Author's response to reviews

Title: Prediction and diagnosis of bladder cancer recurrence based on urinary content of hTERT, SENP1, PPP1CA, and MCM5 transcripts

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Author's response to reviews: see over
Dear Editor,

Thank you for the reviews of our manuscript. Please find our rebuttal to the reviewer’s comments below. All changes to the manuscript have been marked using “track changes”

Best regards,

Assoc. Prof. Lars Dyrskjøt, PhD
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Reviewer: Kiyohide Fujimoto

Reviewer’s report:
Major comments
Overall, the authors took the prospectively collected urine samples as an object of the present study, and consequently there was no further new information comparing the similar previous reports. There are some problems in understanding the results from this study. The authors did not present their inclusion and exclusion criteria; e.g., concomitant upper urinary tract cancer or prior therapy. The present study analyzed fresh cases and recurrent cases. These backgrounds of patients should be manifested and the data should be analyzed in each subgroup classified according to the patient backgrounds.

Author rebuttal: Information about inclusion and exclusion criteria has now been added to the manuscript. Because we intended to validate the robustness of these markers in a regular clinical setting, we included all patients diagnosed with bladder cancer of all stages in the inclusion period. We now write in the manuscript: “All patients diagnosed with bladder cancer were included in the study”. Overview of BCG or MMC installations prior to urine sampling is listed in table 2. Furthermore, we did not do separate analyses of different subgroups of the patients in order to resemble a “real life” test and because there are so few (10 patients only) with primary cancer.

Minor comments
The cost for measurement of biomarkers is one of the important financial problems in considering whether these genetic markers are commercially available and useful in routine clinical practice. Did the authors estimate economical benefit of using these biomarkers alternatively to cystoscopy; how is medical cost reduction estimated in comparison with the cost by regularly-repeated follow-up cystoscopies?

Author rebuttal: The cost of performing cystoscopies is very high. More than 50% of the patients have recurrences and this is a heavy burden to societies estimated to cost EU a sum of 460 mill Euros per year,
calculated as 1000 Euros per visit. It has furthermore been calculated that bladder cancer is responsible for the highest cost per patient from diagnosis to death; in the US calculated to exceed 3.4 billion dollars annually. The cost of performing relative simple PCR assays as described here are very low compared to these cystoscopy costs. We have now added this economical aspect to the manuscript and we now write: “It has been calculated that bladder cancer invokes the highest cost per patient from diagnosis to death; in the US calculated to exceed 3.4 billion dollars annually. Bladder tumor markers may reduce these costs significantly.”

Did the authors assess the influence by contamination of normal urothelial cells in the assay? The quality of the analytical results is very likely affected by the contents of urine sample. Although the authors collected 50ml of urine at regular follow-up visits, 50mL seemed to be little to collect viable cells enough to extract mRNAs. Did the samples provide sufficient amount of mRNAs? What device did the investigators make to obtain the sufficient number of exfoliated cells in the urine?

Author rebuttal: Collection of urine from patients entering outpatient clinics is difficult and collection of large amounts of urine is almost impossible. We collected up to 50 mL of urine from the patients enrolled in this study, and in most cases this resulted in enough RNA for PCR measurements. We were not able to determine the origin of the different cells in the urine samples; however, we did do urine dip stick tests for infection. We did not perform any special procedures in order to obtain the required number of exfoliated cells – we simply used the cells pelleted from a standard urine sample. RNA amounts, pellet size and results of urine dip stick tests are listed in table 2. We have now added the distributions to the table also.

Reviewer: Toru Shimazui

Reviewer’s report:
Because molecular diagnosis using urine samples is probably a promising tool for detection of bladder tumor, this study could be very informative for readers.

Major revisions:
1. Although in Table 3 plenty of marker combinations are shown with similar sensitivity, specificity, PPV, NPV, and p value, it is not persuasive which combination demonstrates the best performance to detect or predict occurrence or recurrence of bladder tumor. To omit cystoscopy, new markers could diagnose not only presence of tumor but also location and morphology of tumor. Therefore, cystoscopy is anyhow inevitable in case that these molecular markers are positive. From a practical point of view, it might be important to construct any combinations, which indicates the best NPV and compensates urine cytology.

Author rebuttal: In the manuscript we mention that the combination of hTERT and cytology gives the best test result. We write: “We combined the markers with cytology in order to improve our test results and found that especially the combination of hTERT and cytology resulted in a sensitivity of 73.3 % and a specificity of 74.1% (Table 3)”. Furthermore, we did try many combinations of the markers and this is listed in additional file 3. We previously included the combination of two markers in table 3, however, this may be misleading and confusing, so this has been deleted now. It is true that markers with the highest NPV would be most desirable – however, the combinations listed all give very similar NPV around 70-73%.

2. In the analyses of recurrence free survival, it is recommended to analyze not only univariate analysis, such as Kaplan-Meier method with Log-rank test, but also multivariate analysis, e.g. proportional hazard analysis.
Author rebuttal: We have now tested a number of variants (i.e. previous stage, previous grade, previous size, previous CIS, previous multiplicity and previous recurrence pattern) using univariate Cox regression analysis. None of these variables showed any significant correlation to recurrence free survival for these non-tumor visits, so there is no need to correct for this in a multivariate Cox regression analysis. Only hTERT, MCM resulted in significant hazard ratios. This has now been included in the manuscript and we now write:“ Univariate Cox regression analysis also showed that both hTERT and MCM5 were significantly associated with recurrence free survival (hTERT: HR=4.6, p<0.001; MCM5: HR=2.7, p=0.03). None of the risk factors for recurrence (i.e. previous stage, grade, size, multiplicity and CIS) were significantly associated with recurrence free survival in this group of patients”.

We have also added the log-rank p-values to the KM plots in figure 2.

Minor revision:
To evaluate predictive value of new molecular markers for non-muscle invasive bladder tumor, T2-4 tumors should be excluded from the analysis. Otherwise, it could be included an additional analysis regarding correlation between these markers and T classification, i.e. tumor stage.

Author rebuttal: Ideally a urine based test for diagnosis and recurrence of bladder cancer should work disregarding tumor stage and grade. In this prospective validation study of urine markers we aimed at testing the markers on all urine samples collected in the study period. We therefore also included the T2-4 tumors in the analysis as this would give us the best idea about the usefulness of the tests in a clinical setting.