Cancer Biomarkers Defined by Autoantibody Signatures to A 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.

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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 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.
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 μg 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 β3GalT, core 3 β3GlcNAc-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 μmol/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 μmol/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 μg/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

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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 from 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 haptons, 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 (GlcNAcβ1-3GalNAc1-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 haptons, 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 β3GnT6, 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 HMFG2 recognized both nonglycosylated (designated M1) and all glycoforms of MUC1, confirming efficient printing of all MUC1 compounds. The glycopeptide-specific mAb SE5 recognizes the immunodominant Tn/STn-MUC1 epitope -GST- with one GalNAc residue in threonine (6Tn-MUC1) or both serine and threonine (9/15Tn-MUC1). In contrast, the carbohydrate hapten antibodies recognizing Tn (GalNAcα1-O-Ser/Thr) mAb (5F4) and STn (NeuAcα2-6GalNAcα1-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 (Galβ1-3GalNAcα1-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. Prevaccination 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 (HGVTSAPDTRPAPOGSTAPPA) 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 Variki 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 hapten. It is well established that non-self carbohydrate structures are immunogenic in man, and natural antibodies to the cancer-associated truncated carbohydrate hapten 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 microbiota (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
not found in the benign conditions, except that antibodies to the truncated core 3 MUC1 glycopeptide epitope was found at lower incidence and lower intensity.\(^5\)

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 C1β3Gal-T enzyme that controls synthesis of the common core 1 O-glycosylation pathway (see Supplementary Fig. S1); however, overexpression of the ST6GalNAc-I sialyltransferase responsible for STn synthesis can also play a role (42, 43). More intriguing is the finding of antibodies to truncated core 3 MUC1 in prostate, breast, and ovarian cancer patients. The core 3 glycosylation pathway is generally believed to be limited to the mucosa of the digestive tract, and truncated core 3 O-glycans have been found in colon cancer (44) in accordance with the expression pattern for the controlling enzyme β3GnT6 (45). Whereas this enzyme is downregulated in colon cancer, little is known about its expression in other types of cancer. Structural studies of O-glycoproteins from breast cancer have not identified core 3-related O-glycans; however, a recent report have shown the existence of cancer-associated truncated glycans with terminal βGlcNAc on glycoproteins and glycolipids in many cancer types, including ductal breast carcinoma (46).

\(^5\) J.W. Pedersen et al., manuscript in preparation.

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**Figure 2.** Detection of cancer-induced autoantibodies to MUC1 O-glycopeptide epitopes by microarray analysis. A, three-dimensional column diagram of IgG antibody reactivity in sera from healthy controls (\(n = 39\)) and newly diagnosed patients with breast (\(n = 26\)), ovarian (\(n = 20\)), and prostate (\(n = 10\)) cancer (Supplementary Table S1). Specific IgG responses were detected in identified breast, ovarian, and prostate cancer patients toward Tn-MUC1, STn-MUC1, T-MUC1, or truncated core 3 O-glycopeptide epitopes. Only very few healthy subjects and cancer patients had IgG antibodies to AOSM and OSM, which are considered Tn and STn hapten antibodies. B, dot-plot analysis of the antibody responses for each glycopeptide target. C, summary of results for each MUC1 glycopeptide with indication of glycan structures and positions within the MUC1 tandem repeat. Values were considered positive if above three times the SD of the average value obtained with sera from healthy individuals. A total of 5 out of the 26 breast cancer patients showed induction of IgG autoantibodies to either Tn, STn, T, or core 3 MUC1 glycopeptides. The same reactivity pattern was seen in ovarian cancer patients with 5 out of 20 patients, demonstrating IgG autoantibodies to one or more of the MUC1 glycopeptides. In contrast, 4 out of 10 prostate cancer patients showed induction of autoantibodies to STn, T, and core 3 MUC1 glycopeptides. In the majority of individuals, no reactivity was seen with Tn and STn MUC2 glycopeptides or AOSM and OSM.
Interestingly, overexpression of β3GnT6 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


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