reactivity with all glycoforms of the glycopeptides, which makes it impossible to discern potential O-glycopeptide-specific antibodies. Carbohydrate-hapten-specific antibodies are generally of the IgM isotype, although examples of anti-STn IgG are found (36). As shown in Fig. 1B, sera from preimmune and vaccinated patients have abundant IgM antibodies to Tn and STn carbohydrate haptens, which exhibited no apparent preference for the peptide backbone. The levels of IgM antibodies to Tn and STn structures were enhanced by the Tn-MUC1 vaccination, but no IgG antibodies to Tn and STn hapten were detected (indicated by lack of reactivity with MUC2 glycopeptides and AOSM/OSM), in agreement with previous ELISA analyses (30). Some IgM antibodies reactive with the unglycosylated MUC1 as well as MUC2 peptides were also observed. Thus, the IgM carbohydrate hapten antibodies made it impossible to discern antibodies with distinct O-glycopeptide specificity. These results stress the importance of using assay methods that allow selective detection of combined glycopeptide epitopes.

The O-glycopeptide array was then used to evaluate the existence of natural autoantibodies in healthy controls and

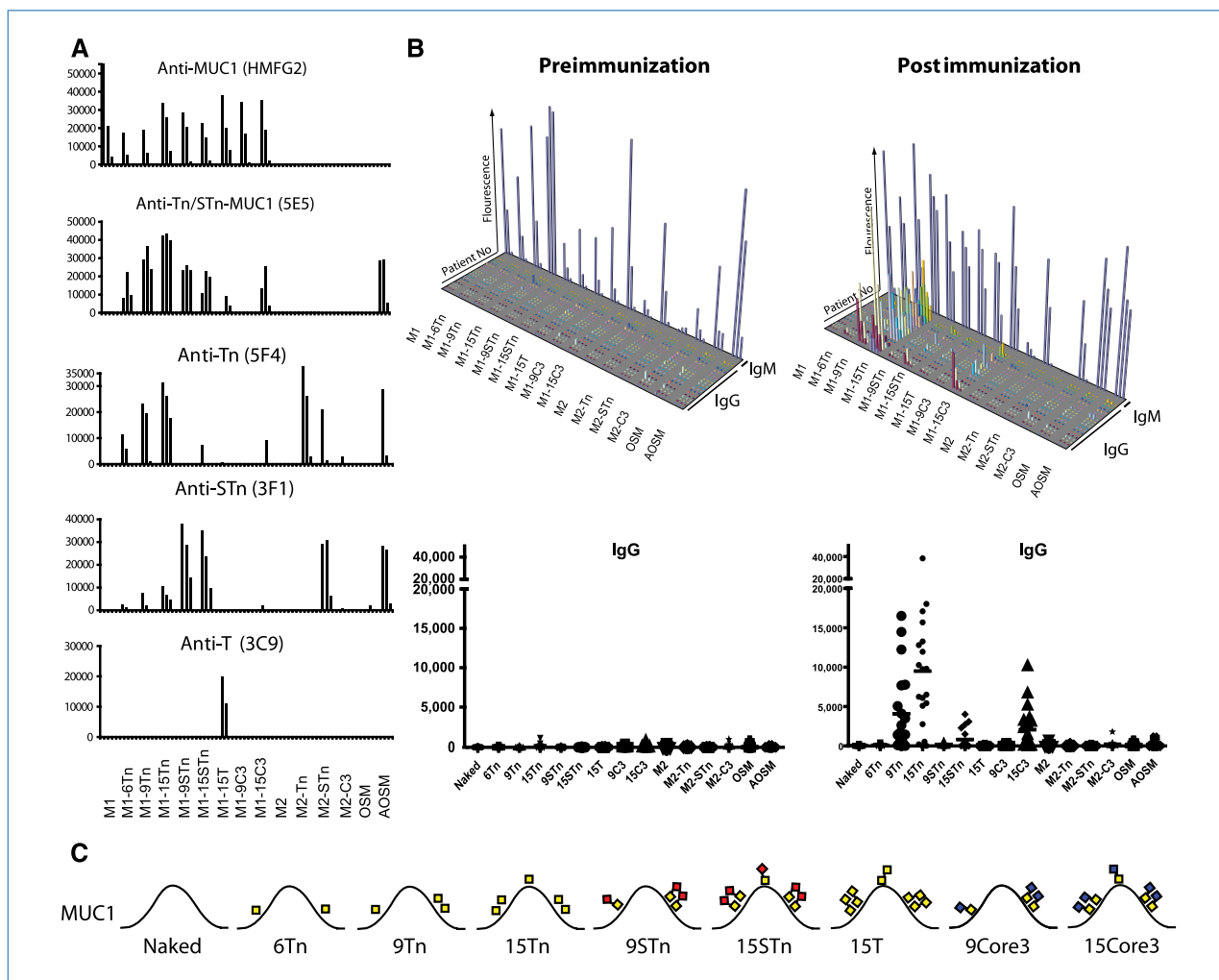


Figure 1. Microarray platform for detection of MUC1 O-glycopeptide-specific antibodies. Immunization of cancer patients with GalNAc-MUC1 break tolerance eliciting glycopeptide-specific antibodies. A, GalNAc-glycosylated variants of MUC1 (6Tn, 9Tn, and 15Tn) were synthesized chemoenzymatically using recombinant polypeptide GalNAc-transferases [GalNAc-T2, GalNAc-T4, and GalNAc-T11; yellow boxes indicate the position of GalNAc in each of the three MUC1 repeats in the produced 60-mer MUC1 (glyco)peptides]. Further elongation was carried out by β 3GnT6, c1Gal-T, or ST6GalNAc-T1, creating truncated core 3, core 1, and STn structures, respectively. MUC2 glycopeptides were synthesized by similar methods. Glycopeptides and control structures were printed on NHS-activated hydrogel slides in quadruplicates at three different concentrations. The glycopeptide array was qualified by incubation with glycan and glycopeptide-specific mAbs detected by Cy3-conjugated secondary antibodies and expressed as fluorescence intensity visualized in column diagram. Anti-MUC1 peptide mAb HMF2 recognized both nonglycosylated (designated M1) and all glycoforms of MUC1, confirming efficient printing of all MUC1 compounds. The glycopeptide-specific mAb 5E5 recognizes the immunodominant Tn/STn-MUC1 epitope -GSTAP- with one GalNAc residue in threonine (6Tn-MUC1) or both serine and threonine (9/15Tn-MUC1). In contrast, the carbohydrate hapten antibodies recognizing Tn (GalNAc α 1-O-Ser/Thr) mAb (5F4) and STn (NeuAc α 2-6GalNAc α 1-O-Ser/Thr) mAb (3F1) react regardless of peptide backbone, as shown by reactivity with the corresponding glycoforms of MUC2 (designated M2) and the Tn and STn glycosylated mucins AOSM and OSM. Finally, the anti-T (Gal β 1-3GalNAc α 1-O-Ser/Thr) mAb reacts specifically with T-MUC1. B, three-dimensional-column diagram (top) and dot-plot presentation (bottom) showing results of analysis of human sera from a clinical trial of a MUC1 glycopeptide vaccine in breast cancer patients ($n = 20$) immunized with 106-mer MUC1 tandem repeat peptide with 25Tn O-glycans conjugated to KLH. The immunogen corresponds to the 15Tn MUC1 60-mer glycopeptide used in this study with Tn residues at all five potential O-glycosylation sites in the 20-mer tandem repeated peptide. The array was incubated with diluted (1:25) serum followed by incubation with IgG- and IgM-specific secondary Cy3-conjugated antibodies. Results from all 20 subjects analyzed for IgG were shown in rows 1 to 20 with glycopeptides as indicated. Prevacination sera showed essentially no IgG antibodies, whereas vaccination with the 25Tn MUC1 vaccine induced IgG antibodies specifically reactive with the 9Tn and 15Tn MUC1 glycopeptides but not unglycosylated MUC1. Results from one subject analyzed for IgM is shown in the last row. Preimmune and vaccinated patients have abundant IgM antibodies to Tn, STn, and T carbohydrate haptens. This underscores the importance of selective detection of IgG responses to identify true combined glycopeptide epitopes. Values above three times the SD of the average values obtained with sera before vaccination were considered positive. A total of 18 out of the 20 subjects who were vaccinated showed induction of IgG autoantibodies to 9Tn and 15Tn MUC1 glycopeptides. These glycopeptides have in common two O-glycans in the immunodominant -GSTAP- epitope, suggesting that this is the reactive epitope. Partial reactivity was seen with STn-MUC1 and core 3 MUC1 in five and seven immunized patients, respectively. No reactivity was seen with Tn and STn MUC2 glycopeptides or AOSM and OSM. C, the graphical presentation of MUC1 glycoforms shows one 20-amino-acid MUC1 tandem repeat sequence (HGVTSAPDTRPAGSTAPPA) with glycans, using symbols as in legend to Supplementary Fig. S1.

newly diagnosed patients with breast, ovarian, and prostate cancer (Fig. 2). Analysis of IgM antibodies yielded broad carbohydrate reactivity without discernable O-glycopeptide specificities in both healthy controls and cancer patients similar to results in Fig. 1. In striking contrast, IgG antibodies from healthy control sera were essentially unreactive with the glycopeptides, whereas specific IgG antibodies to Tn-MUC1, STn-MUC1, and truncated core 3 O-glycopeptides were identified in sera from newly diagnosed breast, ovarian, and prostate cancer patients (Fig. 2A, bottom panels). These cancer-associated IgG antibodies are directed to combination O-glycopeptide epitopes dependent on the glycoform and the peptide sequence, because they did not react with other glycopeptides with the same glycoforms. Three distinct MUC1 O-glycopeptide epitopes with different O-glycan structures (Tn, STn, and core 3) reactive with IgG were identified, and there were different but partially overlapping distributions of these specificities in patients. Cross-inhibition studies confirmed that these were at least partially distinct epitopes in the different patients (not shown). A selection of sera were tested in multiple dilutions (1:25, 1:100, 1:200, 1:1,000, and 1:3,000), demonstrating quite variable titers of antibodies from 1:100 to more than 1:3,000. In most sera, the autoantibody reactivity declined with dilutions over 1:200, whereas a few sera could be diluted 1:3,000 without substantial change in reactivity (not shown). The array analysis did not reveal substantial autoantibodies to unglycosylated MUC1, which is in contrast to some previous reports using ELISA assays (24–27).

Discussion

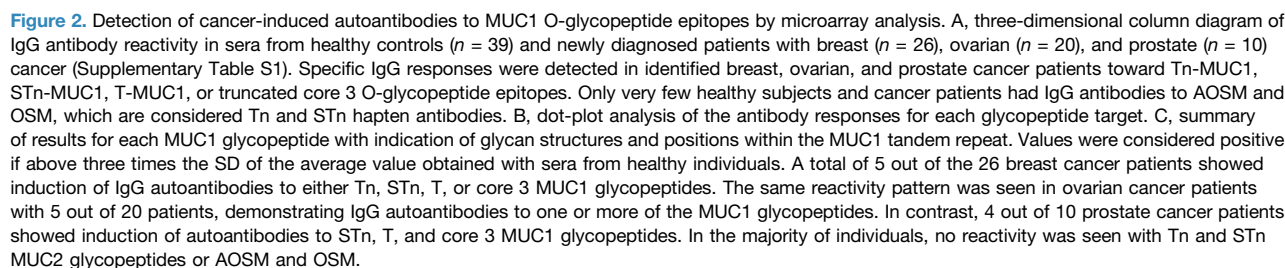
The present study tested and confirmed the hypothesis that autoantibodies to aberrant O-glycopeptide epitopes represent a fruitful source of sensitive biomarkers for early detection of cancer. Cancer-associated IgG autoantibodies to several O-glycopeptide epitopes were identified in MUC1, whereas IgG antibodies to peptide epitopes were not detected. The study therefore clearly supports that autoantibody biomarker discovery strategies should include aberrant posttranslational modifications for greatest success. Chemoenzymatic synthesis of cancer-associated O-glycopeptides in combination with a microarray platform was shown to be a feasible strategy for broader analysis of the entire cancer O-glycopeptidome.

Initially, we found an absence of immature nonsialylated MUC1 and MUC16 glycoforms in serum of cancer patients with elevated mucin levels, suggesting that these glycoforms are removed by immune cells or scavenger receptors recognizing immature uncapped glycans. This is in agreement with Varki and coworkers' (32) "tip of the iceberg" theory, proposing that circulating tumor glycoprotein antigens, including cancer mucins without sialic acids, are removed by scavenger receptors and hence inherently represent insensitive biomarkers. This selective clearance provides a plausible mechanism by which aberrant glycoforms of mucins and O-glycoproteins are presented to and stimulate the immune system (31). MUC1 is also considered a prime candidate for

immunotherapies, and antibody-based therapies may take advantage of targeting the cancer-associated glycoforms of MUC1 as there will be limited inhibition of targeting by circulating antigens.

Detection of autoantibodies to O-glycopeptide combination epitopes, which include both the peptide backbone as well as cancer-associated posttranslational modification hapten structures, is complicated by the presence of rather ubiquitous natural antibodies to the carbohydrate haptens. It is well established that non-self carbohydrate structures are immunogenic in man, and natural antibodies to the cancer-associated truncated carbohydrate haptens T, Tn, and STn are among the most widely studied antibodies (37). Although titers of antibodies to Tn and STn appear to be increased in cancer patients, essentially all individuals have IgM antibodies with some specificity to these structures. Recent studies of the specificity of antibodies to the Globo-H carbohydrate hapten using a novel glycan array show similar results (38). It has been shown that some anti-carbohydrate antibodies result from exposure to related structures found in gut microflora (39); however, expression of aberrant glycosylated glycoproteins in cancer are believed to contribute as well (37). The clinical significance of these antibodies in relation to cancer is largely unknown, but they have not emerged as truly useful biomarkers of cancers. In contrast, antibodies directed to combined O-glycopeptide epitopes, which would have specificity for distinct proteins carrying aberrant cancer O-glycans, are expected to have high affinity, be class switched to IgG, and be absent in healthy individuals (28, 29). We have previously shown that several different Tn-glycopeptides generate glycopeptide-specific IgG antibodies and not carbohydrate hapten antibodies (40). More recently, a protective cancer-specific autoantibody in a spontaneous mouse cancer model was shown to be directed to a Tn-glycopeptide epitope of the mouse orthologue of human podoplanin, OTS8 (41). The current results clearly show that it is possible to detect autoantibodies to O-glycopeptide epitopes on glycopeptide arrays by limiting the detection to IgG subclass without interference by carbohydrate hapten antibodies.

The discovery platform for human autoantibodies was developed on microarray hydrogel slides for high-throughput analysis, for minimum consumption of compounds, and because hydrogel slides provide a remarkably low background for detection of human serum antibodies (35). The array was qualified with a panel of mAbs and lectins and by demonstration of sensitive detection of vaccine-induced IgG antibodies specific for Tn/STn-MUC1. Application of the array on sera with newly diagnosed cancers revealed IgG autoantibodies directed to three distinct O-glycopeptide epitopes, Tn-MUC1, STn-MUC1, and truncated core 3-MUC1, which were not found in two sets of healthy control sera (Fig. 2A, bottom). Future studies are needed to address the occurrence of these autoantibodies in benign diseases of differential diagnostic interest. We have performed a larger study of colorectal cancer in which comparative sera from inflammatory disease controls were available. The identified autoantibodies were



Induction of autoantibodies directed to Tn and STn-MUC1 in breast, ovarian, and prostate cancers are in agreement with our understanding of the expression pattern of MUC1 and the cancer-associated glycoforms Tn and STn. The genetic and biosynthetic basis for expression of these glycans may relate to somatic mutations in *Cosmc*, a chaperone for the enzyme C1 β 3Gal-T enzyme that controls synthesis of the common core 1 O-glycosylation pathway (see Supplementary Fig. S1); however, overexpression of the ST6GalNAc-I sialyl-

⁵ J.W. Pedersen et al., manuscript in preparation.

Interestingly, overexpression of β 3GnT6 in cancer cells suppresses the metastatic phenotype, and mice deficient in this enzyme spontaneously develop colon cancer (47).

In contrast to some previous reports using ELISA assays (24–27), only minimal reactivity was seen against nonglycosylated MUC1. The reason for this discrepancy is not clear at present, but the array analysis shows very weak signals under the cutoff for the analysis in several sera in addition to the one strongly positive ovarian cancer patient. Regardless, the identified MUC1 glycopeptide autoantibody targets produce much more robust signals with high cancer specificity. It is clearly of importance to understand the timing and dynamics of such autoantibodies, as stressed by the lack of MUC1 autoantibodies in serum obtained before vaccination in the 20 treated disease-free breast cancer patients analyzed in Fig. 1. This finding may suggest that the MUC1 glycoform antibodies disappear as the disease progresses and/or after treatment. This would be in agreement with studies of p53 autoantibodies that may reappear at relapse (48). Other studies of autoantibodies to p53 have shown that these may occur before diagnosis of cancer (49), which provide support for using autoantibodies as sensitive and early biomarkers.

In conclusion, our study shows that the aberrantly glycosylated O-glycopeptidome has great potential as a source of targets for cancer-induced autoantibodies. We present what, to our knowledge, is the first evidence of cancer-associated autoantibodies to defined O-glycopeptide epitopes, which should provide bases for further exploration of similar targets. Our proof-of-concept study using MUC1 as a model identified three distinct cancer-associated IgG autoantibodies directed to distinct O-glycopeptide epitopes. The prevalence of the combination of these MUC1 biomarkers in cancer were similar or higher than those found for other identified autoantibodies to protein epitopes, and all have very low prevalence in healthy controls. None of these biomarker targets individually approach levels of sensitivity for clinically useful biomarker assays; however, we envision that these, combined with similar targets from other O-glycoproteins altered in cancer, will eventually reach acceptable levels of sensitivity. The O-glycopeptidome in man is currently poorly understood due to lack of predictive consensus sequence motifs for protein O-glycosylation; however, there are hundreds, if not thousands, of O-glycoproteins in the human proteome. Sites of O-glycosylation is directed by

up to 20 human polypeptide GalNAc-transferases with different substrate specificities, which allow for high control of glycosylation patterns of proteins in cells and at the same time high probability for altered patterns of O-glycan decorations in cancer cells. Our strategy for discovery of further cancer-associated O-glycopeptide epitopes recognized by autoantibodies will be to produce large glycopeptide arrays decorated with O-glycans produced by *in vitro* O-glycosylation with different polypeptide GalNAc-transferases in combination with other glycosyltransferases. This should enable data mining in this large and unexplored field of potential biomarkers. Whereas MUC1 is expressed in many common adenocarcinomas, which correlates with our finding of MUC1 autoantibodies in several types of cancer, we envision that other glycoproteins with more restricted organ- and cancer-type expression patterns may eventually aid in developing disease- and organ-specific autoantibody signatures with high sensitivity and specificity. The concept of autoantibody signatures can be extended to many other posttranslational protein modifications that may be altered in disease and serve as biomarkers.

Disclosure of Potential Conflicts of Interest

M.A. Tarp, U. Mandel, J. Taylor-Papadimitriou, Joy Burchell, and H. Clausen are inventors of a patent application, WO 2008/040362: Generation of a cancer-specific immune response toward MUC1 and cancer-specific MUC1 antibodies. Published 10.04.2008. The other authors declared no potential conflict of interest.

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Supplemental Data

Auto-antibody signatures to aberrant O-glycopeptide epitopes serve as undiscovered biomarkers of cancer

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Results

Detection and glycoprofiling of circulating mucins. Since MUC1 does circulate and serve as a biomarker for late stage cancers, we sought to test if aberrant glycoforms of MUC1 and another prominent cancer biomarker mucin, MUC16, were found in circulation. We developed a highly sensitive ELISA assay (detection limit < 1pg/mL) for detection of the Tn-MUC1 epitope using the cancer-specific monoclonal antibody 5E5. Importantly, sera positive for circulating MUC1 as evaluated with the standard CA15-3 assay were essentially negative for the Tn/STn-MUC1 glycoform (**Fig. S1B**). In contrast, the same assay was used to demonstrate that a number of breast cancer cell lines secreted these glycoforms (**Fig. S2**). Because the Tn/STn-MUC1 glycoform is highly expressed in breast cancers and other cancers overexpressing MUC1 (1), the most likely explanation for the selective absence of Tn/STn-MUC1 in serum is their selective removal by immune cells or scavenger receptors recognizing immature uncapped glycans (2). To further explore this possibility we developed a glycoprofiling ELISA assay using monoclonal antibodies to characterize glycoforms of both MUC1 and MUC16 (detected in the CA125 biomarker assay) produced and secreted by cancer cells and those found in serum. Monoclonal antibodies to mucin core proteins and defined carbohydrate hapten structures were used in combination with an exoglycosidase to demask underlying carbohydrate structures. While cancer cells produce and secrete aberrantly glycosylated glycoforms of MUC1 and MUC16, circulating serum MUC1 and MUC16 only contained fully sialylated glycoforms without exposure of Tn, STn, T and unsubstituted (poly)-N-acetyl-lactosamine (**Fig. S1B and C**). We tested two independent sets of breast cancer sera for MUC1; One set, taken at time of first diagnosis, had no positive sera, and another set, collected at late stages of disease after failed therapies, had 9/22

positive sera with the licensed CA15-3 assay as well as by our in-house capture assay. Complete correlation existed between serum samples in which MUC1 or MUC16 were detected and samples in which exposed T and/or lactosamine after desialylation were detected on captured serum MUC1 and MUC16. There was no detectable expression of unsialylated T or LacNAc (**Fig. S1**), and only the HMFG2/5E5 assay, which has considerably higher sensitivity than the HMFG2/HMFG2 assay in detection of Tn/STn-MUC1 (**Fig. S1B**), detected low levels in two of the 9 MUC1 positive sera. Previous glycoprofiling studies have generally utilized lectins to detect exposed glycoforms on antibody captured serum glycoproteins including MUC1, but the use of monoclonal antibodies with well defined specificities provides greatly improved structural interpretation and eliminates problems with lectin reactivity with capture antibodies (3). These results therefore confirm that circulating MUC1 does not include the aberrant Tn, STn and T glycoforms, which opens the possibility that circulating auto-antibodies directed to such glycoforms participate in their removal in addition to lectin receptors.

Methods

Glycoprofiling MUC1 (CA15-3) and MUC16 (CA125). CA15-3 and CA125 determinations were produced by licensed facilities (Statens Serum Institute, Denmark). Glycoprofiling was performed by a capture ELISA using Nunc-Immuno MaxiSorp F96 plates (Nunc) coated with 1µg/mL HMFG2 (MUC1) or M11 (MUC16) in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Plates were blocked with BSA/Triton X-100 buffer (1% BSA, 1% Triton X-100, 3 mM KCl, 0.5 M NaCl, and 8 mM phosphate buffer (pH 7.4)) for 1 h at RT and incubated with serially diluted sera or spend culture medium and cell lysates from cell lines for 2 hrs at RT followed by washing. When neuraminidase treatment (0.1 U/mL Chlostridium Perfringes VI (Sigma) was performed for 1 hr at 37°C a second blocking step was included for 15 min followed by washing. Culture supernatants of mouse monoclonal antibodies IgM 3C9 (T), 5F4 (Tn) and 1B2 (N-acetyl-Lactosamine) (4;5) were applied for 1 hr followed by rabbit anti-mouse IgM HRP-conjugated antibody (Southern Biotech). Plates were developed with TMB+ one-step substrate system (Dako), reactions stopped with 0.5 M H₂SO₄, and read at 450 nm.

Fig. S1

Profiling O-glycosylation on circulating cancer mucin biomarkers. (A) Scheme depicting common protein O-glycosylation pathways. The simplest structures (boxed) are considered aberrant O-glycans with cancer-associated expression. (B) Glyco-profiling of serum MUC1 from late stage cancer patients by capture ELISA. Values obtained with licensed CA15-3 assay for 22 patients are shown in upper panel with 9 subjects having values above cutoff (>32 AU). HMFG2 represents an in-house ELISA assay using anti-MUC1 MAb HMFG2 as capture and biotinylated HMFG2 for detection. This assay identified the same 9/22 subjects as the licensed CA15-3 assay demonstrating efficient capture of MUC1. Tn/STn represents the selective detection Tn/STn-MUC1 glycoforms by the MAb 5E5 on MUC1 captured by HMFG2. Although the 5E5 assay has greater sensitivity than the biotinylated HMFG2 assay with superior detection of the Tn-MUC1 control (PC), only two sera showed weak reactivity 1-2 fold over background indicating almost complete absence of Tn and STn glycoforms of MUC1 in serum. T and ST represents detection of HMFG2 captured MUC1 by the anti-T MAb 3C9 without and after pretreatment of captured MUC1 with neuraminidase to remove sialic acids. No exposed T was identified, but after neuraminidase treatment all MUC1 positive sera except the one with lowest levels of MUC1 were strongly reactive for T suggesting that circulating MUC1 carries fully sialylated core 1 structures. LacNAc and SLacNAc detected by anti-LacNAc MAb 1B2 shows similar to T and ST selective detection of sialylated type 2 chain lactosamine chains likely associated with core 2 O-glycans and/or N-linked glycans on MUC1. (C) Glyco-profiling capture ELISA assay of MUC16 found in serum from ovarian cancer patients. Values obtained with the licensed CA125 assay for 12 patients are shown in upper panel with 7/12 subjects having values above cutoff (>32 AU). Tn and STn represent our ELISA using anti-MUC16 MAb M11 for capture and anti-Tn MAb 5F4 for detection of Tn without and after pretreatment of captured MUC16 with neuraminidase to remove sialic acids. MUC16 carrying Tn or STn were not identified in any subjects. T and ST represents detection of M11 captured MUC16 by the anti-T MAb 3C9 without and after pretreatment of captured MUC16 with neuraminidase. Without neuraminidase exposed T on MUC16 was not detected, but after treatment most sera with detectable levels of MUC16 were reactive with anti-T MAb 3C9. Similar to T and ST, LacNAc and SLacNAc show selective

detection of sialylated type 2 chain lactosamine chains on MUC16 by anti-LacNAc MAb 1B2. Interestingly, while the assays correlated in terms of positive sera, the relative signal intensities varied quite markedly for each positive serum. Comparing the CA15-3 values with the glycoprofiling results suggests that this assay preferentially identifies MUC1 with SLacNAc structures that indicates a core 2 type O-glycosylation pattern, while the HMFG2 based capture assay showed relatively better detection of the sera which only expressed the core 1 (ST) O-glycosylation pattern.

Fig. S2

Profiling O-glycosylation on mucins produced and secreted from human cancer cell lines.

(A) Glycoprofiling MUC1 in T47D breast cancer cell line with and without neuraminidase treatment to remove sialic acids. T47D produces and secretes Tn-MUC1 glycoforms as detected by 5E5 and 5F4, and neuraminidase treatment had little effect on 5F4 detection suggesting only limited presence of STn. Core 1 glycosylation detected by anti-T antibody 3C9 was mainly found after treatment with neuraminidase suggesting that T47D mainly produces and secretes the sialylated ST glycoform. **(B)** Glycoprofiling MUC16 in OVCAR-3 ovarian cancer cell line. OVCAR-3 produces ST and T core 1 glycoforms as evidenced by slight enhancement in reactivity after neuraminidase treatment by 3C9, while only weak reactivity for Tn and STn glycoforms was detected. Spent culture medium and cell lysates were normalized to equal volumes and tested at initial dilutions of 1:4 and double diluted.

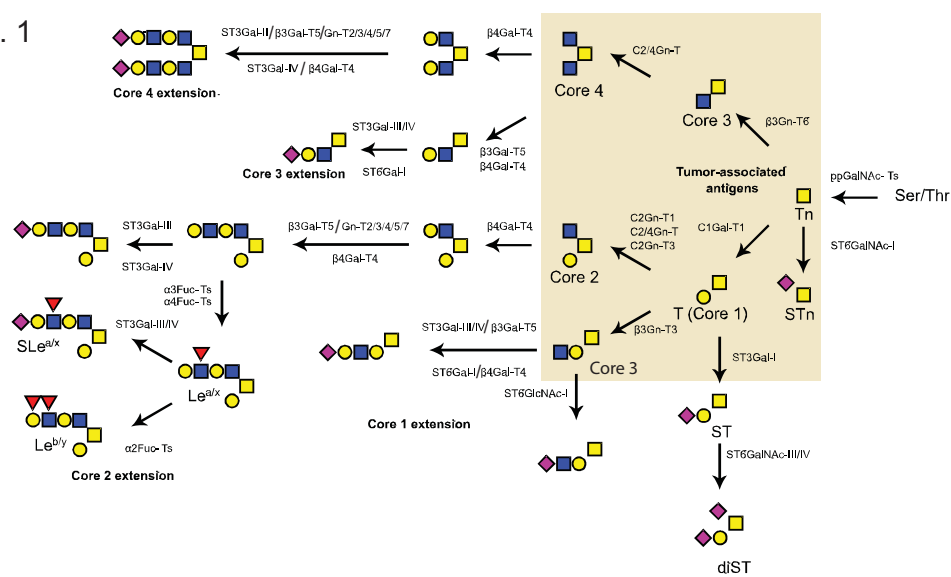
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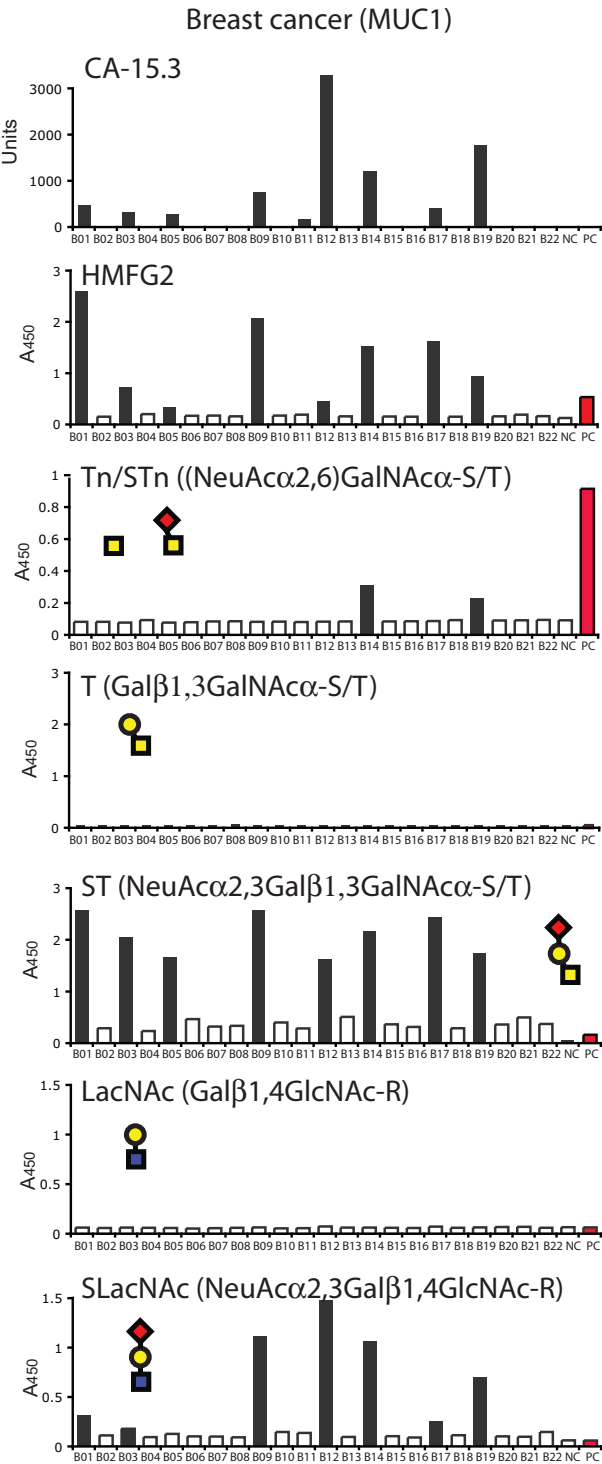
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Supplementary fig. 1

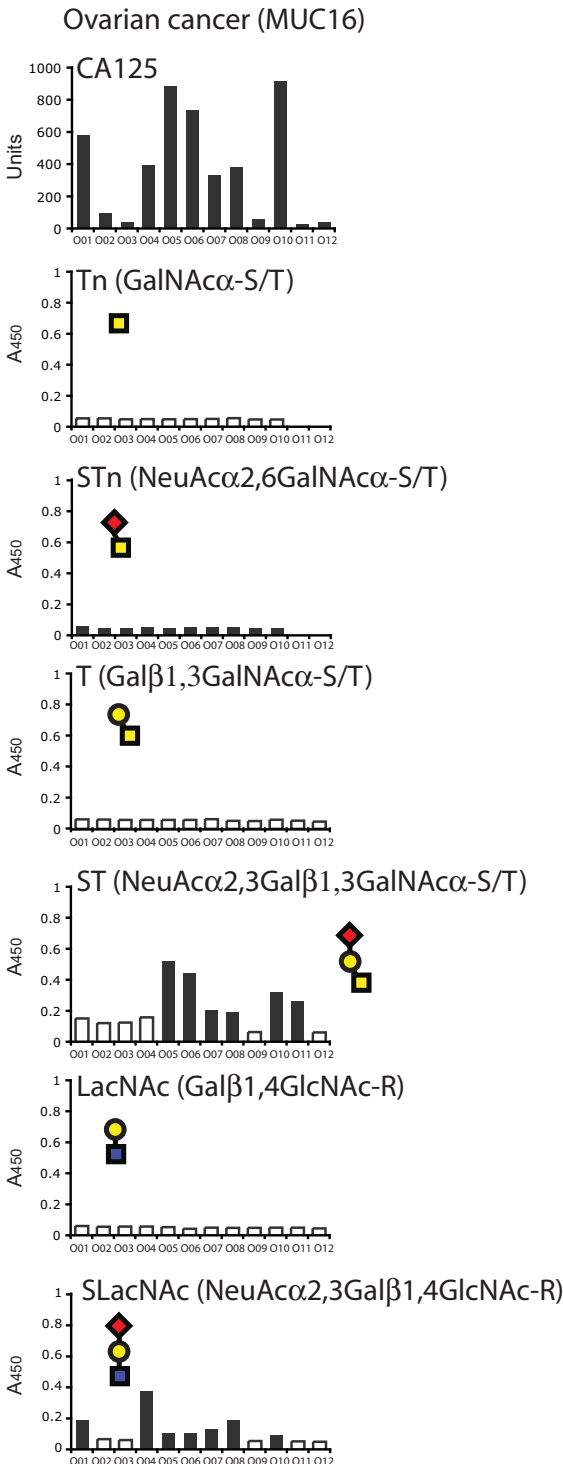
A



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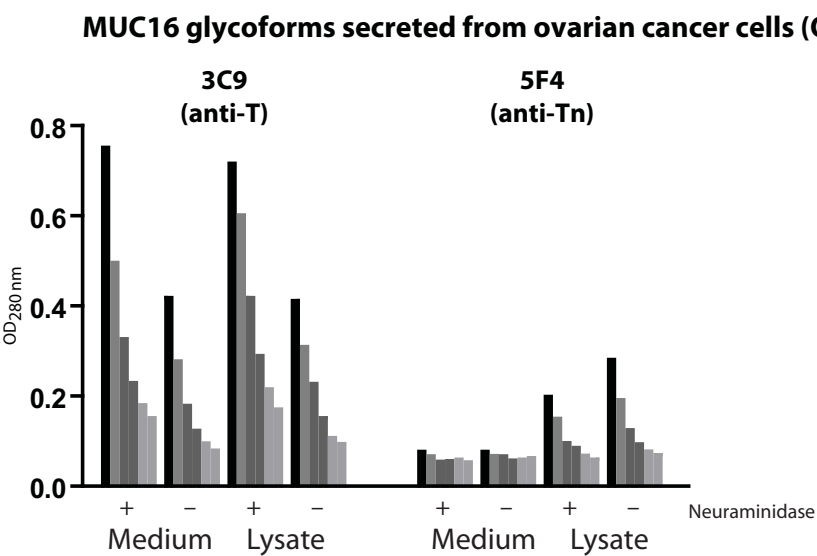
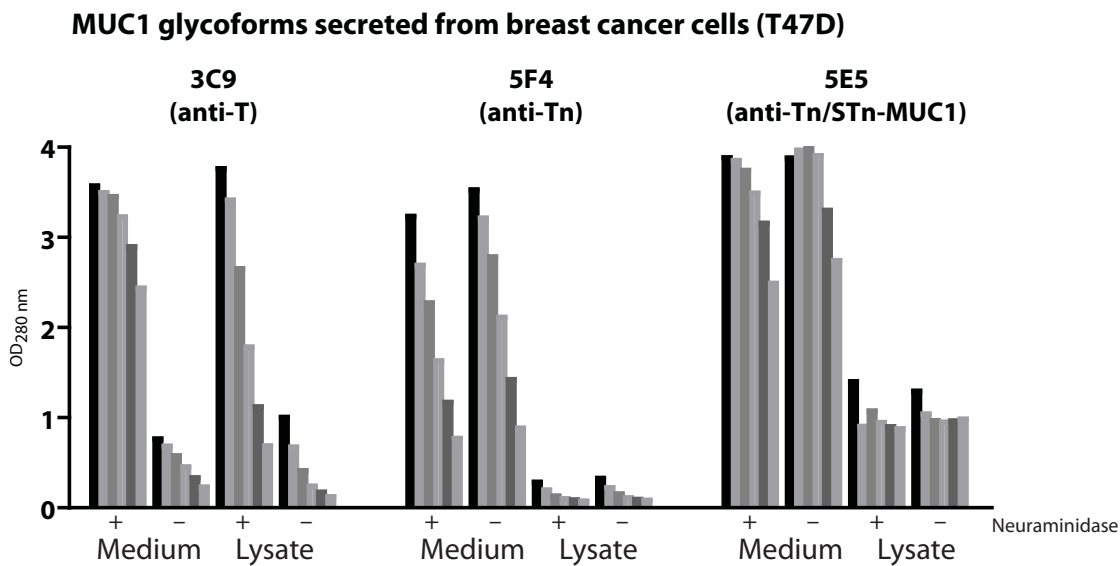


Table: Serum samples used in the study

Breast cancer:

Sample ID	Race	Sex	Age at Excision	Menopausal Status	Clinical Specimen Diagnosis	AJCC/UICC Stage	AJCC/UICC Stage Group
29716A2	Caucasian	F	46	Pre-menopausal	Lobular carcinoma of the breast	T1cNXM0	I
29645A1	Caucasian	F	45	Pre-menopausal	Infiltrating ductal carcinoma of the breast	T1N0M0	I
31573A2	Caucasian	F	56	Post-menopausal	Infiltrating lobular carcinoma of breast	T1N0MX	I
31622A2	Caucasian	F	54	Pre-menopausal	Lobular carcinoma of the breast	T1bNXM0	I
31665A2	Caucasian	F	48	Pre-menopausal	Lobular carcinoma of the breast	T1bNXM0	I
29955A4	Caucasian	F	51	Pre-menopausal	Infiltrating ductal carcinoma of breast	T1N0M0	I
29985A4	Caucasian	F	46	Post-menopausal	Infiltrating ductal carcinoma of the breast	T1cN0M0	I
31358A4	Caucasian	F	53	Menopausal	Invasive lobular carcinoma of breast	T1N0M0	I
42798A1	Caucasian	F	53	Menopausal	Infiltrating ductal carcinoma of breast	T1bN0M0	I
48652A1	Caucasian	F	42	Pre-menopausal	Infiltrating ductal carcinoma of the breast	T1N0M0	I
48741A1	Caucasian	F	51	Pre-menopausal	Infiltrating ductal carcinoma of the breast	T1N0M0	I
71832A1	Caucasian	F	44	Pre-menopausal	Infiltrating ductal carcinoma of the breast	T1cN0MX	I
73191A1	Caucasian	F	53	Menopausal	Infiltrating ductal carcinoma of the breast	T1cN0M0	I
73290A1	Caucasian	F	49	Menopausal	Infiltrating lobular carcinoma of the breast	T1cN0M0	I
58466A2	Caucasian	F	51	no information	Infiltrating ductal carcinoma of the breast	T1cN0MX	I
78014A1	Caucasian	F	49	Menopausal	Lobular carcinoma of the breast	T1cN0M0	I
77913A1	Caucasian	F	41	Pre-menopausal	Infiltrating ductal carcinoma of the breast	T1N0M0	I
39071A1	Caucasian	F	55	no information	Infiltrating lobular carcinoma of the breast	T1N0MX	I
39208A2	Caucasian	F	50	no information	Lobular carcinoma of the breast	T1cN0MX	I
61891A1	Caucasian	F	52	Post-menopausal	Lobular carcinoma of the breast	T1cNXMX	I
31429A4	Caucasian	F	51	Pre-menopausal	Infiltrating lobular carcinoma of the breast	T1N2M0	IIIA
35101B6	Caucasian	F	52	Menopausal	Infiltrating ductal carcinoma of the breast	T1cN2M0	IIIA
45483A1	Caucasian	F	58	Post-menopausal	Infiltrating ductal carcinoma of breast	T1N2M0	IIIA
49210A1	Caucasian	F	54	Pre-menopausal	Infiltrating lobular carcinoma of the breast	T2N2M0	III
51475A1	Caucasian	F	55	Post-menopausal	Infiltrating ductal carcinoma of breast	T4N0M0	IIIB
73162A1	Caucasian	F	55	Menopausal	Infiltrating lobular carcinoma of the breast	T2N3M0	IIIC

Prostate cancer

Sample ID	Ethnicity	Sex	Age at Excision		Clinical Diagnosis (specimen)	AJCC/UICC Stage	AJCC/UICC Stage Group
74147A1	Caucasian	M	62	not applicable	Adenocarcinoma of the prostate	T3aNXXMX	III
58476A2	Caucasian	M	55	not applicable	Adenocarcinoma of the prostate	T3aNXXMX	III
61429A3	Caucasian	M	69	not applicable	Infiltrating adenocarcinoma of the prostate	T3bN0M0	III
61287A3	Caucasian	M	54	not applicable	Adenocarcinoma of the prostate	T4N1M0	IV
64414A6	Caucasian	M	60	not applicable	Infiltrating adenocarcinoma of the prostate	T3bNXMX	III
58010A8	Caucasian	M	70	not applicable	Adenocarcinoma of the prostate	T3aN0M0	III
57964A6	Caucasian	M	62	not applicable	Adenocarcinoma of the prostate	T3aN0M0	III
57979A6	Caucasian	M	55	not applicable	Adenocarcinoma of the prostate	T3bN0M0	III
57966A6	no info	M	69	not applicable	Adenocarcinoma of the prostate	T3bN0M0	III
57980A6	no info	M	53	not applicable	Adenocarcinoma of the prostate	T3aN1M0	IV

Ovarian cancer:

Sample ID	Ethnicity	Sex	Age at Excision	Menopausal Status	Clinical Diagnosis (Specimen)	AJCC/UICC Stage	AJCC/UICC Stage Group
50702A1	Caucasian	F	65	Post-menopausal	Serous cystadenocarcinoma of the ovary	T3cNXMX	IIIC
53288A1	Caucasian	F	36	no information	Serous papillary carcinoma of ovary	T3NXM1	IV
53290A1	Caucasian	F	78	no information	Serous papillary carcinoma of ovary	T3NXM1	IV
71500A1	Caucasian	F	64	Post-Menopausal	Papillary serous adenocarcinoma of the ovary	T3cN0MX	IIIC
38562A3	Caucasian	F	46	Post-menopausal	Serous cystadenocarcinoma of the ovary	T3cNXM0	IIIC
38562A4	Caucasian	F	70	Post-menopausal	Serous cystadenocarcinoma of the ovary	T3cNXM0	IIIC
55299A2	Caucasian	F	72	Post-menopausal	Serous cystadenocarcinoma of the ovary	T3cNXM0	IIIC
55368A2	Caucasian	F	42	Post-Menopausal	Papillary adenocarcinoma of the ovary	T3cN1MX	IIIC
61484A1	Caucasian	F	51	Post-Menopausal	apillary serous cystadenocarcinoma of the ova	T3cNXM0	IIIC
61484A2	Caucasian	F	54	Post-Menopausal	apillary serous cystadenocarcinoma of the ova	T3cNXM0	IIIC
61129A1	Caucasian	F	51	Pre-menopausal	Papillary adenocarcinoma of the ovary	T3cN1M0	IIIC
86147A6	Caucasian	F	63	Menopausal	Papillary cystadenocarcinoma of the ovary	T2N1M1	IV
57290A7	Caucasian	F	56	Post-menopausal	Serous carcinoma of the ovary	T3cNXM1	IV
35733C1	Caucasian	F	53	Post-Menopausal	Serous cystadenocarcinoma of the ovary	T3cN0MX	IIIC
36111B1B ¹	Caucasian	F	68	Post-menopausal	Serous cystadenocarcinoma of the ovary	T3cN0MX	IIIA
36741B1B ¹	Caucasian	F	69	Post-menopausal	Serous cystadenocarcinoma of the ovary	T3cNXM0	III
37756B1A ¹	Caucasian	F	n.i. ²	Post-menopausal	Serous cystadenocarcinoma of the ovary	T3aN1, MX	IIIC
37870B1B ¹	Caucasian	F	76	Post-Menopausal	Serous cystadenocarcinoma of the ovary	n.i. ²	IC
39109B1B ¹	Caucasian	F	61	Post-Menopausal	Papillary adenocarcinoma of the ovary	n.i. ²	IIA
43221B1A ¹	Caucasian	F	n.i. ²	Post-Menopausal	Papillary adenocarcinoma of the ovary	T3cN1M0	IIIC

Healthy controls:³

Specimen ID	Ethnicity	Sex	Age at Excision	Clinical Diagnosis (Specimen)
60955A4	Caucasian	M	42	Normal control donor
61674A8	Caucasian	M	41	Normal control donor
60956A5	Caucasian	M	42	Normal control donor
61147A3	Caucasian	M	46	Normal control donor
61209A4	Hispanic	M	44	Normal control donor
62631A5	Caucasian	M	43	Normal control donor
114932A1	Caucasian	M	44	Normal control donor
63313A7	Caucasian	M	43	Normal control donor
62630A7	Caucasian	M	41	Normal control donor
62589A6	Caucasian	M	46	Normal control donor
63328A6	Caucasian	M	42	Normal control donor
63318A7	Caucasian	M	45	Normal control donor
121093A7	Caucasian	M	45	Normal control donor

130847A5	Caucasian	F	51	Normal control donor
130848A6	Caucasian	F	44	Normal control donor
130850A7	Caucasian	F	57	Normal control donor
130795A8	Caucasian	F	56	Normal control donor
131094A7	Caucasian	F	56	Normal control donor
130800A7	Caucasian	F	56	Normal control donor
130791A8	Caucasian	F	73	Normal control donor
130796A5	Caucasian	F	55	Normal control donor
130839A8	Caucasian	M	46	Normal control donor
130873A8	Caucasian	M	45	Normal control donor
130866A7	Caucasian	F	43	Normal control donor
130864A7	Caucasian	F	40	Normal control donor
130875A8	African Am	F	54	Normal control donor
130845A8	Caucasian	F	58	Normal control donor
130861A7	Caucasian	F	50	Normal control donor
130858A7	Caucasian	F	42	Normal control donor
130862A8	Caucasian	F	48	Normal control donor
130872A8	Caucasian	F	45	Normal control donor
132812A6	Caucasian	F	48	Normal control donor
139266A7	Caucasian	F	49	Normal control donor
139258A8	Caucasian	M	47	Normal control donor
132785A6	Caucasian	M	51	Normal control donor
139212A7	Caucasian	M	57	Normal control donor
130870A6	Caucasian	M	48	Normal control donor
145151A6	Caucasian	F	40	Normal control donor
145155A8	Caucasian	F	42	Normal control donor

1. Sera obtained from The Cooperative Human Tissue Network (CHTN). All other sera obtained from Asterand.

2. n.i. = no information.

3. An additional set of n=33 sera from healthy blood donors from an independant source were also tested.

Age distribution of samples (Average $\pm$ STDEV):

Healthy: Males and females combined: 46.43 $\pm$ 6.4; Males: 44.1 $\pm$ 3.4; Females: 50.4 $\pm$ 8.1.

Breast cancer: 50.5 $\pm$ 4.4. Ovarian cancer: 59.7 $\pm$ 11.8. Prostate cancer: 60.9 $\pm$ 6.6.