Serum Glycan Signatures of Gastric Cancer

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Abstract

Glycomics, a comprehensive study of glycans expressed in biologic systems, is emerging as a simple yet highly sensitive diagnostic tool for disease onset and progression. This study aimed to use glycomics to investigate glycan markers that would differentiate patients with gastric cancer from those with nonatrophic gastritis. Patients with duodenal ulcer were also included because they are thought to represent a biologically different response to infection with Helicobacter pylori, a bacterial infection that can cause either gastric cancer or duodenal ulcer. We collected 72 serum samples from patients in Mexico City that presented with nonatrophic gastritis, duodenal ulcer, or gastric cancer. N-glycans were released from serum samples using the generic method with PNGase F and were analyzed by matrix-assisted laser desorption/ionization Fourier transform-ion cyclotron resonance mass spectrometry. The corresponding glycan compositions were calculated based on accurate mass. ANOVA-based statistical analysis was performed to identify potential markers for each subgroup. Nineteen glycans were significantly different among the diagnostic groups. Generally, decreased levels of high-mannose–type glycans, glycans with one complex type antenna, bigalactosylated biantennary glycans, and increased levels of nongalactosylated biantennary glycans were observed in gastric cancer cases. Altered levels of serum glycans were also observed in duodenal ulcer, but differences were generally in the same direction as gastric cancer. Serum glycan profiles may provide biomarkers to differentiate gastric cancer cases from controls with nonatrophic gastritis. Further studies will be needed to validate these findings as biomarkers and identify the role of protein glycosylation in gastric cancer pathology. Cancer Prev Res; 7(2); 226–35. ©2013 AACR.

Introduction

Gastric cancer is the second most common cause of cancer-related deaths, with nearly 1 million cases and more than 700,000 deaths each year worldwide (1–4). Although the incidence of gastric cancer in industrialized countries has declined markedly over the past 50 years, it remains a major cause of morbidity and mortality, particularly in less developed countries in Asia, Eastern Europe, and Latin America (5). Two main histologic groups of gastric cancer have been recognized, the intestinal (well-differentiated) type and the diffuse type (6). Intestinal type cancer is more common, particularly in the elderly, and it progresses through a series of histologic stages that begins with gastritis and progresses over decades to atrophy (loss of glands), intestinal metaplasia, dysplasia, and finally adenocarcinoma (7).

The observation that gastric cancer may have an environmental etiology, and that it is associated with chronic gastritis, were brought together by the discovery of Helicobacter pylori in 1983 (8). H. pylori infects the gastric epithe-lium of approximately 50% of the world’s population and uniformly causes nonatrophic gastritis, which in some cases progresses to atrophic gastritis and gastric adenocarcinoma. Seroepidemiologic observations now convincingly demonstrate that H. pylori infection is associated with an approximately 6-fold increased risk of gastric cancer (9). These studies have been supported by in vivo experiments in animal models (10, 11), and by large intervention trials to evaluate the effects of H. pylori eradication in prevention of gastric cancer (12). Interestingly, H. pylori infection also causes peptic ulcer disease, but although gastric cancer and peptic ulcer are both associated with H. pylori, they are inversely associated with one another (13). Although the reasons for this are not well understood, patients with ulcer disease provide a valuable comparison group because they...
Glycan Signatures of Gastric Cancer

Materials and Methods

Sample collection

Patients. Human sera were obtained from patients attending the Gastroenterology Unit of the Mexico General Hospital, Secretaria de Salud and the Oncology Hospital, Instituto Mexicano del Seguro Social, both in Mexico City, from October 1999 to July 2002 (50). Patients were at least 30 years old and sought attention because of gastrroduodenal symptoms or because of probable gastric cancer, and were scheduled for endoscopy and gastric biopsy for diagnostic purposes. All participants signed informed consent to participate in the protocol, which was approved by the Research and Ethics Committee of the Hospital General de Mexico, Secretaria de Salud, or the Oncology Hospital at Instituto Mexicano del Seguro Social.

Clinical and histopathologic diagnosis. Gastric biopsies were obtained systematically from 6 defined locations in the gastric antrum, corpus, and transitional zone, and also from the location of a lesion, if one was identified during endoscopy (50). Biopsies from each location were formalin fixed, paraffin embedded, and stained with hematoxylin and eosin for histopathologic evaluation and classification according to the updated Sydney system by a single experienced pathologist (51). Final diagnosis was that of the most severe histologic lesion or based on endoscopy findings in the cases of duodenal ulcer.

Serum samples and H. pylori serology. A 5 mL blood sample was drawn from each patient, and serum was obtained and frozen at −80°C until tested. Serum samples were tested by ELISA for immunoglobulin G (IgG) antibodies against H. pylori whole cell antigens as previously described (52).

Glycan release from human serum

Rapid and reproducible N-glycan profiling was performed using previously described methods (46). Briefly, 50 μL of serum was added to an equal volume of aqueous 200 mmol/L of ammonium bicarbonate containing 10 mmol/L of dithiothreitol solution. The mixture was thermally denatured by alternating between a 100°C and 25°C water bath for 12 cycles of 10 seconds each. Next, 2 μL of PNGase F (New England Biolabs) was added and the mixture was incubated in a microwave reactor (CEM Corporation) for 10 minutes at 20 W and 60°C. Finally, 400 μL of cold ethanol was added and the mixture was placed at −80°C for 1 hour to precipitate the deglycosylated proteins. Following centrifugation, released N-glycans were isolated in the supernatant fraction and dried using a vacuum evaporator.

Purification and enrichment of N-glycans

The purification of released N-glycans was performed by graphitized carbon solid-phase extraction using an automated GX-274 ASPEC liquid handler (Gilson; ref. 46). Initially, the graphitized carbon cartridges (GCC) were washed with a solution of 80% acetonitrile (ACN) in 0.10% trifluoroacetic acid (v/v). Then, GCC were conditioned with pure water.


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seem to have a different host response to H. pylori infection than patients who develop gastric cancer or those who remain asymptatically infected.

Gastric cancer produces no specific symptoms in its early stages when it is surgically curable, and most cases present with locally advanced or metastatic disease, which in the United States has a 5-year survival of less than 26.9% (14). Therefore, early detection and preventive strategies offer the best opportunity to decrease mortality from gastric cancer. Moreover, because most of those infected with H. pylori do not develop cancer or peptic ulcer, and because universal intervention against H. pylori is not practical, and may even be harmful (15), there is a need for biomarkers to identify the subpopulation of those infected with H. pylori who are at most risk for development of gastric cancer. The best currently available biomarker for gastric cancer is a decrease in the ratio of serum pepsinogens I and II (PGI/PGII), which is an improved by using PGI/PGII together with serologic evidence of H. pylori infection (18, 19), but still the positive predictive value is low, and novel biomarkers are needed.

An alternative approach to proteins, which have been the main focus of biomarker studies for decades, is detection of changes in protein glycosylation (20, 21). Glycans are complex bio-oligomers consisting of up to 10 monosaccharide residues that participate in key biologic processes, such as cell–cell interactions, protein folding, and the targeting of degradative lysosomal enzymes. Glycan-based biomarker studies are becoming more common (22–29) as many studies suggest that glycans play key roles in numerous diseases, including cancer and inflammatory diseases (28, 30–32). Mass spectrometry analysis of glycans provides sensitive and accurate detection in many complex biologic matrices, including human serum (33, 34). The majority of serum proteins are glycosylated, and changes in glycosylation are important indicators of health (35). Matrix-assisted laser desorption/ionization (MALDI) Fourier transform ion cyclotron resonance (FT-ICR) has been shown to be a fast and accurate method for glycan detection (30, 36–39), where enzymatic release of glycans yields complex mixtures that can be detected and identified by mass spectrometry (MS; ref. 30–32, 36, 40–49). Here we sought to evaluate the utility of mass spectrometry for detection of native N-glycans in serum as biomarkers to distinguish patients with gastric cancer from those with nonatrophic gastritis; patients with duodenal ulcer were also included because they seem to represent a different biologic response to H. pylori infection.


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After loading aqueous N-glycan solutions onto the cartridge, GCC were washed with pure water at a flow rate of approximately 1 mL/min to remove salts and buffer. Purified and enriched serum N-glycans were eluted into 3 fractions containing 6-mL solutions of 10%, 20%, and 40% ACN in 0.05% trifluoroacetic acid (v/v) in water, respectively. The 3 fractions were then dried using vacuum evaporator and analyzed separately by mass spectrometry.

**Mass spectrometric N-glycan analysis by MALDI FT-ICR mass spectrometry**

Mass spectrometric analysis was performed using methods described previously (30, 31, 46, 49). Briefly, the mass spectrometric analysis was performed using a MALDI FT-ICR mass spectrometry instrument (HiResMALDI; IonSpec Corporation) equipped with a 7.0 T superconducting magnet. A pulsed Nd:YAG laser 2,5-Dihydroxybenzoic acid was used as the ionizing matrix for both positive and negative mode mass spectrometry and a cation dopant (NaCl) was used for neutral oligosaccharide analyses in the positive mode. Five replicate spectrometric analyses were performed for each serum sample.

**Bioinformatics**

The raw mass spectra were imported into an in-house program, the Glycolyzer, which performs a high mass accuracy spectra calibration using 6 N-glycan compositions that are abundant, span the mass range, and are found in all spectra (53). The calibrated spectra were then exported as text files and loaded into the FITCRMS package in R 2.14.2 (54) for additional processing (peak identification, baseline identification, data transformation, and normalization) and statistical analysis.

Peaks were identified in each spectrum using 5 consecutive data points, which when fit with a least squares quadratic function had a coefficient of determination \( R^2 \geq 0.98 \). This approach was previously validated (62) and identifies unique peaks that form a parabolic function. A baseline level was calculated for each spectrum by using a method specifically designed for MALDI FT-ICR spectra, the BXR algorithm (55). Peaks at a given mass were divided into signal peaks and noise peaks by a threshold of 3.798194 times the estimated baseline level at that mass, which is roughly equivalent to being 4.5 SDs above the mean for data following a normal distribution (56).

A logarithmic transformation was used on the glycan signals before statistical analysis. In each fraction separately, all masses that were signal peaks in all 360 spectra (\( N = 72 \times 5 \) replicates per sample) were used as the normalization peaks. In each spectrum, the logarithmic values of these peaks were averaged and that value subtracted from each detected peak in that spectrum. Postnormalization, the replicates for each glycan and each patient were combined into a single value using the maximum of the 5 replicate intensities for each glycan in each patient.

For each glycan, the following 4-way ANOVA model was constructed: \( \log(\text{intensity}) \sim \text{batch} + \text{sex} + \text{age category} + \text{diagnosis} \).

Samples were processed in 6 batches (with 12 samples each) and the samples were selected according to age categories (30–39, 40–49, 50–73, 74–89 years). We initially considered intestinal and diffuse gastric cancer as separate diagnostic categories, but because no statistically significant differences were observed, we combined them to obtain more statistical power. Thus, the final analysis was performed using a 3-category diagnostic variable (nonatrophic gastritis, duodenal ulcer, and gastric cancer). The batches were exactly balanced with respect to diagnosis and roughly balanced with respect to sex and age groups. Between 20 and 50 glycans were detected in each fraction, so multiple testing correction using the method of Benjamini and Hochberg (57) was applied to control the false discovery rate (FDR) in each fraction at 10%. A glycan was declared significantly different among the 3 diagnostic groups if the FDR-adjusted \( P \) value of the \( F \) test was less than 0.1. Once diagnosis was declared statistically significant, differences among the 3 diagnosis classes were identified by the Tukey–Kramer method (58).

**Results and Discussion**

**Clinical sample**

A subset of serum samples was selected from patients with nonatrophic gastritis (\( N = 18 \)), duodenal ulcer (\( N = 18 \)), and gastric cancer (\( N = 36 \)), which included equal numbers of patients with the intestinal and diffuse histologic forms of gastric cancer. Patients with duodenal ulcer were included because they are unlikely to develop gastric cancer and thus might be useful to identify a glycan signature that is protective. Patients with nonatrophic gastritis and duodenal ulcer were age and sex matched, but this was not possible for patients with gastric cancer because they are typically older and predominantly male (Table 1). The overall seroprevalence of \( H. \) pylori was 75% (Table 1). Because patients with nonatrophic gastritis are generally not thought to have disease associated with \( H. \) pylori, which uniformly causes gastritis, and because the prevalence of \( H. \) pylori in Mexico and other Latin American countries is very high (59), patients with nonatrophic gastritis in effect served as healthy controls.

**Serum N-glycan profile**

Figure 1 shows representative MALDI spectra of the serum N-glycans identified in serum of patients with gastric cancer, including neutral glycans from the 10% (A) and 20% (B) ACN fractions analyzed by positive mode MALDI MS, and acidic glycans obtained from 40% ACN fractions (C) analyzed in negative mode. Neutral glycans consisting of only Hex and HexNAc and mono-fucosylated complex type glycans were dominant in the 10% and 20% fractions, whereas glycans containing sialic acid residues were dominant in the 40% ACN fraction. The full list of isolated serum glycans is shown in Supplementary Table S1. The majority of the glycans found were complex and high-mannose–type N-glycans in the range of Hexose (Hex)\(_{3-10}\) N-Acetylgalactosamine (HexNAc)\(_{2-6}\). Fucose (Fuc)\(_{0-3}\). Sialic Acid
(NeuAc)_{0–2} as typically observed in serum glycan studies (32, 43, 44, 47). Interestingly, 4 masses were observed that would correspond to fucosylated high-mannose–type glycans with the compositions HexNAc\_2: Hex\_6–9 Fuc\_1. Fucosylated high-mannose–type glycans have not been reported in serum but have been observed in tissue from patients with colon cancer (60) and on a placental protein (61), but the presence of these structures in human serum has not been reported. However, no tandem mass spectra confirming the presence of these unusual structures could be obtained, and we are left only with accurate masses. We have not included these signals for further statistical analysis.

**Candidate glycan markers for gastric cancer**

Glycan peaks were determined quantitatively in the 10%, 20%, and 40% fractions according to accurate masses, using methods described previously and confirmed by tandem mass spectrometry (30, 31, 49). Of the serum glycans detected (Supplementary Table S1), only those identified in at least 2 patients are included in the statistical analysis. ANOVA (56, 62) was used to identify disease-specific glycans. Table 2 lists the mean and 95% confidence intervals (CI) for the difference between nonatrophic gastritis and each of duodenal ulcer and gastric cancer on the raw scale for the unique glycans that showed statistically significant differences (FDR 10%) for diagnosis. Overall, the ANOVA demonstrated significant differences for 19 glycans. Three statistically significant glycans were detected in both 10% and 20% ACN fractions (166.622, 1770.662, and 1786.657), although the results were similar (Table 2).

Because the results for the intestinal and diffuse forms of gastric cancer were similar (Supplementary Fig. S1), with no statistically significant differences between the 2 types of cancer, all patients with gastric cancer were combined to increase statistical power. For both gastric cancer and duodenal ulcer, the mean differences with nonatrophic gastritis were calculated, together with 95% CIs for the difference obtained using the Tukey–Kramer method. The results plotted on a log2 scale are shown in Fig. 2, which represents diagrammatically the data for the unique glycans shown in Table 2. Putative structures corresponding to the molecular masses are provided in Table 2. In total, 6 glycans were shown to be present at significantly altered levels in the serum of duodenal ulcer cases compared with nonatrophic gastritis controls, whereas the levels of 15 glycans were

<table>
<thead>
<tr>
<th>Diagnostic category</th>
<th>N</th>
<th>Mean age</th>
<th>M</th>
<th>F</th>
<th>H. pylori (+)</th>
</tr>
</thead>
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<tr>
<td>Nonatrophic gastritis</td>
<td>18</td>
<td>46</td>
<td>10</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>18</td>
<td>53</td>
<td>9</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal</td>
<td>18</td>
<td>68</td>
<td>12</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Diffuse</td>
<td>18</td>
<td>57</td>
<td>12</td>
<td>6</td>
<td>15</td>
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<tr>
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<td>72</td>
<td>56</td>
<td>43</td>
<td>29</td>
<td>54</td>
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Table 2. Statistically significant glycans and corresponding putative structures

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<th>[M]</th>
<th>ACN</th>
<th>Category composition</th>
<th>Putative structure</th>
<th>P</th>
<th>FDR adj</th>
<th>Duodenal ulcer %</th>
<th>95% CI</th>
<th>Gastric cancer %</th>
<th>95% CI</th>
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<td>1234.426</td>
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<td>0.093</td>
<td>-43.6</td>
<td>-69.6, 4.5</td>
<td>-37.5</td>
<td>-63.4, 6.6</td>
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<td>0.044</td>
<td>-37.9</td>
<td>-60.4, -2.7</td>
<td>-33.3</td>
<td>-54.8, -1.6</td>
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<td>High mannose 6Hex, 2HexNAc</td>
<td></td>
<td>0.008</td>
<td>-47.3</td>
<td>-67.4, -14.9</td>
<td>-45.8</td>
<td>-64.2, -18.0</td>
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<td>0.025</td>
<td>-30.2</td>
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<td>-28.6</td>
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<tr>
<td>1437.510</td>
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<td>0.008</td>
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<td>-43.0</td>
<td>-59.8, -19.4</td>
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<td>0.004</td>
<td>16.4</td>
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<td>80.8</td>
<td>24.7, 162.0</td>
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<td>42.8</td>
<td>6.7, 91.1</td>
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<td>0.008</td>
<td>-32.8</td>
<td>-54.3, -1.4</td>
<td>-39.3</td>
<td>-56.5, -15.3</td>
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<td>35.2</td>
<td>-3.7, 89.9</td>
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<td>-14.0</td>
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<td>-48.3, -8.8</td>
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<td></td>
<td>0.008</td>
<td>29.8</td>
<td>2.5, 64.4</td>
<td>35.9</td>
<td>10.7, 66.7</td>
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<td>0.030</td>
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<td>69.9</td>
<td>14.2, 152.7</td>
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<td>0.044</td>
<td>-21.8</td>
<td>-44.7, 10.5</td>
<td>-28.4</td>
<td>-47.7, -4.7</td>
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</tr>
<tr>
<td>1770.662</td>
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<td>-23.9</td>
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<td>0.008</td>
<td>-18.1</td>
<td>-45.1, 22.1</td>
<td>-40.5</td>
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<td>-45.5, 17.1</td>
<td>-35.5</td>
<td>-53.7, -10.2</td>
<td></td>
</tr>
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</table>

(Continued on the following page)
shown to be altered significantly with gastric cancer. No differences were found between gastric cancer and duodenal ulcer, and changes in glycan expression were almost uniformly in the same direction for both duodenal ulcer and gastric cancer, suggesting that the different clinical pathways of these 2 disease states are not associated with different glycan signatures. Most glycans that differed significantly between duodenal ulcer or gastric cancer, compared with nonatrophic gastritis, showed a decrease in abundance (e.g., 1396.488, 1421.520, 1437.510), although significant increases were sometimes apparent (e.g., 1462.547 and 1519.588; Table 2).

**Differential glycan groups**

The serum glycans that differed between gastric cancer cases and nonatrophic gastritis controls may be divided into 4 groups based on their structural features: high-mannose–type glycans, glycans with one complex type antenna, mannose (Man), N-acetylgalactosamine (GlcNAc), fucose (Fuc), hexose (Hex), sialic acid (NeuAc), N-acetylhexosamine (HexNAc).
bigalactosylated biantennary glycans, and nongalactosylated biantennary glycans. This is further illustrated in Fig. 3. Although the high-mannose–type glycans, glycans with one complex type antenna, and bigalactosylated biantennary glycans show decreased levels in gastric cancer cases compared with nonatrophic gastritis, increased levels of nongalactosylated biantennary glycans are observed in gastric cancer cases.

Recent studies from our group have focused on the development of biomarkers for the detection of several types of cancer, including ovarian (53, 63) and lung (64) cancer. The altered patterns observed with ovarian cancer are very similar to the changes that occur with gastric cancer. Particularly, using MALDI FT-ICR mass spectrometry analysis it was observed that several neutral glycans (including high-mannose–type glycans) were decreased in ovarian cancer, with the exception of a small group of nongalactosylated glycans (53). Using nLC-MS technology, decreased levels of neutral glycans, especially the high-mannose–type glycans, could be confirmed, but increased levels of nongalactosylated glycans were not observed (63). These results suggest that the profile observed is not exclusive for gastric cancer, but is more likely a sign of cancer in general. Indeed, other groups have reported similar differential patterns for gastric cancer as well as lung and ovarian cancer (29, 65–68).

One of the glycan groups that showed differential glycosylation patterns with gastric cancer is the group of high-mannose–type glycans. These glycans are biochemically interesting, as they are the precursor glycans for the "more mature" complex and hybrid-type glycans. Glycan biosynthesis starts in the endoplasmic reticulum, where a glycan precursor consisting of the glycan core (man\(_3\)GlcNAc\(_2\)) decorated with 6 mannose and 3 glucose residues is built from monosaccharides. The glycan precursor is then transferred to the protein, and subsequently the glucose residues are removed, to form a high-mannose–type glycan. When the protein is then transferred to the golgi, the mannose residues may be enzymatically removed, allowing the synthesis of hybrid and complex type glycans. Increased levels of high-mannose–type glycans have been associated with tumor cells and tissue (44, 69), and it has been speculated that this is caused by premature termination of the glycosylation synthesis.

In this study, however, gastric cancer was associated with lower levels of high-mannose–type glycans in serum. The higher abundance serum proteins are most likely produced in the liver or B cells, but not in the cancer tissue itself, and the glycosylation synthesis may be very effective, thus reducing the number of high-mannose–type glycans (70). Another possibility is that protein abundances in the serum of patients with cancer change. As not every protein is decorated with the same types of glycans, it is possible that the relative abundance of proteins carrying high-mannose–type glycans is decreased, thus resulting in the lowered levels of high-mannose–type glycans observed.

Immunoglobulin G and gastric cancer glycosylation patterns

In this and other studies, increased levels of nongalactosylated biantennary glycans have been reported to be associated with cancer. It is well known that these types of glycans are nearly exclusively (95% of the abundance) found on IgG, the glycoprotein of highest abundance in serum. Recently, glycosylation patterns of the Fc (constant fragment) portion of IgG was analyzed in gastric cancer cases and healthy controls (71). Increased levels of nongalactosylated glycans were observed on IgG, suggesting that the increased levels of these glycans described here are likely because of altered glycosylation on IgG. It is widely known that the N-glycans on the IgG Fc region have an important effect on the structure of the IgG molecule and its
binding properties to the Fe-γ receptor (72), and the altered glycosylation observed in gastric cancer may thus be a way to modify the immune response. Increased levels of nongalactosylated N-glycans on the IgGs have also been associated with autoimmune and other inflammatory diseases. Because chronic inflammation is one of the characteristics of cancer (73), it is likely that the increased levels of nongalactosylated glycans observed in serum of gastric cancer cases are related to the inflammatory response. However, further studies are needed to determine which is case and which is consequence.

Conclusion

This study shows the differential human serum N-glycosylation patterns in gastric cancer cases compared with controls. Differences were observed in 4 groups of glycans, such as the high-mannose-type glycans, glycans with one complex type antenna, bigalactosylated biantennary glycans, and nongalactosylated biantennary glycans. These glycans potentially provide predictive signatures for the detection of gastric cancer. Further studies are needed to identify disease pathways from a glycomics point of view, and provide new leads for the development of biomarkers for the early detection of gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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54. Barkauskas D. FTICRMS: programs for analyzing fourier transform-ion cyclotron resonance mass spectrometry data R package. 2012:version 0.9.


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