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Relative Quantification and Higher-Order Modeling of the Plasma Glycan Cancer Burden Ratio in Ovarian Cancer Case-Control Samples

Elizabeth S. Hecht[†], Elizabeth H. Scholl[‡], S. Hunter Walker[†], Amber D. Taylor[†], William A. Cliby[§], Alison A. Motsinger-Reif[‡], and David C. Muddiman^{*,†}

[†]W.M. Keck FTMS Laboratory for Human Health Research, Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States

[‡]Bioinformatics Consulting and Service Core, Bioinformatics Research Center, Department of Statistics, North Carolina State University, Raleigh, North Carolina 27695, United States

[§]Division of Gynecologic Surgery, Mayo Clinic, Rochester, Minnesota 55905, United States

Abstract

An early-stage, population-wide biomarker for ovarian cancer (OVC) is essential to reverse its high mortality rate. Aberrant glycosylation by OVC has been reported, but studies have yet to identify an *N*-glycan with sufficiently high specificity. We curated a human biorepository of 82 case-control plasma samples, with 27%, 12%, 46%, and 15% falling across stages I–IV, respectively. For relative quantitation, glycans were analyzed by the individuality normalization when labeling with glycan hydrazide tags (INLIGHT) strategy for enhanced electrospray ionization, MS/MS analysis. Sixty-three glycan cancer burden ratios (GBRs), defined as the log₁₀ ratio of the case-control extracted ion chromatogram abundances, were calculated above the limit of detection. The final GBR models, built using stepwise forward regression, included three significant terms: OVC stage, normalized mean GBR, and tag chemical purity; glycan class, fucosylation, or sialylation were not significant variables. After Bonferroni correction, seven *N*-glycans were identified as significant ($p < 0.05$), and after false discovery rate correction, an additional four glycans were determined to be significant ($p < 0.05$), with one borderline ($p = 0.05$). For all *N*-glycans, the vectors of the effects from stages II–IV were sequentially reversed, suggesting potential biological changes in OVC morphology or in host response.

Keywords

N-linked glycosylation; INLIGHT; ovarian cancer; relative quantification; cancer biomarker; human plasma

*dcmuddim@ncsu.edu. Phone: 919-513-0084.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The authors declare no competing financial interest.

INTRODUCTION

Ovarian cancer (OVC) is the most lethal gynecologic malignancy with a 70% mortality rate, and results in approximately 15 000 deaths annually in the United States.(1) Much of the mortality is due to a lack of effective early stage screening options, which, when combined with nonspecific symptoms, results in most cases presenting with advanced-stage disease.(2) Though multiple biomarker candidates, such as HE-4(2, 3) and CA-125,(2, 4, 5) have been identified through a combination of proteomics and immunohistology studies, they lack the specificity and sensitivity, even when used in combination,(6) for population-wide diagnostics.

Glycosylation expression is tightly regulated, and aberrant regulation is a hallmark of cancers localized to the ovary,(7–9) breast,(10, 11) pancreas,(12, 13) and others.(14, 15) As one of the only cotranslational protein modifications, glycosylation has been shown to affect key biological processes including protein folding,(16–18) enzymatic activity,(19–21) cell signaling,(22–24) and cell adhesion.(25, 26) Specifically for OVC, mesothelin-MUC16-mediated metastasis is mediated by *N*-glycosylation, for example.(27) Other secreted glycoproteins have also been shown to have a poorly understood association with the progression of the cancer(27, 28)

The *N*-linked glycome offers unique quantification challenges because glycan synthesis is nontemplate based and structures are diversified isomerically.(29–31) Combined with separation and ionization challenges posed by their hydrophilicity, glycan quantification is contingent on derivatization workflows to mediate these effects.(32–34) Consequently, standard mass spectrometry (MS) measurement techniques (spectral counting, area-under-the-curve) have limited efficacy and large analytical variability when applied to glycomics.(34, 35)

The individuality normalization when labeling with isotopic glycan hydrazide tags (INLIGHT) strategy was designed to address these challenges and achieves accurate, enhanced (~4-fold) nanoliquid chromatography (LC) electrospray ionization (ESI)–MS relative quantitation of glycans.(36–38) *N*-linked glycans are derivatized via hydrazine chemistry at the reducing sugar with a native (NAT) or $^{13}\text{C}_6$ stable-isotope labeled (SIL) 4-phenethylbenzohydrazide (P2PGN) reagent. The reaction is time efficient, preserves >95% of labile sialic acid moieties, and does not introduce solvent or salt artifacts into the electrospray.(33, 39) This study is the first application of the INLIGHT strategy for quantification of glycan compositions in a biological repository.

Recently, significant correlations between late-stage OVC and core fucosylated, bisecting, or Lewis-type sialylated *N*-glycans were reported.(7, 8, 40–42) However, these studies were unsuccessful in identifying a robust and specific predictor for early-stage OVC. Clinically, OVC biomarkers must carry a specificity-sensitivity value great than 99.9% due to the low annual incidence (~22 000 cases/year).(43) Given that global changes in the secreted glycome could provide noninvasive markers for OVC, we sought to identify these in a case-control study spanning early to late-stage specimens (Figure 1).

EXPERIMENTAL PROCEDURES

Experimental Design and Statistical Rationale

Plasma samples were obtained from 164 paired OVC case-control females at the Mayo Clinic Division of Gynecologic Surgery (Rochester, MN) (IRB #08-005749) and transferred to North Carolina State University for *N*-glycan analysis (IRB #000-00-330). All control patients (*N* = 82) had benign pathology diagnosed surgically, including endometriosis, benign ovarian tumors, cysts, or tubal disease (Supplemental Table 1). Matched case controls were made on the basis of age, menopausal status, and plasma draw date. Epithelial OVCs underwent laparotomy and full surgical staging (I–IV) and were further stratified by substage according to the 2009 International Federation of Gynecology and Obstetrics (FIGO) surgical staging criteria. Plasma was processed in triplicate and in seven randomized batches for LC–MS glycan analysis.

N-Glycan Extraction and Purification from Human Plasma

N-linked glycans were purified from human plasma according to Walker et al.(38) Briefly, 50 μ L of plasma was diluted to 200 μ L in 10 mM DTT and 100 mM aqueous ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO). Proteins were heat denatured for 2 min at 100 °C and deglycosylated by peptide-*N*-glycosidase F (glycerol-free PNGase F, 15.3 IUB mU, New England BioLabs, Ipswich, MA) over 18 h at 37 °C. Digestion was quenched by the addition of 800 μ L of chilled ethanol (Sigma-Aldrich) followed by a 1 h chill at –80 °C. The mixture was centrifuged at 13 200 rpm for 30 min (–9 °C), and the supernatant was dried in a vacuum concentrator. Glycans were reconstituted in 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich) in HPLC-grade water (Fisher Scientific, Waltham, MA). Samples were purified by graphitized carbon solid phase extraction (SPE) columns (Alltech Extract-Clean Carbograph Columns, Grace Discovery Sciences, Columbia, MD), vacuum-dried, and stored at –20 °C.

INLIGHT Derivatization of *N*-Glycans

The INLIGHT strategy was used to derivatize purified *N*-glycans as previously described. (38) NAT or SIL P2PGN reagent was synthesized in-house according to two different schemes previously reported.(37, 44) Briefly, samples were tagged with 200 μ L of 1 mg/mL P2PGN in 75:25 methanol/acetic acid at 56 °C for 3 h. The sample was dried to completion in a vacuum concentrator at 55 °C to quench the reaction. Tagged glycans were resuspended in 200 μ L of HPLC-grade water containing 5% acetonitrile (Fisher Scientific) and centrifuged for 5 min at 14 000 $\times$ *g* to remove excess tag. The supernatants of each case-control pair were mixed in a 1:1 ratio and queued for LC–MS analysis.

NanoRPLC–MS/MS Analysis

A cHiPLC–Nanoflex system (AB Sciex, Framingham, MA) in the vented column configuration was coupled to an EASY–nLC II system (Thermo Fisher Scientific, Waltham, MA). Ten microliters of duplexed case-control sample was loaded (2 μ L/min) onto a dual analytical C18 column (ChromXP C18–CL, 3 μ m, 120 Å, 75 μ m ID, 15 cm, AB Sciex) and emitted through a 10 μ m PicoTip (New Objective, Woburn, MA). Glycans were separated at

a flow rate of 275 nL/min in mobile phase A (MPA) (98% water/2% acetonitrile/0.2% formic acid) and mobile phase B (MPB) (2% water/98% acetonitrile/0.2% formic acid, respectively). The gradient elution increased MPB accordingly: 0–1 min (2%), 1–2 min (2–22%), 2–22 min (22–35%), 22–23 min (3–90%), 23–30 min (90%), 30–31 min (90–2%), 31–39 min (2%).

The RPLC system was coupled via a zero-dead-volume union tee to a Q-Exactive mass spectrometer (Thermo Fisher Scientific). Ions were generated at an emitter voltage of 2.25 kV, heated inlet capillary of 225 °C, and S-Lens RF of 45. Precursor ion spectra (700–1900 m/z range) were obtained at a resolving power (RP) of 70 000 (FWHM at $m/z = 200$), automatic gain control (AGC) of 1×10^6 , and a maximum injection time (IT) of 250 ms. MS/MS spectra were acquired in data-dependent acquisition (DDA) mode for the top five ions, placed on an exclusion list for 25 s, and fragmented at 20% normalized collision energy (NCE) in a higher energy collision dissociation (HCD) cell. MS² spectra were obtained at a RP of 17 500, AGC of 2×10^5 , a maximum IT of 120 ms, and an isolation window of 4.0 m/z . Each sample was analyzed by MS in triplicate.

Processing of MS Spectra

Raw MS spectra were processed using the Quan Browser module (Thermo Xcalibur 2.2 SP1.48 software) as described.(38) An in-house human plasma SIL and NAT INLIGHT glycan composition database (Supplemental Table 2) was used to identify glycans by accurate mass (mass measurement accuracy (MMA) of ± 5 ppm). Compositions are given using standard nomenclature: mannose (Man/M), N-acetyl hexosamine (HexNac/N), fucose (Fuc/F), and sialic acid (NeuAc/A). For each glycan, the area under the extracted ion chromatogram (XIC) was integrated, corrected for molecular weight overlap between NAT/SIL pairs, and normalized per spectrum.

RESULTS

N-Glycan Relative Quantification by LC–MS

Across all case-control samples, 71 N-glycan compositions were identified by LC–MS analysis (Supplemental Table 2). Glycans present in greater than or equal to 90% of spectra and detected above an abundance of 1×10^5 were included in the data set. The molecular weight overlap corrected and normalized areas of the remaining 63 glycans showed significant left-skewed distributions. Gaussian distributions were achieved by taking log₁₀-transforms of the raw areas (Supplemental Figure 1). Box-and-whisker plots were generated to identify outliers, and a single sample from stage II was removed from all subsequent analysis (Supplemental Figure 2).

Multivariate Analysis of the Glycan Cancer Burden Ratio

Glycan cancer burden ratios (GBRs) were calculated as the log ratio between cancer and control patients per glycan and averaged over each triplicate MS injection (Supplemental Figure 3). Fixed effects models were constructed against GBR in R v3.1 (Supplemental Methods). The full linear model included ten independent variables and showed significant differences in six glycan compositions (Bonferroni adjusted p -value < 0.05) (Supplemental

Table 3). Exogenous variables could be roughly divided into three categories corresponding to sample descriptors (i.e., age, batch, tagging), glycan indicators (i.e., class, fucosylation, normalized mean GBR), and OVC characteristics (i.e., OVC stage and CA-125 level). A hypothesis-driven approach was used to select seven secondary interactions for testing (Supplemental Table 4); however, none of these effects was significant (chi-square test, $F > 0.05$).

The reduced model was constructed using stepwise forward regression methods that selected for the set of variables yielding the lowest Bayesian information criterion (BIC) value (Supplemental Figure 4). Quantile–quantile plots were used to verify the significance of the full and reduced model responses against the null hypothesis that they reflected a Gaussian distribution of random noise (Supplemental Figure 5). The reduced GBR model included terms for OVC stage, normalized mean GBR, and tagging. To gain sensitivity to differences in the homeostatic concentrations of *N*-linked glycans released from circulating glycoproteins, normalized mean GBR was taken as the ratio of the average abundance between case-control samples. Tagging was included as a variable to account for differences in the purity of the NAT and SIL reagents, which were made in-house from two different synthetic schemes.(37, 44) Variables reflecting global changes to the composition of *N*-glycans, including branching and fucosylation, were not significant (Supplemental Figure 6) and were not included in the model. The plasma repository was validated by correlating CA-125 levels with OVC stage. As expected, CA-125 concentrations were positively correlated with advancing OVC stage (Supplemental Figure 7). There were no significant correlations GBRs and CA-125 protein concentrations in plasma.

From using the most conservative Bonferroni adjusted p -value, seven *N*-glycans were found to have highly significant correlations with OVC stage ($p < 0.05$) (Table 1). From using the false-discovery-rate (FDR) cutoff, an additional three *N*-glycans were identified as significant ($p < 0.05$), with a fourth glycan falling on the borderline ($p = 0.0502$), for a total of 11 biomarker candidates. By using stage I as a reference, the percent change in the GBR was calculated for stages II–IV (Table 1, Figure 2A,B). The average change in the GBR between the first and second stage of OVC was 11%, with a maximum percent increase of 40%. In all cases, the effects predicted in stage III were reversed in stage IV. For example, a glycan significantly decreased in stage III (relative to stage I) was found at increased relative concentrations in stage IV (Figure 2B). Globally, when averaged over all OVC stages, seven of the glycan biomarker candidates had median levels up-regulated at the 95% confidence interval (Figure 2A).

Partial separation of isomers was achieved by LC; however, since full resolution could not be obtained, the total areas under the curves were modeled. MS/MS analysis can provide limited information about the linkages of each glycan through identification of diagnostic MS² fragments. Though the GlcNac–GlcNac–Fuc fragment and Fuc neutral mass loss was searched for in the MS/MS spectrum, it was not observed, which neither confirms or denies the possibility of core fucosylation on H₅N₅F₁A₂, H₆N₅F₁A₁, and H₃N₅F₁ species. Likewise, localization of sialic acids proved to be particularly challenging due to the coelution of multiple isomers. Human biology constrains the types of saccharide units and the backbone linkages allowed. This narrows the possible structures for a given

composition, which was determined by accurate mass and confirmed by MS/MS data. Database searching of compositions through the Consortium for Functional Glycomics (CFG) composition engine(45) generated information about the most common structures observed in humans and those with previous associations in cancer. Using this tool, we predicted the most likely compositions for each of the significant glycans (Table 1). Each structure was either significantly branched, contained a bisecting GlcNAc, or was of mannose variety.

Global Analysis of Plasma Repository

Hierarchical clustering was used to visualize similarities between the glycans on a dendrogram. Correlations were calculated by a Lance-Williams updating formula on the dissimilarity matrix using the hclust package in R. Six main clusters were identified at a height of $y = 1.2$ on the dendrogram (Figure 3). Of the 11 biomarker candidates, 70% belonged to a single cluster: $H_5N_5F_1A_2$, $H_6N_5F_1A_1$, H_4N_5 , $H_8N_7A_2$, H_3N_5 , $H_7N_6F_1A_2$, and $H_6N_5A_1$. Within the cluster, the glycans fell on three clades within 0.5 distance of each other. The remaining significant glycans fell into two clusters. Attempts were made to discern further patterns between GBR and OVC stage using principle component analysis. Covariance matrices were calculated on both GBR against OVC stage (I–IV) and raw glycan abundances against sample diagnoses (control, OVC stage I–IV). Despite the control group showing less variance than the OVC samples, there were no significant differences between the classes (Supplemental Figure 8).

Associations of CA-125, GBR, and OVC Stage

CA-125 protein levels did not have a significant association with GBR; however, when modeled individually against OVC substage, age, and batch, it was an excellent predictor of OVC using both standard CA-125 cutoff values (CV) per stage (35 kU/L) or based on cutoff values determined via the model, lending confidence to our statistical analysis. Receiver operating characteristic (ROC) curves were constructed using CV values or determined by a training/testing cohort. These models were in good agreement with themselves, with area under the curve (AUC) values of 0.92 and 0.93, respectively (Supplemental Figure 9), and in good agreement with literature values (95% specificity AUC values ranging from 0.877–0.921(46, 47)).

ROC curves were also calculated for a panel of markers including CA125 and the 11 candidate glycans. The inclusion of N-glycans as a copredictor with CA-125 decreased the sensitivity/specificity of the response, resulting in an AUC value of 0.83. When tested individually, glycans had a maximum AUC value of 0.64 (Supplemental Table 5).

DISCUSSION

OVC presents with nonspecific symptoms or is asymptomatic, making curation of early stage plasma and tissue repositories for biomarker research particularly challenging and leading to high mortality rates. Of the 82 case-control samples analyzed, 40% belonged to stages I or II. However, these groups could be further classified by FIGO staging to ten subtypes (IA–IV) and carried different serous, serous borderline, clear cell, or mucinous

diagnoses (Supplemental Table 1). In addition, the benign controls encompassed a variety of diagnoses (Supplemental Table 1), adding variability across the matched pairs. These assignments added too many degrees of freedom to be included as variables in the model but would have decreased the heterogeneity within OVC groups and reduced the standard errors of effects.

Purification of the *N*-glycome was completed on undepleted plasma to limit sample preparation variability between case-control samples. Previous research has shown that there is no significant difference between the abundances of the majority of *N*-glycans in whole versus depleted plasma samples,(48) and thus we did not expect to lose sensitivity in the samples. Eleven *N*-glycans were found to be significant after FDR and Bonferroni adjustment when their GBRs were modeled against OVC stage. Variables accounting for the degree of fucosylation, sialylation, complexity, or hybridization were not significant in the GBR model. This finding is in direct contradiction with two highly similar studies completed on human serum glycans.(49, 50) However, those studies selected patients with only serous adenocarcinomas (diagnosed in only 66% of this repository), suggesting that the global activity of these glycosyltransferases, including various mannosidases, α 2–6 and α 2–8 sialyltransferase, and β 1,6 *N*-acetylglucosaminyltransferase, may be affected by different types of OVC. Additionally, differences between these studies may result from the type of analysis used. This study used the INLIGHT technology, which offers advantages in terms of the glycan ionization efficiency, compared to native species, and stable isotope labeled quantification compared to area under the curve approaches.(44)

OVC stage was selected as a significant variable and included in the GBR models. When the estimates for the indicator variables were individually examined, no effects for stage II were considered significant (Figure 2B). Though this may be a result of the small sample size of stage II ($N = 10$ case-control pairs), it is worthwhile to consider the magnitude of the changes in abundance. The average absolute percent change in the GBR, relative to stage I, was 11%, 32%, and 44%, in stages II–IV, respectively. In the scientific community, detection of a two-fold change is generally accepted as the minimum to quantify biological changes from disease. In this study, we found that changes as low as 17% were statistically significant ($p < 0.05$, F-test). The INLIGHT strategy had been previously reported to detect biological differences in duplexed samples down to 20%.(38) Though the changes in stage II may be real, the analytical variability may mask these changes. Interestingly, we found that the up- or down-regulation of the significant *N*-glycans in stage III was reversed in stage IV. This differs from several OVC, or the closely related triple-negative breast cancer, studies focused on late-stage or nonplasma specimens that showed a global up- or down-regulation of a glycan composition.(51–57) Since CA-125 levels systematically increase with progressing stage, it is unlikely that this glycoprotein is responsible for the changes in glycan abundance observed.

Two mechanisms could explain the change in the vectors of the glycan effects. First, altered glycosylation may reflect biological changes in the progression of OVC tumors and the surrounding organs. There is a clear stratification in the localization of stage I tumors (inside the ovary) versus II–IV, which has spread to local tissues, including the uterus and fallopian tubes, and then distal tissues. It should be noted that modeling and hierarchical clustering of

samples grouped into “early” (I and II) and “late-stage” (III and IV) showed no improvement in ROCs. However, other changes, such as the development of ascites, or consequently hypoalbuminaemia are specific to individual patients/cancers, and this information was not available. These conditions would certainly effect the glycoprotein secretome and perhaps the cell glycosylation machinery. An alternative hypothesis to explain the alternative glycan burden ratios would relate to changes in the immune response to metastasis or immune suppression coincident with advanced-stage cancer. It is common for OVC patients to become anergic in the late stage.(58) Biological stimulation and suppression of cytokine, peptide, and interleukin molecules,(59, 60) which regulate tumor growth, may be related to the trends observed in the glycan burden ratio. However, there is little precedence in the literature to provide a biological explanation, and follow-up experiments are needed.

The *N*-glycan compositions found to be statistically significant after Bonferroni correction have some precedence for regulation by OVC and related cancers. Aberrant regulation of H₈N₂(49) and H₆N₅F₁A₁(51) was identified in ovarian and triple-negative breast cancer, respectively. In choriocarcinoma cell lines, a cancer of the uterus, H₅N₅F₁A₂, containing a bisecting GlcNAc, was uniquely observed compared to normal cell lines.(61) H₅N₅F₁A₂ was expressed abnormally in hepatocytes,(62) which could be indicative of liver secretions triggered in response to OVC. Interestingly, we could not find any studies in cancer that identified the H₈N₇A₂ glycan, making it a potentially novel candidate.

CONCLUSIONS

This study offers new insights regarding the progression of OVC and its effects on glycosylation. It identified at least four *N*-glycan compositions that were significant after Bonferroni correction that have previously not been shown to have an association with OVC. It also suggests that future work on OVC biomarkers, either by composition or class, may need to be confined to specific subtypes (i.e., clear-cell, serous, etc.) to reach the required specificity for population wide testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Figure 2.

Figure 3.

Table 1

N-Glycan Structure ^{ab}	Glycan Composition	Model Effects of OVC Stage on Log ₁₀ (GBR)					p-value	
		I	II	III	IV	FDR	Bonferroni
H ₅ N ₅ F ₁ A ₂	0	-0.033	0.170	-0.285	0.00059	0.00059
H ₆ N ₃ F ₁ A ₁	0	-0.045	0.155	-0.295	0.00073	0.00184
H ₄ N ₅	0	-0.048	0.124	-0.290	0.00073	0.00218
H ₈ N ₇ A ₂	0	-0.214	0.181	-0.249	0.00194	0.00777
H ₆ N ₅ F ₂ A ₃	0	0.078	-0.133	0.272	0.00198	0.01259
H ₈ N ₂	0	0.012	-0.095	0.060	0.00198	0.01358
H ₃ N ₅	0	0.005	0.118	-0.271	0.00198	0.01389
H ₃ N ₅ F ₁	0	-0.010	-0.079	0.202	0.00666	0.05328
H ₅ N ₄ A ₁	0	0.013	-0.076	0.094	0.02425	0.22516
H ₇ N ₆ F ₁ A ₂	0	0.034	0.175	-0.174	0.02425	0.24248	

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N-Glycan Structure ^{ab}	Glycan Composition	Model Effects of OVC Stage on Log ₁₀ (GBR)				p-value	
		I	II	III	IV	FDR	Bonferroni
H ₆ N ₅ A ₁	0	-0.070	0.042	-0.154	0.05015	0.55166