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Site-Specific Fucosylation Analysis Identifying Glycoproteins Associated with Aggressive Prostate Cancer Cell Lines Using Tandem Affinity Enrichments of Intact Glycopeptides Followed by Mass Spectrometry

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Abstract

Fucosylation (Fuc) of glycoproteins plays an important role in regulating protein function and has been associated with the development of several cancer types including prostate cancer (Pca). Therefore, the research of Fuc glycoproteins has attracted increasing attention recently in the analytical field. Herein, a strategy based on lectin affinity enrichments of intact glycopeptides followed by mass spectrometry has been established to evaluate the specificities of various Fuc-binding lectins for glycosite-specific Fuc analysis of nonaggressive (NAG) and aggressive (AG) Pca cell lines. The enrichment specificities of Fuc glycopeptides using lectins (LCA, PSA, AAL, LTL, UEA I, and AOL) and MAX extraction cartridges alone, or in tandem, were evaluated. Our results showed that the use of lectin enrichment significantly increased the ratio of fucosylated glycopeptides to total glycopeptides compared to MAX enrichment. Furthermore, tandem use of lectin followed by MAX increased the number of identifications of Fuc glycopeptides compared to using lectin enrichment alone. LCA, PSA, and AOL showed stronger binding capacity than AAL, LTL, and UEA I. Also, LCA and PSA bound specifically to core Fuc, whereas AOL, AAL, and UEA I showed binding to both core Fuc and branch Fuc. The optimized enrichment method with tandem enrichment of LCA followed by MAX (LCA-MAX) was then applied to examine the Fuc

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Notes

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Supporting Information

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Materials and methods; workflow of the optimization of enrichment methods; workflow for further validation of the AOL specificity by enrichment from flow through sample; representative MS/MS spectra of a tryptic intact Fuc glycopeptides; OPLS-DA score scatter plot and S-plot; the ECM-receptor interaction, PI3K–AKT signaling pathway, and focal adhesion from KEGG mapper analysis (PDF) Raw search data for lectin specificity comparison (XLSX)

Raw search data for large scale profiling of Fuc glycopeptides from Pca cell lines (XLSX)

List of differentially expressed Fuc glycopeptides between AG and NAG Pca cell lines (PDF)

glycoproteomes in two NAG and two AG Pca cell lines. In total, 973 intact Fuc glycopeptides were identified and quantified from 252 Fuc proteins by using the tandem-mass-tags (TMT) labeling and nanoliquid chromatography–mass spectrometry (nanoLC–MS/MS) analysis. Further data analysis revealed that 51 Fuc glycopeptides were overexpressed more than 2-fold in AG cell lines compared to NAG cells. The analysis of protein core fucosylation has great potential for aiding our understanding of invasive activity of AG Pca and may lead to the development of diagnostic approaches for AG Pca.

Graphical Abstract

Glycosylation of proteins is one of the functionally important protein modifications, playing a crucial role in a variety of cellular processes, such as cell adhesion, receptor activation, tumor invasion, metastasis, and inflammatory response.^{1–4} Fucosylation (Fuc), especially core Fuc branching, is one of the most common glycosylation-based modifications that has been reported to be involved in the development of diseases such as cancer and inflammation.⁵ Accumulated studies have revealed differing Fuc levels in patient biological samples among normal, benign neoplasms, cancer, and other diseases.^{6–17} For instance, serum Fuc haptoglobin levels have been shown to be significantly increased in chronic pancreatitis (CP) patients when compared to healthy volunteers (HV) and further increased in pancreatic ductal adenocarcinoma (PDAC) patients. In addition, serum core Fuc haptoglobin levels are significantly higher in CP patients compared to HV and PDAC patients.¹³ Furthermore, the serum levels of Fuc fetuin A was found to decrease in liver cirrhosis patients when compared to hepatocellular carcinoma patients or healthy controls.¹⁴ With evidence of aberrant Fuc in disease, core Fuc glycoproteins have been used as biomarkers in clinical diagnostics, as evidenced by core Fuc glycoform of α fetoprotein (AFP-L3) gaining approval by the FDA as a diagnostic biomarker in hepatocellular carcinoma.¹⁸

Because of the biological implications of Fuc glycoproteins, research of Fuc glycoproteins has attracted increasing attention in the analytical field. There are two major strategies for Fuc glycoproteins analyses: (1) targeted Fuc proteins measurement by ELISA, western/lectin blot, or mass spectrometry (MS)^{6,13,14,16,19,20} and (2) large-scale Fuc glycoproteomes

profiling by MS analysis.^{8,10,18,21–24} The second approach may be more comprehensive as it facilitates the understanding of underlying mechanisms associated with tumorigenesis and metastasis as well as the discovery of novel potential biomarkers for early clinical diagnosis. Currently, large-scale Fuc glyco-proteomics analyses often employ a divide-and-conquer approach that profiles glycans or deglycosylated peptides separately, utilizing enzymatic or chemical methods to selectively cleave either the glycan or glycopeptide.^{8,10,18,20–25} However, a key drawback of this strategy is that information linking the site of glycosylation to its respective glycan structure is lost, and aspects such as glycan microheterogeneity at specific glycosites cannot be examined.²⁶ Therefore, strategies to profile the large-scale glycoproteomes, including Fuc, by analyzing intact glycopeptides directly are needed.

The abundance of Fuc glycopeptides is relatively low when compared to that of nonglycosylated peptides and non-Fuc glycopeptides generated after tryptic digestion of protein samples. Further confounding analysis, coelution of non-Fuc peptides during mass spectrometry analysis can mask the spectra of Fuc-glycopeptide spectra, and thus specific enrichment of Fuc glycopeptides is preferred. Lectins are proteins that can recognize specific glycan moieties and have been extensively used to selectively enrich glycosylated proteins/peptides to profile unique glycoforms.^{27,28} Lectin enrichment at protein-level captures lectin-binding glycoproteins and their associated proteins, while lectin enrichment at the peptide-level generally provides a specific enrichment of glycans at specific glycosylation sites.^{29,30} To date, the *Lens culinaris* agglutinin (LCA, LCH) lectin is often used to enrich Fuc glycopeptides for large-scale Fuc glycoproteomes profiling.^{8,10,18,21–24,31} In addition to LCA, other lectins, including *Pisum sativum* agglutinin (PSA),³² *Aleuria aurantia* lectin (AAL),³³ *Lotus tetragonolobus* lectin (LTL),³⁴ *Ulex europaeus* agglutinin I (UEA I),³⁵ *Aspergillus oryzae* lectin (AOL),³⁶ *Bryothamnion triquetrum* lectin (BTL),³⁷ *Rhizopus stolonifer* lectin (RSL),³⁸ *Cephalosporium curvulum* lectin (CSL),³⁹ and *Pholiota squarrosa* lectin (PhoSL),³² have also been reported to be fucosyl-specific. Previously, the specificity of the lectins LCA, PSA, AAL, and AOL has been evaluated using frontal affinity chromatography,^{40,41} whereas an assay measuring the inhibition of red blood cell agglutination to assess lectin specificity was utilized to assess the specificity of LTL and UEA I.⁴² However, the specificities of these lectins when utilized to enrich intact glycopeptides via their glycan structure has yet to be evaluated.

Prostate cancer (Pca) is the most commonly diagnosed cancer in men, both in the United States and worldwide.⁴³ Prostate-specific antigen, a glycoprotein with an N-linked glycosylation site, is an FDA-approved serum biomarker for prostate cancer screening and is used for the detection of Pca in high risk populations.^{44,45} However, serum prostate-specific antigen concentration has a low diagnostic sensitivity and specificity for Pca and cannot be used reliably to differentiate nonaggressive (NAG) from aggressive (AG) Pca tumor types, which can result in unnecessary biopsy procedures.^{46–48} Recently, tremendous efforts have focused on the discovery of novel biomarkers to improve the detection of Pca, particularly the identification of aberrant expression of protein glycoforms.^{16,17,49–53} We have previously reported the expression of α (1,6) fucosyltransferase (FUT8), an enzyme that catalyzes the transfer of fucose from GDP-fucose to N-linked type complex glycopeptides, was elevated in prostate cancer tissue samples as well as in the aggressive Pca cell line, PC3, compared less aggressive to LNCaP cells.⁵⁴ Furthermore, using a lectin AAL-based

immunoassay to detect Fuc glycoforms of glycoproteins in serum samples of Pca patients, we showed that the ratio of Fuc prostate-specific antigen was significantly increased in AG Pca in comparison to NAG Pca.⁵⁵ In another study, examining the differences of global protein and glycoprotein expression profiles, we showed that expression of Fuc glycopeptides was increased in PC3 cells relative to LNCaP cells.⁵⁶ Overall, these results indicate that systematic analysis of the Fuc glycoproteomes in AG Pca and NAG Pca may identify differences in Fuc glycoforms expression and lead to the development of applicable glycoprotein markers with increased specificity and sensitivity for stratifying Pca patients.

In this study, we aimed to develop a method to evaluate the specificity of various Fuc-binding lectins when utilized for the enrichment of intact Fuc glycopeptides for glycosite-specific fucosylation analysis in Pca cells. For this analysis, we examined four widely studied cell lines representative of NAG and AG states of prostate tumor progression: LNCaP and LAPC4, which are androgen-dependent, and PC3 and DU145, which are androgen-independent cell lines.⁵⁷ We first evaluated the enrichment specificities of intact Fuc glycopeptides using six lectins (LCA, PSA, AAL, LTL, UEA I, and AOL) and MAX extraction cartridges separately or in combination, followed by nanoliquid chromatography–mass spectrometry (nanoLC–MS/MS) analysis. To identify Fuc intact glycopeptides and obtain Fuc glycosite-specific glycan information, we used our in-house developed glycopeptide analysis software, GPQuest.⁵⁸ The optimized enrichment strategy was then paired with tandem-mass-tag (TMT) quantitative analysis to profile the Fuc glycoproteomes derived from the four Pca cell lines (see Figure 1).

MATERIALS AND METHODS

Chemicals and Reagents

Agarose bound lectins including LCA, PSA, AAL, LTL, UEA I were purchased from Vector Laboratories (Burlingame, CA). Lectin AOL-biotin conjugate was purchased from TCI America (Portland, OR). Oasis MAX 1 cm³ extraction cartridges and Sep-Pak C18 cartridges were from Waters (Milford, MA). Other materials were listed in the Supporting Information.

Cell Lines and Culture Conditions, Protein Extraction, and Trypsin Digestion

This section was described in the Supporting Information.

Intact Fuc Glycopeptides Enriched by Lectins and MAX

All lectins except AOL lectin were purchased in agarose bound form. The agarose bound AOL was prepared from lectin AOL-biotin conjugate according to the manufacturer's instructions for AOL-biotin conjugate and streptavidin agarose. Before use, each agarose bound lectin was washed by TBS buffer (pH 7.4) to remove sugar added to stabilize the lectin. The detailed enrichment protocols of lectin specificity comparison and large scale profiling were described in the Supporting Information.

TMT Labeling

The enriched dried down samples were reconstituted in 50 μL of 200 mM HEPES (pH 8.5) and vortexed well to mix. Each TMT 10plex reagent was dissolved in 41 μL of ACN, and 20 μL was added into each of its corresponding mass label sample tubes. These samples were mixed well and incubated for 1 h at room temperature. TMT channels 127C and 128C were used to label two replicate LAPC4 samples in order to determine the analytical reproducibility. TMT channels 126, 129C, and 130C were used to label LNCaP, PC3, and DU145, respectively (see Figure 1b). After TMT labeling, the five channels of tagged peptides were combined and purified by C18 cartridges. Then, the combined sample was dried and resuspended into 2% ACN/0.1% FA solution for nanoLC–MS/MS analysis after centrifuging for 10 min at 13 000 rpm.

NanoLC–MS/MS Analysis

The samples were separated utilizing a Dionex Ultimate 3000 RSLC nano system (Thermo Scientific) with a PepMap RSLC C18 column (75 $\mu\text{m} \times 50 \text{ cm}$, 2 μm , Thermo Scientific) protected by an Acclaim PepMap C18 column (100 $\mu\text{m} \times 2 \text{ cm}$, 5 μm , Thermo Scientific). MS analysis was performed using a Thermo Q Exactive mass spectrometer (Thermo Scientific). The LC conditions and MS parameters were described in the Supporting Information.

Data Analysis

For intact glycopeptide identification, the data were searched using an in house developed glycopeptide analysis software, GPQuest 2.0.⁵⁸ The GPQuest software detects spectra from intact glycopeptides in raw LC–MS/MS data by selecting oxonium ion-containing spectra and matches them to glycopeptide library using MS/MS ions. The glycan modifications at specific glycosylation site are then determined by additional MS/MS ions and the mass of precursor ion. The software is available for download with request from our Web site (biomarkercenter.org). The databases for searching were the human glycosite database for prostate cancer and human N-glycan database which contains 6640 and 277 entries, respectively. The parameters for mass tolerance of MS1 and MS2 were 10 and 20 ppm, respectively. The spectrum containing oxonium ion m/z 204.08 was chosen for further searching. Results were filtered based on the following criteria: (1) the false discovery rate (FDR) less than 1%, (2) containing 3 or more assigned b/y ions of peptides, (3) the total intensity of all these b/y ions more than 10.0% against the total intensity of the spectrum, and (4) all the peptide spectrum matches (PSMs) out of these criteria were removed. The intensities of reporter ions of TMT were exported for quantitation of glycopeptides. The multivariate statistics, orthogonal partial least-squares-discriminate analysis (OPLS-DA), was performed on the SIMCA-P 14.1 software (Umetrics AB, Umeå, Sweden).

RESULTS AND DISCUSSION

Evaluating Enrichment Strategy for Fuc Glycopeptides

Although lectins display a select specificity for certain glycoconjugates, nonspecific binding may occur when lectin affinity is used alone for enrichment of glycopeptides from complex

samples.⁵⁹ Oasis MAX, a commercially available hydrophilic-based strong anion exchange column, has been shown to have high efficiency for enrichment of glycopeptides.⁶⁰ To assess and identify the optimal enrichment strategy for Fuc glycopeptides, we examined six lectins (LCA, PSA, AAL, LTL, UEA I, and AOL) and MAX extraction cartridges individually as well as in tandem. Figure S1 shows the workflow of the enrichment methods for Fuc glycopeptides, with six enrichments by different lectin individually, six by lectin-MAX (tandem enrichment using lectin followed by MAX), one MAX only, and six MAX-lectin (tandem enrichment using MAX followed by lectin). The intact glycopeptides from each strategy were preanalyzed by a nanoLC-LTQ Orbitrap velos mass spectrometer (Thermo Scientific). The number of spectra containing oxonium ion m/z 204.08 was used to evaluate the intact glycopeptide capacity of different enrichment strategies. Few spectra containing the m/z 204.08 were found in the six MAX-lectin enriched samples (data not shown). Samples enriched by the other strategies (lectin alone, lectin-MAX, and MAX alone) contained more spectra of m/z 204.08 were further investigated by Q Exactive MS analysis according to the conditions described in NanoLC-MS/MS Analysis section, with the exception of utilizing a HCD NCE of 29%. Using the Q Exactive mass spectrometer enabled higher quality spectra and was more suitable for confident identification of intact glycopeptides and heterogeneity at specific glycosylation sites.

MS/MS spectra from intact glycopeptides have unique fragmentation signatures following HCD fragmentation, including oxonium ions (m/z 138, 163, 204, 274, 292, and 366), peptide-related ions, and peptide + HexNAc fragment ions, which yields information that can be integrated to identify glycopeptides.⁵⁶ The generated MS/MS spectra data were searched by GPQuest and the number of unique intact glycopeptides identified by the different enrichment methods is shown in Figure 2 and Table S1. We found that lectin enrichment could increase the ratio of identified Fuc glycopeptides to total glycopeptides when compared to MAX enrichment alone, observing ratios up to 100% when using the lectins LCA, PSA, AOL, and AAL, whereas we observed a ratio is 13.9% for MAX alone (Figure 2a). Furthermore, lectin-MAX enrichment could increase the number of identified intact glycopeptide significantly, despite the ratio of Fuc glycopeptides decreasing slightly in some lectins (Figure 2b). One possible reason for increased identification number by tandem use of lectin and MAX may be that the additional cleanup by the MAX column removed contaminants that would be detected with sensitive MS instrumentation. With these results, we concluded tandem enrichment using lectin followed by MAX was better than MAX alone and lectin alone on enrichment of Fuc glycopeptides.

Lectin Specificity on Intact Fuc Glycopeptides

After obtaining the optimal enrichment method (lectin-MAX), the number of identifications and specificity of Fuc glycopeptide enrichment was evaluated for each of the six lectins. Among these lectins, we identified more Fuc glycopeptides in the LCA-MAX enrichment, followed by AOL-MAX and PSA-MAX. The ratios of Fuc glycopeptides to total glycopeptides for LCA-MAX, PSA-MAX, and AOL-MAX were 72.1%, 94.7%, and 91.7%, respectively. PSA-MAX showed the highest Fuc ratio but the least identification number while opposite result was obtained by LCA-MAX (see Figure 2b). The characteristic ions for core Fuc (PepHexNAcFuc ion) and for branch Fuc (HexHexNAcFuc ion) were used for

investigating the specificity of lectin on core or branch Fuc in lectin-MAX samples. Figure 3a,b was the representative spectra for core and branch Fuc that were enriched by LCA-MAX and AOL-MAX, respectively. The number of Fuc glycans containing each characteristic ion enriched by different lectins are shown in Figure 3c. Our results showed that core Fuc was the major Fuc glycosylation in the Fuc glycans enriched by LCA-MAX and PSA-MAX when compared to other lectins (AAL, AOL, and UEA I) or MAX alone (see Figure 3c). These results indicated that LCA and PSA bound preferentially to core Fuc, whereas AOL, AAL, and UEA I exhibited broad specificity to Fuc glycans.

Next, we considered the binding capacity and specificity on core Fuc, analyzing the number of identified Fuc glycans where using LCA-MAX, PSA-MAX, AOL-MAX, and MAX alone enrichments. As shown in Figure 3d, the numbers of Fuc glycans in LCA-MAX, PSA-MAX, AOL-MAX, and MAX alone were 18, 5, 13 and 15, respectively. There were 2 glycans in common in these four samples; 5 glycans were common in LCA-MAX and PSA-MAX; 4 glycans were in common in LCA-MAX and AOL-MAX; 3 glycans were in common in PSA-MAX and AOL-MAX. Interestingly, all the glycans from PSA-MAX were covered by LCA-MAX, while 6 glycans were unique in AOL-MAX, suggesting that LCA and PSA have similar specificities for core Fuc, whereas AOL displayed a distinct core Fuc binding profile compared to LCA. To investigate the additional specificity of AOL relative to LCA, the peptides derived from the various of Pca cell lines were first enriched by LCA, with the unbound flow-through subjected to enrichment with AOL (see Figure S2). Following nanoLC-MS/MS and GPQuest analysis, we found 8 Fuc glycans uniquely identified in AOL-MAX enriched sample. All these unique glycans either had more than 1 fucose or 1 fucose with 2 sialic acids (data not shown), which indicated that the lectin AOL had a higher affinity for glycans with an additional Fuc branching compared to LCA.

Large Scale Profiling of Fuc Glycopeptides from Pca Cells by LCA-MAX

From the results described above, we selected tandem enrichment method using LCA followed by MAX (LCA-MAX) for large scale profiling of core Fuc glycoproteomes in the various Pca cell lines, with evidence of higher capacity and specificity of LCA to core Fuc glycopeptides. TMT labeling was implemented following subsequent LCA and MAX enrichment for quantitative analysis of Fuc glycopeptides from the four different cell lines (see Figure 1b).

The TMT labeled peptides were mixed and three technical replicates were analyzed using a Q Exactive mass spectrometer as previously reported.⁸ More than 14 929 oxonium ion-containing spectra (containing m/z 204.08) were detected in each run. Figure S3 shows an example of MS/MS spectra for the triply charged precursor (m/z 1055.18), which is identified as the GlcNAc2Man3Fuc1 glycoform at the site of DNATEEEILVYLEK. In this case, most of the peaks in the MS/MS spectrum could be annotated with specific fragment ions, including TMT reporter ions, oxonium ions, and the fragment ions of the peptide. Because of the lysine residue at the C-terminal, both termini of this peptide were labeled by the TMT tag. The fragment ions of peptide and peptide + HexNAc were observed in this spectrum, containing one or two TMT tags, such as $Y_1^+ [K + TMT + H]^+$ and $Y_{14}^+ [peptide + 2TMT + H]^+$. Oxonium ions (m/z 138, 163, 168, 186, 204, and 366) from the glycan

structure could be readily observed due to the relative weakness of glycosidic bonds (vs peptide bonds) being easily fragmented (see Figure S3a,b). As shown in Figure S3c, the analytical reproducibility of this method was confirmed by investigating the abundances of TMT channels 127C (127.13) and 128C (128.13) which were used to label two biological replicates derived from LAPC4 cells.

The spectra from all three runs were searched by GPQuest, and the results were filtered using a FDR less than 1% and containing 3 or more assigned b/y ions that possessed more than 10.0% intensity in total for a certain spectrum. The numbers of Fuc glycan, Fuc glycosite, intact Fuc glycopeptide, and Fuc protein identified in three runs were 52 ± 3 , 243 ± 11 , 598 ± 20 , and 183 ± 12 , respectively. By combining all three runs, 973 unique intact Fuc glycopeptides from 252 unique Fuc proteins were identified from the four Pca cell lines (see Figure 4a and Table S2). The results indicated the optimized method was efficient for extraction of intact Fuc glycopeptides. Among the identified intact Fuc glycopeptides, 86.6% Fuc glycopeptides contained one fucose and 10.2% Fuc glycopeptides contained 2 fucoses, whereas 2.9% Fuc glycopeptides had 3 fucoses. Evidence of more than 3 fucoses in the intact glycopeptides were very rare in our data set, identifying only 0.3% Fuc glycopeptides with 4 or more fucoses (see Figure 4b). For Fuc proteins, 61.1% were identified by 2 or more intact Fuc glycopeptides, whereas 38.9% were identified from one Fuc glycopeptides. Previously reported approaches for Fuc protein study removed the entire or partial glycan structure with PNGase F or Endo F3 prior to identification with MS.^{8,10,18,21–24} These approaches, however, provide limited information on fucosylation at specific glycosylation sites of glycoprotein. The intact glycopeptide-based strategy employed in this study provides information about the glycosylation event at the glycosites of peptides as well as the fucosylation profile of the entire glycan, which is potentially useful to illustrate the functionality of distinct of fucosylation branching on proteins of interest.

Differentially Expressed Fuc Glycopeptides in the AG and NAG Pca Cell Lines

The ratio of identified Fuc glycopeptides in different samples (cell lines) were calculated using TMT report tags. The intensity of each Fuc glycopeptide was divided by the average intensity of two biological replicate LAPC4 cells to obtain the ratio for comparative analysis between the cell lines. To identify the differentially expressed Fuc glycopeptides, the ratio of 973 Fuc glycopeptides (variables) between NAG (LNCaP and LAPC4) and AG (PC3 and DU145) cell lines were assessed by orthogonal partial least-squares-discriminant analysis (OPLS-DA), which is widely applied in metabolomics and demonstrated as a powerful tool for the analysis of multidimensional data.^{61–64} As indicated by these results, Fuc glycopeptides in NAG and AG cell lines were well described by OPLS-DA ($R^2(Y) = 0.997$) with high predictive ability ($Q^2(Y) = 0.886$) that showed obvious separation between two groups (see Figure S4). An S-plot was further created from the OPLS-DA model to investigate the Fuc glycopeptides related to the statistically significant differences between the NAG and AG Pca cell lines. As shown in Figure S5, the “end points” of the S-plot defined unique Fuc glycopeptides that differentiated the AG from NAG cell lines. Furthermore, the variable importance in projection (VIP) score was used to assess the significance of the selected Fuc glycopeptides (variables) in OPLS-DA models. The higher the VIP value (exceeding 1.0) the more significant is the variable in the complex analysis

when comparing difference between two or more groups.⁶⁵ As a result, a total of 276 Fuc glycopeptides with the VIP-value exceeding 1.0 were screened out as discriminant variables (expressed differentially) between NAG and AG groups. The differentially expressed Fuc glycopeptides identified in less than two runs and with less than 2-fold change between NAG and AG groups were excluded. The application of this stringent filter resulted in the identification of 51 Fuc glycopeptides from 26 Fuc proteins that were found to be upregulated in AG compared to NAG cell lines (Table S3).

To gain insight into biological impact of these differentially expressed Fuc proteins, our results were subjected to Kyoto Encyclopedia of Gene and Genome (KEGG) Mapper pathway analysis. KEGG analysis revealed these proteins are associated with the pathways of ECM–receptor interaction, PI3K–AKT signaling pathway, and focal adhesion (Figures S6–S8). From the ECM–receptor interaction pathway, 13 Fuc glycopeptides from integrin alpha-2 ($\alpha 2$), integrin alpha-3 ($\alpha 3$), and integrin beta-1 ($\beta 1$) were upregulated in AG cell lines (Table S3). Integrins are transmembrane receptors that facilitate cell-extracellular matrix (ECM) adhesion. Upon ligand binding, integrins activate signal transduction pathways that mediate cellular signals including regulation of the cell cycle, organization of the intracellular cytoskeleton, and movement of new receptors to the cell membrane.⁶⁶ Chang et al. showed that the increase in fucosylation of integrin $\beta 1$ could activate integrin $\beta 1$ in J82 human bladder cancer cells.⁶⁷ It has also been reported that core fucosylation is essential for the functions of integrin $\alpha 3\beta 1$ -mediated cell migration and signaling, wherein deletion of core fucosylation on integrin $\alpha 3\beta 1$ could down-regulates its function.⁶⁸ These observations are consistent with our previous report that FUT8 overexpression in LNCaP cells increased Pca cell migration, while loss of FUT8 in PC3 cells decreased cell motility⁵⁴ and our results in this study indicate this could be integrin-mediated.

The epidermal growth factor receptor (EGFR), which has been shown to play critical roles in metastases of several cancers including prostate cancer was found to be highly fucosylated in AG cells compared to NAG prostate cancer cells. In addition to responses to growth factor stimulation, EGFR is known to regulate several biological functions that include cell growth and differentiation.⁶⁹ Gu et al. found that the core Fuc on EGFR is essential for the binding of the EGF to its receptor which is required for EGFR-mediated intracellular signaling.⁷⁰ In addition, hyper fucosylation on EGFR promotes EGF-mediated cellular growth and decreases sensitivity to tyrosin kinase inhibitors.⁷¹ Finally, we identified three overexpressed Fuc glycopeptides derived from aspartyl/asparaginyl beta-hydroxylase (HAAH), one of which was found to be significantly differentially expressed between AG and NAG in this study. Overexpression of HAAH has been associated with a variety of human cancers, with higher levels of immunoreactivity mainly occurring in infiltrating, or metastasizing, tumors including prostate cancer.⁷² Together, these results suggest that increased fucosylation plays an important functional role in the invasive activity of the AG cell lines.

CONCLUSIONS

In this study, we established a strategy to selectively enrich core Fuc glycopeptides and evaluated alterations in fucosylation profiles between NAG and AG Pca cell lines. We first illustrated that tandem enrichment pairing lectin and MAX column enrichment was optimal

for high yield of Fuc glycopeptides. Next, we explored the specificities of various fucose-binding lectins, showing LCA, PSA, and AOL displayed stronger binding capacities than AAL, LTL, and UEA I. Furthermore, LCA and PSA bound specifically to core Fuc, whereas AOL, AAL, and UEA I bound both core Fuc and branch Fuc. Among these lectins, LCA was the optimal lectin for enrichment of core Fuc glycopeptides and was utilized in our tandem enrichment strategy to profile the Fuc glycoproteomes of NAG and AG Pca cell lines. In total, 973 Fuc glycopeptides from 252 Fuc proteins were identified and quantified, with 51 Fuc glycopeptides from 26 Fuc proteins found to be upregulated more than 2-fold in AG cell lines compared to NAG cells. KEGG pathway analysis revealed the overexpressed Fuc peptides were derived from proteins associated with the PI3K–AKT signaling pathway, focal adhesion, and ECM–receptor interaction. Interestingly, in the AG Pca cells, 13 Fuc glycopeptides derived from three integrins (integrin $\alpha 2$, integrin $\alpha 3$, and integrin $\beta 1$) displayed increased core Fuc, with a functional impact in ECM–receptor interaction. Our study not only determined the specificities of Fuc-binding lectins but also established a workflow of tandem Fuc glycopeptide enrichment to investigate Pca cell lines to identify core Fuc glycoproteins that could have diagnostic value for AG Pca.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Workflow of optimization of enrichment methods and comparison of specificities of six Fuc-binding lectins (a) and large scale identification and quantification of intact Fuc glycopeptides from four Pca cell lines using LCA-MAX enrichment (b).

Figure 2.

Number of identifications of unique total glycopeptides and Fuc glycopeptides from the mixture of four Pca cells peptides enriched by MAX or lectin alone (a) and tandem using lectin followed by MAX (b). Different enrichment methods were operated in the same conditions. The ratio of Fuc glycopeptide against total glycopeptide was shown by the green line.

Figure 3.

Comparison of lectin specificities on core or branch Fuc by characteristic ion analysis and overlap of Fuc glycans between different lectins. Representative MS/MS spectra of core Fuc glycopeptides from LCA-MAX sample (a) and core + branch Fuc glycopeptides from AOL-MAX sample (b). The identification number of unique Fuc glycans containing the core and branch Fuc ions from different enrichment samples (c) and the overlap of Fuc glycans between MAX, LCA-MAX, PSA-MAX, and AOL-MAX enrichment samples (d). The core Fuc characteristic ion is PepHexNAcFuc (+) ion, and the branch Fuc ion is HexHexNAcFuc (+) ion.

Figure 4.

Results of large scale profiling of Fuc glycoproteomes from four Pca cell lines. (a) The identification number of Fuc glycans, glycosite containing peptides, Fuc glycopeptides, and Fuc proteins from three runs. The number from bottom to the top is the number uniquely identified from the first, second, and third run. (b) Distribution of intact Fuc glycopeptides containing different numbers of fucose.