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Comparing Urinary Glycoproteins among Three Urogenital Cancers and Identifying Prostate Cancer-Specific Glycoproteins

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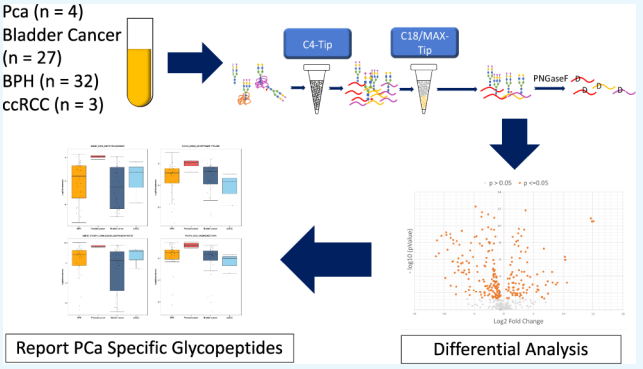
ABSTRACT: Prostate cancer, bladder cancer, and renal cancers are major urogenital cancers. Of which, prostate cancer is the most commonly diagnosed and second leading cause of cancer death for men in the United States. For urogenital cancers, urine is considered as proximate body fluid to the tumor site for developing non-invasiveness tests. However, the specific molecular signatures from different urogenital cancers are needed to relate changes in urine to various cancer detections. Herein, we utilized a previously published C4-Tip and C18/MAX-Tip workflow for enrichment of glycopeptides from urine samples and evaluated urinary glycopeptides for its cancer specificity. We analyzed 66 urine samples from bladder cancer (n = 27), prostate cancer (n = 4), clear cell renal cell carcinoma (ccRCC, n = 3), and benign plastic hyperplasia (BPH, n = 32) and then compared them with a previous publication that reported glycopeptides associated with aggressive prostate cancer (Gleason score ≥ 8). We further demonstrated the cancer specificity of the glycopeptides associated with aggressive prostate cancer. In this study, a total of 33 glycopeptides were identified to be specifically differentially expressed in prostate cancer compared to other urogenital cancer types as well as BPH urines. By cross-comparison with our previous urinary glycoproteomic dataset for aggressive prostate cancer, we reported a total of four glycopeptides from glycoproteins DSC2, MGAM, PIK3IP1, and CD55, commonly identified to be prostate cancer-specific. Together, these results deepen our understanding of the urinary glycoproteins associated with urogenital cancer types and expand our knowledge of the cancer specificity of urinary glycoproteins among urogenital cancer progression.



INTRODUCTION

Prostate cancer, bladder cancer, and renal cancers are major urogenital cancers. Of which, prostate cancer is the most commonly diagnosed and second leading cause of death for men in the United States.[1] Despite the fact that the prostate, bladder, and kidney are part of the urogenital system, the molecular profiles of the tumors can differ widely.[2] Thus, it is critical to identify molecular signatures, which can differentiate different urogenital cancer types.

Protein glycosylation is one of the most common and diverse post-translational modifications.[3,4] It has been reported that as many as half of the proteins in the human body are glycosylated.[5] Glycoproteins play a significant role in many biological functions, and aberrant protein glycosylation has been reported to relate to different diseases, including various cancer types,[6,7] rheumatoid arthritis,[8] and heart disease.[9] Recently, a study has reported that COVID-19 patients with severe symptoms have increased levels of IgG afucosylation compared to patients with mild symptoms.[10] Therefore, identifying key contributors between glycoproteins found in the urinary tract and the progression of urogenital cancers could be instrumental for our understanding of urogenital cancer biology.

For urogenital cancers, urine is considered an attractive source for discovering potential markers for cancer detection for its proximity to the tumor site, non-invasiveness, and ease of collecting specimens. Moreover, compared to human serum or blood, the urine proteome is viewed as a sample source with a less dynamic range in protein concentration as well as being less dominated by plasma proteins like albumin, immunoglobulins, or fibrinogens. These make urine an ideal biological specimen for developing tests for the detection of urogenital cancer. However, urine also contains inorganic salts, urea, and other biomolecules that can confound glycoproteomics analysis. Traditionally, urine samples need to undergo sample pre-processing techniques, such as protein precipitation, buffer exchange, ultrafiltration, or ultracentrifugation. In our previous publication, we reported a comprehensive workflow[11,12] that can

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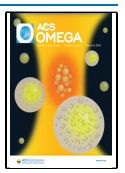

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harvest the urine glycoproteome without additional sample preprocessing in a high-throughput manner. Here, we adopted the aforementioned workflow to evaluate the urinary glycoproteome related to prostate cancer (PCa, $n = 4$) in comparison to bladder cancer ($n = 27$), clear cell renal cell carcinoma (ccRCC, $n = 3$), and benign prostatic hyperplasia (BPH, $n = 32$). A total of 33 glycopeptides were identified with specificity for prostate cancer relative to other cancer types and BPHs. We compared this to our previous published work, which discovered 79 aggressive PCa (Gleason score ≥ 8)-associated glycopeptides (corresponding to 66 glycoproteins), and identified four glycopeptides from glycoproteins DSC2, MGAM, PIK3IP1, and CD55 that were specifically expressed in prostate cancer urine samples, further indicating that these glycopeptides were associated with PCa rather than other urogenital cancer types.

MATERIALS AND METHODS

Chemicals and Materials. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. An Eppendorf 96-well plate, matrix D.A.R.T.s tips (20−300 μL), and automation reservoirs were obtained from Thermo Fisher Scientific (Hudson, NH). Oasis Mix Mode Anion eXchange (MAX) columns were purchased from Waters Corporation (Milford, MA). MS-grade trypsin was from Promega Corporation (Madison, WI). MS-grade LysC was from FUJIFILM Wako Chemicals. C4 reverse phase resin was purchased from Separation Method Technologies (Newark, DE) and Fisher Scientific (Waltham, MA). The polyethylene sheet has a median pore size of 15−45 μm (thickness = 1.57 mm or 0.062 in.; Interstate Specialty Products; Sutton, MA).

Sample Preparation. Human urine samples for this study were collected and analyzed under the approval by the Johns Hopkins Institutional Review Board. First Void Urine was collected from bladder cancer ($n = 27$) donors, benign prostatic hyperplasia ($n = 32$), prostate cancer ($n = 4$), and ccRCC ($n = 3$) and stored at −20 °C. The description of the urine samples is included in Table S1. The urine sample was subjected to spinning at 3000*g* for 10 min followed by extraction of supernatant and disposal of sediments.

Sample Preparation and IGP Enrichment Using a Versette Automated Handling System. All the samples were handled using a Thermo Fisher Scientific Versette Liquid Handling System with one cycle (aspiration/dispense) performed in approximately 2 min. All urine samples started with the same volume (500 μL).

The global proteomic sample processing procedure is derived from Clark et al.,[11,13] which is termed C4-Tip. Briefly, C4-Tips were conditioned with 50% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) followed by 0.1% TFA for 10 cycles each. Urinary proteins were acidified with 20% formic acid to make the pH < 3 and subsequently bound to C4-Tips for 30 cycles and paused for 1 min between each aspiration/dispense. Urinary protein-bound C4-Tips were then washed with 0.1% TFA and 50 mM triethyl ammonium bicarbonate (TEAB) to adjust the pH value (10 cycles each). Reduction of proteins was performed with 10 mM TCEP bond-breaker and 15 mM iodoacetamide in the dark for 20 cycles each. Protein digestion was carried out with Lys-C for 30 cycles and then followed by trypsin digestion for another 120 cycles (18 μL per 500 μL of urine samples for trypsin (0.5 mg/mL) and 9 μL per 500 μL of urine samples for Lys-C (1 mg/mL)) in a digestion buffer of 50 mM TEAB and 30% ACN. The digested peptides were eluted with 50% ACN in 0.1% TFA twice to ensure that all the peptides were collected (10 cycles each).

Intact glycopeptides were isolated from the peptide mixture according to our previously published automated method.[12,13] Samples were dried and reconstituted in 0.1% TFA while C18/MAX-Tip conditioning was performed using (1) ACN, (2) 100 mM triethyl ammonium acetate, (3) 95% ACN + 1% TFA, and (4) 0.1% TFA (sequentially, 10 cycles each) as described in the previous publication.[14] Reconstituted samples were bound onto C18/MAX-Tip (20 cycles) and washed with 0.1% TFA for 10 cycles. The global peptides were eluted from the C18/MAX-Tip with 95% ACN in 0.1% TFA (3 × 10 cycles). The IGPs were sequentially enriched with 50% ACN + 0.1% FA (3 × 6 cycles) and dried down.

For the removal of *N*-glycans, half of the intact glycopeptides (equivalent to 250 μL of starting urine volume) were dissolved in 98 μL of 100 mM TEAB with 2 μL of PNGaseF. The enzymatic glycan removal was incubated overnight in 37 °C. The de-*N*-glycopeptide and glycan mixtures are subjected to C18-cleanup via the StageTip method.[15] After removal of *N*-glycans, 1/5 of the de-*N*-glycopeptide (equivalent to 50 μL of starting urine volume) was subjected to DIA MS analysis with the addition of 1 μL of index retention time (iRT) peptides.

Nano-LC−MS/MS Analysis. For urinary glycoproteomic analysis, 10% of the total de-*N*-glycopeptides enriched from 500 μL of urine was analyzed by MS regardless of their initial urine protein concentration.

All the samples were analyzed by a Thermo Exploris 480 mass spectrometer (Thermo Scientific, USA) in a DIA mode. Peptides were separated with a 28 cm × 75 μm C18 column on an Easy-nLC 1200 at a flow rate of 300 nL/min with a 110 min linear gradient (from 6 to 30% B over 85 min, A = 3% ACN + 0.1% formic acid, B = 90% ACN + 0.1% formic acid). Full MS scans were acquired over the mass range of 400−1000 at a resolution of 120,000, AGC target set at 1×10^6, and a max injection time of 60 ms. MS2 scans were performed with a set of 50 overlapping windows covering the precursor m/z range of 400−1000 with a fixed isolation width of 12 m/z. The AGC target was set as the same as the full MS scan, while the resolution was set at 15,000, maximum injection time set as 25 ms, and NCE at 30%.

Database Search and Statistical Analysis of Glycopeptide DIA Data. DIA raw files of 66 urine samples were searched in Spectronaut using the directDIA analysis mode for quantitative analysis of the de-*N*-glycopeptides (also referred to as glycopeptides). A mass tolerance strategy for both MS1 and MS2 was set as dynamic, and the correction factor was set as one. Calibration mode was set as automatic, and the system default mass tolerance strategy was set for both MS1 and MS2. A local (non-linear) regression for the source-specific iRT to retention time was applied, and exclusion of deamidated peptides for RT regression was set as false. Cross-run normalization was not selected, and all quantified glycopeptides were filtered with a precursor Q-value cutoff of 0.01 (which corresponded to an FDR of 1%). After the de-*N*-glycopeptides were identified and quantified, normalization was performed on the glycopeptide intensity to the total protein amount of the individual urine samples.[16]

Statistical Analysis. A Mann−Whitney U test was used to calculate the *p*-value between any two groups of samples. A glycopeptide was considered significantly expressed at *p*-value < 0.05 and fold change > 2, except when conducting differential

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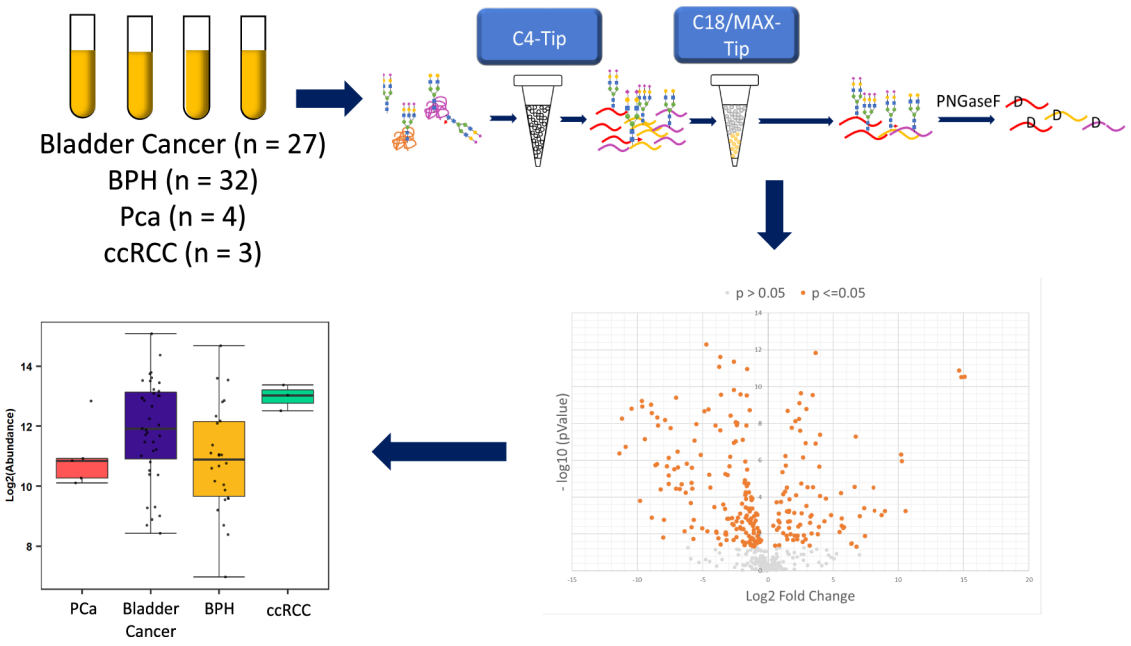

Figure 1. Workflow for quantitative analysis of urinary glycopeptides associated with different urogenital cancers. A total of 66 samples were processed using the C4-Tip and C18/MAX-Tip workflow, and differential analysis was carried out to obtain significantly up/downregulated glycopeptides.

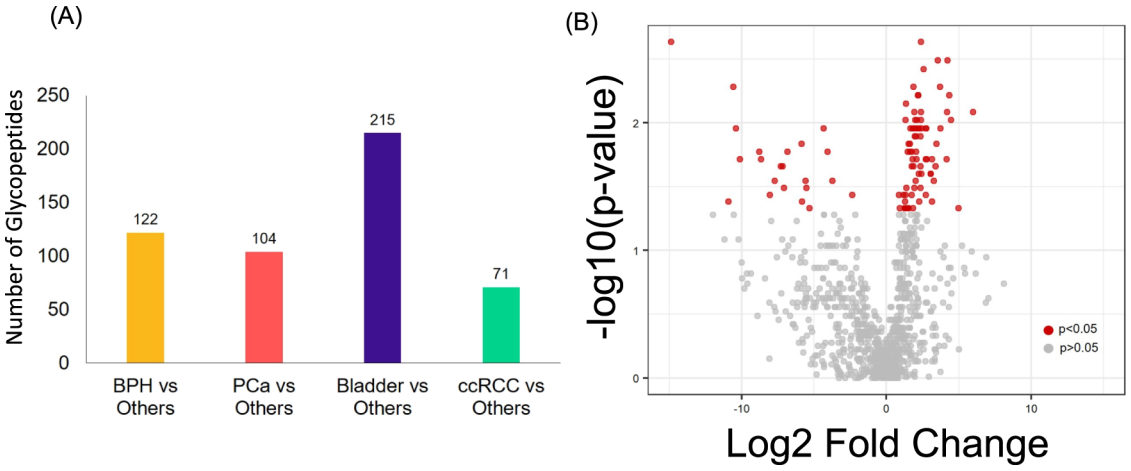

Figure 2. Differential analysis to discover cancer-associated glycopeptides. (A) Sixty-six urine samples were grouped as different cancer types (or benign), and differential analysis was conducted to identify glycopeptides significantly up/downregulated (with p-value < 0.05, fold change > 2). (B) Differential analysis for prostate cancer cohort against benign prostatic hyperplasia to identify glycopeptides associated with prostate cancer.

analysis for prostate cancer against ccRCCs, p-value < 0.06 was used.

RESULTS

Overall Experimental Workflow and Overview of the Quantified Glycopeptides. To ensure high repeatability of glycopeptide enrichment, we followed the experimental workflow implemented previously[11,12,16] (Figure 1). Briefly, all the urine samples (n = 66, Table S1) were first digested with a C4- and C18/MAX-Tip to isolate intact glycopeptides. Removal of glycans was preceded by PNGase F digestion on half of the starting volume of the glycopeptides followed by the C18 stage tip to separate the glycans from the de-*N*-glycopeptides. One-fifth of the de-*N*-glycopeptide eluate (equivalent to 50 μL starting urine volume) was subjected to LC−MS/MS analysis via DIA mode. The glycopeptides were identified and quantified from the DIA raw file of each urine sample by using Spectronaut (version 15.1). Normalization was performed on glycopeptides to the total protein amount in the individual urine sample.[16] Across 66 urine samples, 872 glycopeptides originating from 485 glycoproteins were identified and quantified with an FDR < 0.01 for both proteins and peptides (Table S2).

Assessment of Glycopeptides Associated with Different Urogenital Cancer Types. To assess the differences between one urogenital cancer type and the other urogenital cancer types (including BPH), differential analysis was conducted where the glycopeptide expression profiles of one cancer type were compared to those from others (i.e., BPH vs all others, PCa vs all others, bladder vs all others, and ccRCC vs all others). We found that 122, 104, 215, and 71 glycopeptides were significantly up/downregulated (p < 0.05) with >2-fold changes for BPH, PCa, bladder, and ccRCC samples relatively to other cancer types (Figure 2A), principal component analysis was also

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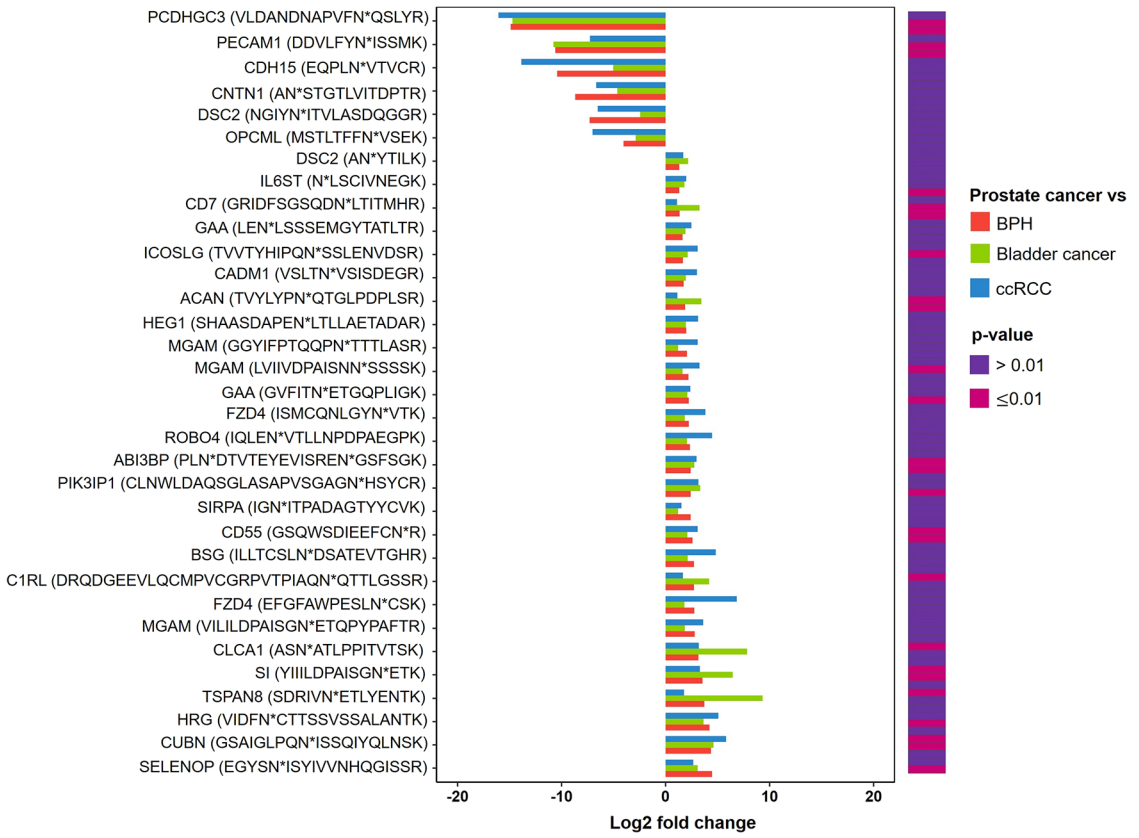

Figure 3. Differential analysis to discover prostate cancer-specific glycopeptides. A total of 96 glycopeptides associated with prostate cancer were identified and further evaluated by differential analysis against (1) bladder cancer and (2) ccRCC urine specimens. We observed 33 glycopeptides displaying prostate cancer specificity with the same trend of up- or downregulation when compared against different urogenital cancer types as well as benign prostatic hyperplasia.

conducted to assess the separation of prostate cancer with other cancers/BPH (Figure S3).

Quantitative Analysis of Urinary de-*N*-Glycopeptides to Identify Cancer-Specific de-Glycopeptides. To identify glycopeptides that were associated with prostate cancer, we conducted differential analysis for the prostate cancer samples against the BPH samples. Among the 872 glycopeptides identified and quantified, 76 were significantly upregulated ($p < 0.05$) and 22 were significantly downregulated (Figure 2B). Among the 98 differentially expressed glycopeptides, 96 glycopeptides had at least twofold changes (Table S3). We found that glycopeptides from glycoproteins, PCDHGC3 (protocadherin gamma-C3), DSC2 (desmocollin-2), LRP10 (low-density lipoprotein receptor-related protein 10), MGAM (maltase-glucoamylase), CNTN1 (contactin-1), and ENPEP (glutamyl aminopeptidase), displayed more than 10-fold increase in PCa relative to BPH (Table S3). All of these glycoproteins are reported to have mRNA expression in prostate, bladder tissues, or both based on the RNA-seq dataset provided on GeneCards.[17]

For significantly up/downregulated glycoproteins, PCDHGC3 is a member of the protocadherin gamma gene cluster. The protocadherin gamma gene cluster has an immunoglobulin-like organization, which suggests that a novel mechanism may be involved in their regulation and expression.[18] The protein expression for PCDHGC3 is found to be exclusively expressed in prostate, urine, urinary bladder, ovary, testis, and gallbladder based on the integrated proteomics expression in Protemics DB.[19] Interestingly, PCDHGC3 gene has also been found to be highly methylated only in carcinomas, but not in earlier stages, and has been proposed to act as a driver for the progression from adenoma to carcinomas in colorectal cancers.[20] DSC2 is a member of the desmocolin protein subfamily. Desmocolins are cadherin-like glycoproteins and are major components of the desmosome. Desmosomes are found in high concentrations in cells that are subjected to mechanical stress.[21] Elevated DSC2 expression has been reported in prostate cancer cells compared with RWPE-1 cells recently.[22] Moreover, inhibition of DSC2 also promoted the proliferation, migration, and invasion while suppressing the apoptosis of LNCaP cells and PC-3 cells.[22]

LRP10 is a low-density lipoprotein receptor protein, and it is found to have higher RNA expression in prostate cancer compared to bladder cancer, renal cancers, and testis cancer based on the data annotated by the Human Protein Atlas.[23,24] LRP10 gene has also been reported to be hypermethylated in prostate cancer.[25] MGAM is a maltase-glucoamylase, which is an enzyme that plays a role in the digestion of starch. It is reported to have lower mRNA expression in prostate cancer tissues compared to non-tumorous prostate tissues.[26] However, it is also reported that glycopeptide (glycosite at N827) expression for MGAM is significantly upregulated in aggressive prostate cancer (Gleason score $\geq$ 8) compared to non-aggressive prostate cancer (Gleason score = 6).[16] In our dataset, we found five glycopeptides from MGAM being significantly upregulated in prostate cancer compared to BPH with more than twofold changes (Table S3), suggesting that glycopeptide analysis could provide more insight to protein or glycopeptide

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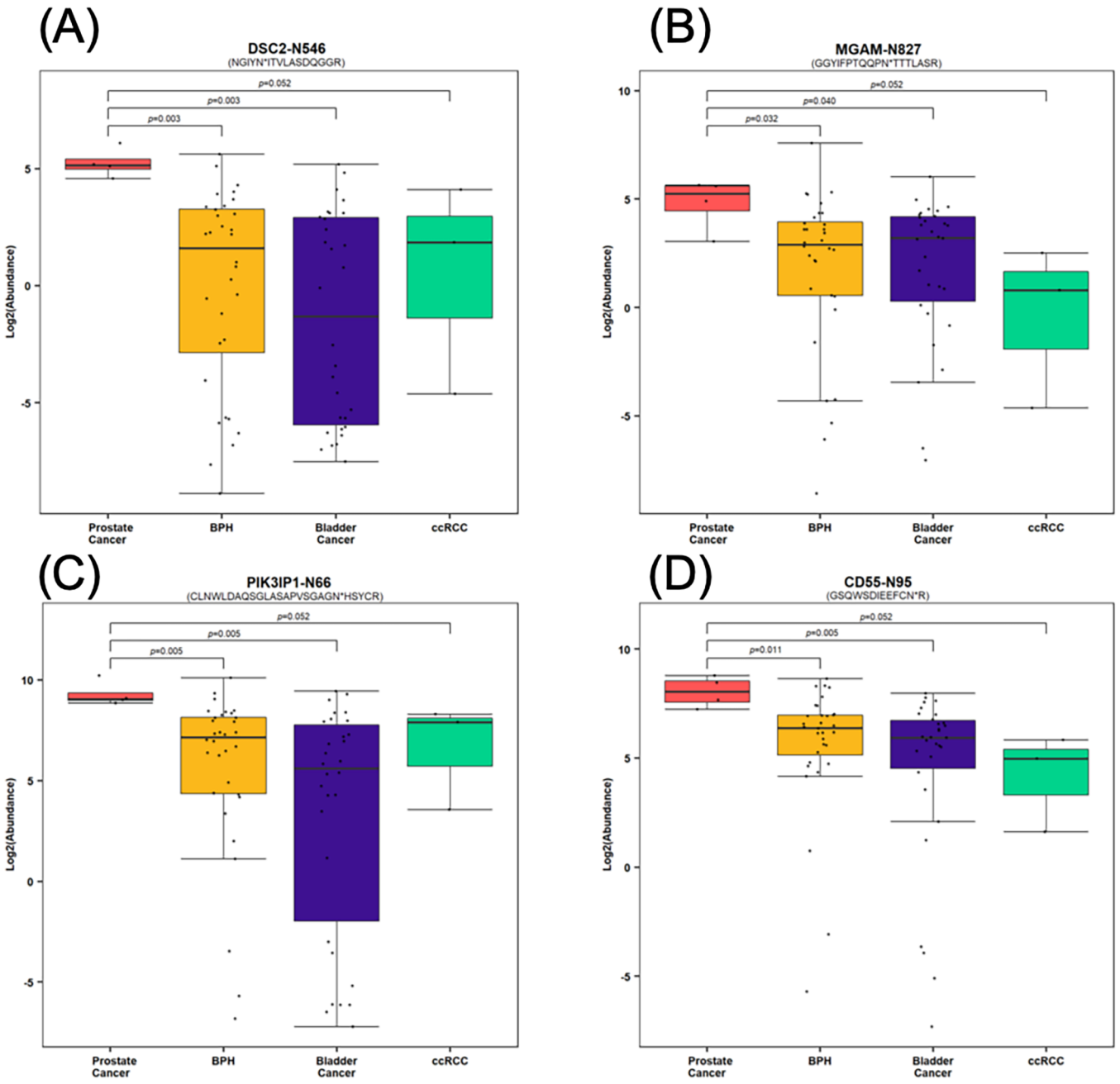

Figure 4. Expression of commonly identified glycopeptides that are prostate cancer-specific and associated with aggressive prostate cancer. Box plot of glycopeptides from (A) DSC2 N546, (B) MGAM N827, (C) PIK3IP1 N66, and (D) CD55 N95 commonly found to be up- or downregulated to distinguish prostate cancer specificity.

expression of certain cancer types than mRNA expression. CNTN1 is a member of the immunoglobulin superfamily. It is reported to have higher RNA expression in prostate cancer than in renal cancers, bladder cancer, and testis cancer based on the data annotated by the Human Protein Atlas.[24] CNTN1 is also reported to be significantly upregulated in prostate cancer tissues compared to normal adjacent tissues, and the knockdown of CNTN1 inhibited proliferation, migration, invasiveness, and epithelial-mesenchymal transition in prostate cancer cells.[27] ENPEP is an integral member protein that is capable of upregulating blood pressure and can regulate blood vessel formation and enhance tumorigenesis in some tissues. It is worth noting that ENPEP, along with ANPEP, DPP4, and TMPRSS2, is found to be candidate co-receptors for the coronavirus SARS-COV-2.[28] ENPEP is also reported to be upregulated in urinary extracellular vesicles in renal cancer patients compared to prostate cancer patients.[29]

Quantitative Differential Analysis of Urinary de-*N* Glycopeptides to Determine Prostate Cancer-Specific Glycopeptides. To further evaluate whether the glycopeptides associated with prostate cancer is specific to prostate cancer instead of being commonly observed in another urogenital cancer type, we conducted differential analysis between prostate cancer and bladder cancer and between prostate cancer and ccRCC (Table S4). Among the aforementioned 96 prostate cancer-associated glycopeptides, 33 displayed significant changes when compared to both bladder cancer samples and ccRCC samples (Table S5) with an estimated FDR of <0.056

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based on label permutation (n = 500). We found 27 and six glycopeptides (from the 33 glycopeptides) to be upregulated and downregulated, respectively (Figure 3).

The 33 glycopeptides showed the same expression patterns when compared to BPH, bladder cancer, and ccRCC urine specimens, suggesting that these glycopeptides are more likely to be prostate cancer-specific glycopeptides. The remaining 63 glycopeptides (out of 96 glycopeptides) only showed significant up/downregulation in prostate cancer relative to BPH, suggesting that these glycopeptides may not be prostate cancer-specific glycopeptides; however, these glycopeptides may be associated with ≥2 aforementioned urogenital cancer types.

For the glycopeptides specifically associated with prostate cancer, we observed glycopeptides from glycoprotein PECAM1 (platelet endothelial cell adhesion molecule), PCDHGC3, CDH15 (cadherin-15), DSC2, ICOSLG (ICOS ligand), ACAN (aggrecan core protein), and CNTN1 displaying more than fivefold changes. PECAM1 is a member of the immunoglobulin superfamily and is likely involved in leukocyte migration, angiogenesis, and integrin activation.[30] PECAM1 makes up a large portion of endothelial junctions, and the protein encoded by this gene is found on the surface of platelets, monocytes, and some types of the T cells.[30] Interestingly, RNA expression for PECAM1 is found to be highly expressed in renal cancers compared to urothelial cancer, prostate cancer, and testis cancers based on the TCGA dataset; however, in our dataset, the glycopeptide (glycosite at N84) from PECAM1 demonstrated significant upregulation in prostate cancer relative to bladder cancer, ccRCC, and BPH samples. CDH15 is a member of the cadherin superfamily that encodes calcium-dependent intercellular adhesion glycoproteins. Estimated protein expression for CDH15 is found to be possessed only in the urine, frontal cortex, and pancreatic juice from Proteomics DB. Hypermethylation of the CDH15 gene is found to associate with hepatocellular carcinoma.[31] ICOSLG is an inducible T cell co-stimulator ligand, which acts as a co-stimulator signal for T-cell proliferation and cytokine secretion and potentially plays a role in mediating local tissue responses to inflammatory conditions.[32] Recently, protein expression for ICOSLG has been found to be elevated in patients with BPH and has also been reported to not be a specific marker for high-risk prostate cancer (Gleason score = 9) using a high-multiplex immunoassay.[33] Although our dataset does not have enough high-risk prostate cancer to further validate this observation, we found one glycopeptide (ICOSLG N70) that was significantly upregulated, which could be prostate cancer-specific glycopeptides. This could provide more insight that glycoproteomic analysis using LC−MS/MS can unveil additional insights compared to other proteomic analysis strategies. ACAN is a member of the aggrecan/versican proteoglycan family. The ACAN protein is a major part of the extracellular matrix in cartilaginous tissues. It was reported that mRNA expression for ACAN was present in prostate tumors, while ACAN was usually not detected in normal prostate tissues or BPH.[34]

Determining Glycopeptides with Altered Expression for Distinguishing Prostate Cancer from Bladder Cancer Samples and Benign Prostatic Hyperplasia. We compared the expression profiles of the abovementioned 33 glycopeptides with our previous work of which 79 urinary glycopeptides were found to be associated with aggressive prostate cancer.[16] Four glycopeptides identified in the current study as prostate-specific were also identified in our previous publication, including glycopeptides from DSC2, MGAM, PIK3IP1 (phosphoinositide-3-kinase-interacting protein 1), and CD55 (complement decay-accelerating factor) (Figure 4). CD55 is a glycoprotein that is involved in the regulation of the complement cascade. CD55 has been reported to be upregulated in prostate cancer tissue specimens via tissue microarray analysis.[35] It is also reported that CD55 can inhibit the complement-mediated lysis in prostate cancer cell lines, which includes PC-3 and DU145 cells.[36] PIK3IP1 has been reported to be significantly elevated in the urine of prostate cancer patients compared to patients with BPH.[37] PIK3IP1 is also the targeted genes for miRNA-32, in which this miRNA has been reported to have a higher expression in castration-resistant prostate cancer compared to benign prostate cases.[38,39]

In total, nine glycopeptides from six glycoproteins were commonly identified by the current study and our previous publication (Table S6). The glycopeptide expressions for these nine glycopeptides are shown in Figure 4 and Figure S1. Together, the four commonly identified glycopeptides demonstrated that these glycopeptides were not only associated with aggressive prostate cancer but also able to distinguish between prostate cancer and other urogenital cancer types. As for the nine glycopeptides reported, two of them were from protein DSC2 (glycopeptide DSC2 N392 and DSC N546), and three of them were from glycoprotein MGAM (MGAM N827, MGAM N458, and MGAM N1323). This could suggest that multiple glycopeptides from the same glycoprotein were simultaneously upregulated in prostate cancer; however, without global proteomic data evaluating the global peptide expression for these glycoproteins, we could not assess the upregulation in the protein level for these glycoproteins in prostate cancer compared to other urogenital cancer types.

DISCUSSION

Urogenital cancer, in particular, prostate cancer, is still one of the leading causes of death in men in the United States as of 2020. For prostate cancer, the Gleason scoring system has been used to evaluate the risk of mortality and probability of metastasis, where a Gleason score of ≥7 is viewed as aggressive prostate cancer, and a Gleason score of less than 7 is non-aggressive prostate cancer. On the other hand, BPH patients, bladder cancer patients, or even ccRCC patients can express similar onset symptoms as prostate cancer patients such as blood in the urine, pain or burning sensation during urination, inability to urinate, or enlarged prostate for BPH and prostate cancer patients. In type II diabetes cases, BPH can also raise the likelihood of bladder cancer occurence.[40] For these urogenital cancers that display similar onset symptoms, it is crucial to develop a strategy that can differentiate these cancers from one another for accurate early detection, diagnosis, and efficient downstream treatment.

In this study, we evaluated urinary glycopeptides enriched from different urogenital cancer types, as well as BPHs, for determination of upregulating glycopeptides that were specific to prostate cancer but not in other urogenital cancer types. We identified 33 urinary glycopeptides from a total of 872 urinary glycopeptides that could differentiate between prostate cancer and other urogenital diseases. The prostate cancer-specific glycopeptides were then cross-compared with our previous work, which reported 79 urinary glycopeptides associated with aggressive prostate cancer. In which, four glycopeptides were commonly identified between the two studies. On the glycoprotein level, nine glycopeptides from six glycoproteins

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were commonly identified. A glycopeptide FLN*ESYK from glycoprotein ACPP has been previously reported to show the best performance for distinguishing between aggressive and non-aggressive prostate cancer, which is downregulated in aggressive prostate cancer. In this study, we also detected the same glycopeptide from ACPP. Even though the current data set was not ideal for determining the discriminating power of ACPP toward prostate cancer in comparison to BPH and other urogenital cancer types, we found that the median expression of FLN*ESYK from ACPP was lower in prostate cancer than in BPH and bladder cancer (Figure S2). It is worth noting that other factors or comorbidities could also simultaneously affect urinary glycosylation, for instance, lower urinary tract symptoms, diabetes, hematuria, cardiovascular diseases, or hypertension. With the general characteristics of our cohort being narrow and aimed toward urogenital cancers and benign prostatic hyperplasia, it is likely that patients with these urogenital cancers or BPH could also carry a urogenital disease that can potentially simultaneously dysregulate urinary glycosylation.

It is also worth mentioning that RNA expression does not necessary correlate with protein expression, and protein expression also does not directly correlate with glycopeptide expression. For example, RNA expressions of PECAM1, PCDHGC3, and MGAM have been reported to be more expressed in renal cancers than in prostate cancer based on the TCGA dataset. However, in our dataset, the glycopeptide expressions for the PECAM1 N84, PCDHGC3 N245, MGAM N827, MGAM N458, and MGAM N1323 were all highly expressed in the prostate cancer urine specimens compared to urine samples of other cancer types. A hypothesis for this phenomenon is that certain glycosylation sites or glycoforms were highly expressed in prostate cancer, but the protein expression or gene expression remained the same, or even downregulated. Fucosylated PSA is a good example; while the serum PSA level alone may not be able to distinguish between aggressive prostate cancer and non-aggressive, the serum-fucosylated PSA has been repeatedly reported to improve the detection power of aggressive prostate cancer from non-aggressive prostate cancer[41,42] or differentiate between prostate cancer and BPH.[43]

CONCLUSIONS

In this study, we reported 33 glycopeptides that were specific to prostate cancer by analyzing a cohort composed of 66 urine specimens from three different urogenital cancers and BPH. Among the 33 differentially expressed glycopeptides, glycopeptides from glycoproteins DSC2, MGAM, PIK3P1, and CD55 were discovered in our previous study, which were associated with aggressive prostate cancer. The current study suggests that a glycoproteomic approach via LC−MS/MS could provide additional insights to glycoproteins specifically associated with prostate cancer as compared to bladder cancer, ccRCC, and BPH. For the limited amount of samples included in this study, the cohort for ccRCC ($n = 3$) and prostate cancer ($n = 4$) is too small to produce statistically significant results, and indeed, a validation study using a larger sample size is needed to further confirm our results; nevertheless, our data still suggest that urine as an appealing sample source for conducting a large cohort glycoproteomics study to distinguish prostate cancer from other urogenital cancer and could potentially discover other glycopeptides that are specifically associated with different cancer types.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c05223.

Additional supporting information describing the sample cohort of urogenital cancers (Table S1), glycopeptide expression quantified using Spectronaut DirectDIA analysis of the urogenital sample cohort (Table S2), differentially expressed glycopeptides for prostate cancer against benign prostatic hyperplasia urine specimens (Table S3), differential analysis to distinguish prostate-specific glycopeptides prior to a *p*-value filter (Table S4) and after *p*-value filter (Table S5), comparison between 33 prostate cancer-specific glycopeptides and 79 glycopeptides previously reported to associate with aggressive prostate cancer (Table S6), and comparison of tissue proteomic data from Zhou et al. (Table S7) (XLSX)

Glycopeptide expression of commonly identified glycoprotein between this current study and our previous published work (Figure S1), expression profile of glycopeptide FLN*ESYK from glycoprotein ACPP (Figure S2), and principal component analysis to distinguish glycoproteomic signatures among sample cohorts (Figure S3) (PDF)

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Notes

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