Author's response to reviews

Title: Urinary Levels of Hepatocarcinoma-intestine-pancreas/Pancreatitis-associated Protein as a Diagnostic Biomarker in Patients with Bladder Cancer

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Author's response to reviews: see over
Dear Dr. C. Rosser

Thank you for your letter of 15 June, recommending revision. We have carefully considered your comments and here would like to revise the manuscript. The major points, which we have changed in this revised version of manuscript, are following:

(1) According to the suggestion of Reviewer #2, we excluded the data obtained from patients with upper tract tumors throughout the manuscript, and accordingly we have changed text, title, Fig. 1A, 3A, 5 and Table 2.

(2) We have re-examined the Western blotting to show multiple samples of bladder cancer demonstrating the increase of HIP/PAP protein according to the comment by Reviewer #2. We have exchanged the figure (Fig. 1A).

(3) As both reviewers pointed out the possibility that patients with urinary tract infections or hematuria might cause a false positive with the test. Although it is impossible to rule out the possibility, but all of hematuria non-cancer samples were negative, and four of six non-interstitial severe cystitis patients were also negative. We have discussed about those points in the Discussion section.

We have attached the point-by-point response to the concerns. Please find the following pages.

We think the revised version of the manuscript was improved very much by the helpful comments of the reviewers.

We are looking forward to hearing from you about the decision.

Sincerely

Dr. Hiroshi KIYAMA
Responses to Reviewer #1

Comment 1
Information on the study cohort needs to be mentioned in the abstract.
Response
We have added patient characteristics in the abstract.

Comment 2
Page 4, Background. Authors state that BTA/NMP22 tests are unable to correctly predict bladder tumors. These biomarker tests can detect UC with performances better than voided urine cytology. They are not perfect, particularly when detecting low stage/grade UC, but the literature shows that NMP22 achieves sensitivity/specificity values close to those achieved by HIP/PAP in this study. The performance of these biomarkers needs to be more fairly described.
Response
We agree with this comment, and accordingly we have changed the text as follow ‘In particular, NMP22 has been widely used as a urine marker for BCa of good sensitivity in comparison with urine cytology. Numerous studies have demonstrated that NMP22 has a superior sensitivity (46–88% and 40–85%, respectively) to urine cytology. However, there remains differences among reports yet, and the sensitivity is not fully satisfactory.’ (Page 4, line 10-13)

Comment 3
Page 5, the authors describe the fact that they have reported higher HIP/PAP levels in patients with painful bladder syndrome/interstitial cystitis. The authors state that this was the basis for investigating HIP/PAP in UC, but the utility of HIP/PAP as a UC biomarker may be severely compromised because many patients with urinary tract infections may be falsely positive with this test. With this knowledge, there should have been an effort to include UTI cases in the cohort, but of the 119 control cases, only 6 have cystitis. This limitation needs to be discussed.
Response
We have added the following sentences in Discussion according to the reviewers’
comments. ‘In this study, we investigated six cases of non-interstitial severe cystitis patients in the group of benign urological disease, and two in the six cases were false positive. Similar to the cases of other urinary markers such as NMP-22 and BTA, the false positive for HIP/PAP may be possibly identified in patients with benign inflammatory conditions.’ (Page 12, line 10-14)

**Comment 4**
There is no mention of correlation of HIP/PAP with hematuria. The other common interference with urinary tests is the presence of blood in the urine. Since HIP/PAP is present in serum/plasma (Mol Cell Proteomics 2004, 3(4):311-326) the investigation for such correlation is pertinent. Was there any correlation of HIP/PAP with blood in cancer and/or controls? If not tested, this limitation needs to be discussed.

**Response**
We have added the following sentences in Discussion. ‘In addition, to eliminate the potential impact of hematuria in this ELISA kit, we investigated two cases of patients with Nutcracker syndrome in the group of benign urological disease. Although the above two patients had gross hematuria, the concentration of HIP/PAP in the urine of both two cases was less than 8.5 pg/mL. We therefore assume that this ELISA kit is not affected by hematuria.’ (Page 12, line 14-18)

**Comment 5**
NMP-22 tests usually state that they should be performed on fresh urine samples. In this retrospective study, samples were processed and frozen for storage. This may explain the weaker performance of NMP22 in this study than described in the literature. This needs to be considered and commented on.

**Response**
Both urinary NMP-22 and BTA levels were measured and the cytology was examined using freshly voided urine immediately after the samples were obtained. We thus re-write the sentences properly. (Page 6, line 9-10)

**Comment 6**
The legend of Figure 3A indicates that ‘Bars represent median levels’; however
there are no bars in Figure 3A.

Response
This was our mistake. We have added median bars in Figure 3A.

Responses to Reviewer #2

Comment 1
ABSTRACT: The methods section requires more details as to patient numbers, patient conditions, cell lines, etc.
Response
The other reviewer also pointed this out, and we have added the patient characteristics in the abstract.

Comment 2
The word superficial is used throughout the manuscript. The correct terminology would be non-muscle invasive bladder cancer.
Response
We apologize for the use of ambiguous words. We re-wrote the superficial bladder cancer to the non-muscle invasive bladder cancer throughout the manuscript.

Comment 3
Should stress the preliminary nature of these results.
Response
According to this suggestion, we have added the following sentences in Conclusion. ‘A large scale studies will be needed to establish the usefulness of this biomarker.’

Comment 4
BACKGROUND: The first sentence “Bladder cancer is the most common urothelial carcinoma is confusing. I think you mean to say Urothelial carcinoma (formerly transitional cell carcinoma) is the most common bladder cancer.
Response
According to the reviewer’s comments, we re-wrote the sentence as follows. ‘Urothelial carcinoma (UC) is the most common Bladder cancer (BCa).’ (Page 4, line 2)
Comment 5
Next statistics from 2008 are given. Can you provide statistics from 2011 or 2012?

Response
To our knowledge, this reference appears to be the newest.

Comment 6
Previously the authors demonstrated HIP/PAP is increased in inflammatory conditions of the bladder. Does this distract from HIP/PAP as a diagnostic marker since inflammatory conditions will be positive? Perhaps patients with standard inflammatory conditions (cystitis) should be added as a separate control group.

Response
In consideration of this comment, we have added the following sentences in Discussion. ‘In this study, we investigated six cases of non-interstitial severe cystitis patients in the group of benign urological disease, and two in the six cases were false positive. Similar to the cases of other urinary markers such as NMP-22 and BTA, the false positive for HIP/PAP may be possibly identified in patients with benign inflammatory conditions.’ (Page 12, line 10-14)

Comment 7
METHODS: In the text state the break down of the benign controls. The healthy volunteers may offer little information since their numbers are so low.

Response
As we mentioned in the Abstract according to this reviewer’s comment, this study is not a comprehensive large size cohort, but trying to suggest the advantage and possibility of the use of HIP/PAP as a marker of the urotherial cancer to the researchers in this field as quick as possible. At this moment we thus would like not to expand the scale and not to take time for getting further informed consents, which will take significant time to obtain from many volunteers. We thus would like this reviewer to understand about keeping the size.

Comment 8
Subjects with bladder and upper tract tumors are included. Though these are UC, the
molecular make-up are different. Please delete subjects with upper tract tumors and only report subjects with bladder cancer.

**Response**

According to this comment, we have re-made Figure 1A, 3A, 5 and Table 2, and changed text.

**Comment 9**

Western blot was performed to illustrate HIP/PAP expression. I think this is ok for the cell lines, but for the human specimens immunohistochemical staining should be employed to confirm its presence and to determine the location of the HIP/PAP. Furthermore more than 1 tumor should be analyzed.

**Response**

This comment is quite reasonable, but regrettably, the reactivity of our antibody was suitable for the Western blot but not for immunohistochemistry. In terms of the sample number, we have increased sample numbers and re-examined Western blotting in Fig. 1A according to the suggestion.

**Comment 10**

How did urinary hemoglobin levels affect assay? As you know urinary blood can adversely affect NMP-22 and BTA. Were HIP/PAP levels normalized to urinary protein or urinary creatinine? Urinary levels/concentrations may vary widely throughout the day due to hydration status, voiding patterns, etc.

**Response**

The other reviewer also pointed out this issue. As we mentioned in the response to Reviewer #1, we have added the following sentences in Discussion. ‘In addition, to eliminate the potential impact of hematuria in this ELISA kit, we investigated two cases of patients with Nutcracker syndrome in the group of benign urological disease. Although the above two patients had gross hematuria, the concentration of HIP/PAP in the urine of both two cases was less than 8.5 pg/mL. We therefore assume that this ELISA kit is not affected by hematuria.’ (Page 12, line 14-18)

We have analyzed HIP/PAP levels normalized to urinary creatinine. The data between un-normalized to urinary creatinine were not different significantly in all examination of this study. Furthermore, the previous paper of our party also analyzed urinary
HIP/PAP levels in the same way. (Urology 2010, 75(4):933-937)

Comment 11
Urinary supernatants were frozen and analyzed. Is it possible to reliable measure NMP-22 from previously frozen samples? NMP-22 manufacturers say its not reliable. BTA is ok. Please consider reporting by STARD and REMARK criteria for biomarkers.

Response
This was also pointed out by the first reviewer. Both urinary NMP-22 and BTA levels were measured and the cytology was examined using freshly voided urine immediately after the samples were obtained. We thus re-write the sentences properly. (Page 6, line 9-10)

Comment 12
RESULTS: Was there a positive correlation with HIP/PAP with grade as there was with stage? How about to size of tumor?

Response
We have shown the correlation between urinary HIP/PAP levels and T stage on Figure.3B. Bladder cancer group divided into two groups; tumor size \( \geq \) 3cm or < 3cm. As this reviewer expected, there was a positive correlation.

Comment 13
Please include the levels of HIP/PAP as it relates to low-risk and intermediate risk as well as low progression, intermediate and high progression.

Response
We have mentioned about this in this paper. ‘With regards to the recurrence-risk classification of non-muscle invasive bladder cancers, urinary levels of HIP/PAP in the low risk group were significantly lower than those in intermediate risk group (P=0.0002) (Fig. 4A).’ (Page 10, line 11-13)

Comment 14
Only sensitivity of cytology is reported. This is a limitation.

Response
We understand the limitation, and simply mentioned about this in the text. ‘Though
urine cytology had a sensitivity of 40.8% (data not shown), we could not calculate specificity, PPV and NPV because few control patients underwent urine cytology.’ (Page 11, line 9-11)

Comment 15
DISCUSSION: Please include a paragraph on limitations of your study. Is it possible to compare and contrast your data to that of Geng et al.?
: Please stress these results are preliminary.
Response
As this reviewer mentioned and we responded in some comments, we understand this paper is not a wide scale cohort, and we also think better to mention about the limitation in Discussion. We thus have added the following sentences in Conclusion. ‘A large scale studies will be needed to establish the usefulness of this biomarker.’ (Page 14, line 10-11)

In terms of the comparison of our data to Geng et al, we think it is hard to compare and contrast because their study focused on Reg-I, another family member of HIP/PAP, and the design of our study is different from that of them.

Comment 16
FIGURE 1: Delete normal ureter and ureteral tumor. Add normal bladder (perhaps you may obtain from a patient undergoing prostatectomy. Better yet, IHC should be performed to demonstrate staining and location of staining.
Response
According to your comments, we have deleted ureter samples in Fig. 1A. Furthermore, as we mentioned in Comment 9, we have increased the sample numbers.

Comment 17
FIGURE 2: Nice figure but does not add much. Please convert to works and add to Methods section stating ELISA is specific for HIP/PAP (data not shown).
Response
This figure is particularly important when you deal with Reg family proteins. Because Reg family members have very similar structures, and many of previous paper did not precisely explain about the specificity of the antibodies, which brought about many
Comment 18
FIGURE 3: Please split the control group to healthy volunteers and benign conditions.
Response
We would like to leave this. Because we could not examine the significance of the experiment due to lack of healthy volunteer. This was also mentioned in Comment 7.

Comment 19
Median bars are undetectable. Please make more pronounced.
Response
This was our mistake. We have added median bars in Figure 3A.

Comment 20
FIGURE 4: Please add high recurrence to (A).
Response
We have added to the following sentence on page 10 line 13-14. ‘In this study, high recurrence-risk group did not exist.’

Comment 21
Most Figures could benefit from being re-labeled for clarity. This goes in line with grammar and formatting mentioned above.
Response
We completely clarified all figures as you mentioned.

Comment 22
FIGURE 5: Can BTA be included?
Response
BTA is a qualitative test. So BTA was not available for ROC analysis.

Comment 23
TABLE 1
Please compare numbers, sex, age to controls and tumors. G1, G2 and G3 are now
reported as low grade and high grade.

Response
As you mentioned G1, G2 and G3 are now reported as low grade and high grade. In this study recurrence-risk and progression-risk scores are calculated for each patient according to the EORTC definition. These factors comprise tumor grade, tumor stage, tumor size, number of tumor, earlier recurrence rate, and the presence of carcinoma in situ. Tumor grade use G1, G2 and G3 instead of low grade and high grade. So we use G1, G2 and G3 in this paper.

Comment 24
What is LOH? Please write out. What is NGB? Please write out.

Response
We have added to the List of abbreviations in page 14-15 of this manuscript

Comment 25
Please perform multivariate analysis? Controlling for various factors was HIP/PAP the best diagnostic marker? Controlling for various factors was HIP/PAP the best prognostic factor?

Response
We think those should be carried out in the following comprehensive expanded studies near future.