Peer Review File

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Review Comment

The article, entitled: "Urinary Glycan Biomarkers in Prostate Cancer" attempts at giving an overview of the status of urinary glycan profiles/ molecules, as non-invasive biomarkers for prostate cancer diagnosis, monitoring, prognosis, and treatment.

Unfortunately, the structure and content of this article fail to critically describe how the urinary glycan biomarkers have been evaluated in the context of prostate cancer management and what is the impact of these findings. Consistent presentation of biomarker accuracy per context of use is also missing. Moreover, the technical part is poorly described, without any hint on which platform could be better suited according to each clinical purpose. Last but not least, the selected biomarker studies seem to be randomly included, since there are no consistent criteria (proper validation, high number of samples, proper controls used, promising diagnostic/ prognostic performance etc.).

Response: Thank you for the pointing suggestion. We agree with the comments. We revised as the reviewer suggested.

Detailed comments:
Comment 1: The most important limitation is that after this review, there are no clear conclusions. You should indicate which are the best biomarkers for each clinical context of use, for example early diagnosis, prediction of disease progression and prediction of treatment depending on the disease localization and tumour expansion.

Response: Thank you for the pointing suggestion. We agree with the comments. We added clear massage in the conclusion section. Also, we added the table (Table 1) to address this suggestion.

Comment 2: A summary of the FDA approved biomarkers and/or those commercially available is missing. This sets the stage for critical assessment of the urinary glycan biomarkers and how close are to clinical implementation.

Response: Thank you for the pointing suggestion. We agree with the comments. We added the summary of the FDA approved biomarkers and/or those commercially availability. Unfortunately, no urinary glycan biomarkers are approved by FDA. Also, there was commercially available measuring tools but those need custom technique, tools, and machines.

Page 12 line 265

5. Summary of urinary glycan biomarkers and the information of the Food and Drug Administration (FDA) approved biomarkers and/or those commercial availabilities.

In this narrative review, we showed potential urinary glycan biomarkers for PC detection and aggressive disease (Table 1). Of those, urinary fucosylated PSA levels are a promising biomarker for PC detection and aggressiveness among the aberrant PSA glycosylation. Urinary CGNT1 in the post-massage urine can be useful for the prediction of the extracapsular extension after radical prostatectomy. However, no FDA approved urinary glycan biomarker is available. Also, urinary glycan biomarker analyses were carried out using a custom technique, tools, and machines, while those are commercially available. Therefore, there is a significant hurdle between the urinary glycan analysis and clinical implementation. Therefore, urinary glycan analysis is far from clinical implementation. Further studies and methodological improvements are necessary to overcome these limitations.

Comment 3: Please state the inclusion criteria for selecting the biomarker studies, for example validation in c
independent cohort, number of samples, proper controls used, promising diagnostic/ prognostic performance etc.
Response: Thank you for the pointing suggestion. We agree with the comments. We added the inclusion criteria in the text.

Page 5 line 96

3. Methods

Search methods for identification of studies

PubMed online database was accessed for research on Aug 10th, 2020. Searches were performed using the keywords: “prostate cancer”, “urine”, and “glycan”. Each identified abstract was independently evaluated by two authors. All studies were independently evaluated and selected the consistent criteria such as independent cohort, a proper number of samples and controls, clinically meaningful outcomes, and promising diagnostic/prognostic performance. This study was performed according to the ethical standards of the Declaration of Helsinki and approved by the ethics review boards of the Hirosaki University School of Medicine (authorization number: 2019–001 and 2019–099).

4. Result of study screening

We identified 38 studies and excluded 30 studies that did not meet the inclusion criteria. Finally, we included 8 studies in this narrative review (Fig. 3). Studies were classified into 5 categories such as 1) aberrant PSA glycosylation, 2) urinary glycoproteins, 3) exosome, 4) glycosyltransferases, and 5) hyaluronic acid. The number of studies for PC detection, aggressive disease, and both of them were 6 (80,97-101), 1 (77), and 1 (79), respectively.

Comment 4: The description of the biomarkers does not give any useful information. There is no consistency in the presentation and important information is missing, such as: p values, type of sta-

tistical analysis, context of use, AUC estimates, % sensitivity and specificity, negative and positive predictive value. Moreover, there is no critical assessment of the findings.
Response: Thank you for the pointing suggestion. We agree with the comments. We added p values, AUC estimates, % sensitivity and specificity, and critical assessment of the findings. Also, we added the table (Table 1) to address this suggestion.

Page 6 line 118
They investigated Lewis-type or core-type fucosylated PSA (PSA-AAL) and core-type fucosylated PSA (PSA-PhoSL) in from urine in 69 patients who suspected PC (20 patients without PC and 49 patients with PC) and found urinary fucosylated PSA was significantly decreased in the men with PC compared with the men without PC ($P = 0.026$ and $P < 0.001$, respectively). Also, both PSA-AAL and PSA-PhoSL were significantly associated with the Gleason scores of the biopsy specimens ($P = 0.001$, and $P < 0.001$, respectively). The area under the receiver-operator characteristic curve (AUC) value for the prediction of cancers of Gleason score ≥ 7 was 0.69 ($P = 0.0064$) for urinary PSA-AAL and 0.72 ($P = 0.0014$) for urinary PSA-PhoSL. They developed an optimum logistic regression model to predict the probability of detecting cancers with a GS ≥ 7 in biopsy was obtained as $P = [1 + \exp (1.247 + 4.56 \times PSAD – 0.00448 \times PSA\text{-}AAL – 0.0493 \times PSA\text{-}PhoSL)]^{-1}$. Using this model, the AUC value for the prediction was 0.82 (95% CI 0.72–0.92, $P < 0.0001$) with the sensitivity and specificity of the model at the best cutoff value were 74.1% and 81.5%, respectively (Table 1). Although the biological mechanism leading to decreased urinary fucosylated PSA level in urine remains unclear, decreased urinary fucosylated PSA level may be a potential marker for aggressive PC.

Page 6 line 133
They investigated 61 benign prostate hyperplasia (BPH) urine samples and 38 prostate cancer urine samples. After the immunoprecipitation and in-gel protein digestions, the peptides and N-glycopeptides generated from the chymotrypsin digestion were analyzed with an LC-MS. The normalized Hex5HexNAc4NeuAc1dHex1 (H5N4S1F1), monosialylated, sialylated, and unfucosylated glycoforms showed significant differences between BPH and PC. The ROC curve and the AUC of those glycoforms showed significant differences in PC detection with sensitivity and specificity of 87.5% and 60%, respectively (Table 1). This result suggests the unfucosylated glycoforms of PSA were potential urinary glycan biomarkers in PC, in opposition to the results from Fujita et al (79). One reason for this discrepancy might be the methodological differences between the lectin-antibody ELISA detection and LC-MS detection. Furthermore, the preparation of urine samples greatly influences the outcomes of downstream analyses. For example, urinary Tamm-Horsfall Protein (uromodulin) interferes with urinary assays and forms contaminant precipitates in the urine. Therefore, urinary aberrant PSA glycosylation needs further study to apply the clinical practice.

Page 7 line 152
They found no significant difference in S2,6PSA levels between the biopsy negative patients and PC patients with Gleason score 6 ($P = 0.364$), between the biopsy negative patients and PC patients with Gleason score 7 ($P = 0.116$), and between the biopsy negative patients and PC patents with

Gleason score 8 or more (P = 0.276). Also, they found no relationship was found between S2,6PSA and prostate cancer aggressiveness. These results may suggest the limited utility of S2,6PSA alone in urine to detect PC. The ratio of S2,3PSA and S2,6PSA needs to be investigated because these 2 glycoforms are associated with each other during the PC progression. Therefore, this finding needs to be interpreted with caution because of the small sample size and limitation measurement of PSA glycoforms. Currently, urinary fucosylated PSA levels are a promising biomarker for PC detection and aggressiveness among the aberrant PSA glycosylation.

Page 8 line 165
Capillary electrophoresis is a technique that separates molecules via an electric field according to size and charge. Several capillary electrophoresis-based systems for urinary glycan analysis are available, such as the Gly-Q system (Fig. 4) and the multicapillary electrophoresis-based ABI3130 sequencer. Vermassen et al. (99) evaluated urinary N-glycosylation profiles in post-prostate massage urine using capillary electrophoresis and demonstrated differences between patients with PC and benign prostate hyperplasia. Also, they developed a urinary glycoprofile marker (ratio of non-fucosylated bi-, tri-, and tetra-antennary glycan structures on total triantennary glycan structures divided by the prostate volume), and showed the potential to differentiate benign prostate hyperplasia from PC with the AUC, sensitivity, and specificity of 0.77, 90%, and 47%, respectively (Table 1). The updated analysis showed similar performance of the urinary glycoprofile marker in the patients with a gray zone (Table 1). The predictive accuracy of the urinary glycoprofile marker was significantly better than that of serum PSA (P<0.001) (80). A Capillary electrophoresis system can analyze glycoprotein in urine; however, limited evidence is currently available. Also, we need to combine some glycans (such as urinary glycoprofile marker) to detect PC. Further large-scale studies are necessary to address the use of capillary electrophoresis-based analysis to identify urinary glycan PC biomarkers.

Page 10 line 207
They investigated post-digital rectal examination urine from 35 patients before underwent radical prostatectomy and detected GCNT1 by an anti-GCNT1 monoclonal antibody, followed by a horseradish peroxidase (HRP)-conjugated antibody. The GCNT1 expression (P = 0.006) was highly correlated to the extracapsular extension of PC in a logistic regression analysis with the AUC value of 0.7614 (Table 1). Of urinary glycan markers, GCNT1 may be a potential predictive marker for tumor recurrence after radical prostatectomy.

Page 10 line 223
ROC analysis for hyaluronic acid and hyaluronidase had a significant predictive ability for PC with AUC of 0.65 (70% sensitivity and 55.2% specificity) and 0.69 (65% sensitivity and 53.9% specificity), respectively (Table 1).

Comment 5: The description of the technological platforms is vague, often not mentioned at all. Important references are missing for example Paragraph “Potential biomarkers in urine” lines 85-93.

Response: Thank you for the pointing suggestion. We agree with the comments. We added the description of the technological platforms in the figure legends. Also, we added references in those parts.

Comment 6: Table 1, does not present any meaningful evidence on the application of glycosyltrasferases as urinary biomarkers in PCa. Regulation trend is missing, along with the references and other important information like clinical context of use and accuracy.

Response: Thank you for the pointing suggestion. We agree with the comments. We removed previous Table 1 and revised it.

Comment 7: There are several syntax and typographical errors throughout the text, like for example:

- Line 25 “the diagnose”
- Line 30 “common usage in PC”
- Line 37 “associated with the diagnosis and aggressive of PC”’

Please make sure that you have a full comprehensive read of your manuscript by a fluent English speaker.

Response: We apologize for errors. We fixed these parts. This manuscript was fully checked by a native speaker.