Author's response to reviews

Title: Expression and biological-clinical significance of hTR, hTERT and CKS2 in washing fluids of patients with bladder cancer.

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

I am submitting the revised version of manuscript entitled “Expression and biological-clinical significance of hTR, hTERT and CKS2 in washing fluids of patients with bladder cancer” by L. Mezzasoma et al.

Please, below find the point-by-point responses to the reviewers concerns and clarifications to editorial requests. All changes made to the manuscript have been highlighted in red colour.

REFEREE 1 (Daniele Calistri):

Minor Essential Revisions:

1) Introduction. Comment: “References 17-22, cited on page 4, are not only related to articles that deal with hTERT mRNA expression only but also to telomerase activity. The text should be changed to reflect this.”. Response: This is indeed correct and changes have been accordingly made (please, see pag. 4, lines 4-6).

2) Materials and Methods. Comments: a) Were the patients at the first diagnosis of BC? Response: Yes, the patients were at the first diagnosis of BC, as now added to the revised version of the manuscript (please, see pag. 5, lines 10-11).

b) Were there any cases of CIS in the superficial cancer group? Response: No cases of CIS were present in the superficial cancer group. In fact, as already described in the manuscript, this group included only superficial low grade [pTa (n = 24), pT1 (n = 2)] (please, see pag. 5, line 15-16).

c) The authors state that controls comprised individuals with no history of BC and also individuals undergoing follow up for BC. This population could perhaps have been better
selected. Response: This population could perhaps have been better selected, however, since, at the time of sampling, all subjects were BC free, we included them in the control group.

d) Page 5, line 4: what were the clinical indications of the control subgroup; are the authors referring to hematuria/irritative symptoms or a previous history of cancer? Response: for the clinical characteristics of the control group, please, see pag. 5, lines 17.

3) Results and Discussion. Comments: a)”There were 36 patients with bladder cancer; among these, 26 had a superficial low-grade tumor and 10 high-grade invasive cancers. The series is probably too small to permit correct statistical comparisons between these two subcategories”. Response: There was a different behavior of study markers with respect to cancer stage. It is of great interest to find out markers discriminating between superficial and invasive bladder cancers. Thus, we reported our findings based on stage comparisons but we also stated clearly in the discussion that these results are based on a few cases and should be regarded solely as a clue for further investigation. However, a statement highlighting this concept has been added also in the “Results” section (please, see pag. 9, lines 13-14).

b) “The approach proposed in bladder washing is invasive and it is not clear what the final aim of the authors is for the schedule proposed. Why do the authors not try to evaluate the accuracy of these biological markers in voided urine?” Response: The aim of the study was to investigate the biological role of hTR, hTERT and CKS2 in BC development and progression. For this reason, we used samples particularly rich of exfoliated tumor cells, as bladder washings are. In consideration of the observed significant changes in the expression levels of the three considered genes between BC bearing patients and controls, we also decided to evaluate their possible role as molecular markers of BC diagnosis and progression. In consideration of the obtained results, it will certainly be our interest to evaluate in the future, the accuracy of these biological markers in voided urine in order to find a non invasive
diagnostic/prognostic tool. All these aspects have been now more clearly explained in the revised manuscript (please, see pag. 4, lines 16-19 and pag. 14, lines 13-15).

c) “The expression of hTR is lower in cancer patients than in healthy individuals and, among tumors, in high-grade than in low grade lesions. The authors discuss this aspect in the third paragraph of the Discussion but it would be an interesting to compare their findings with studies in the literature, which, conversely, observe an increased expression of telomerase subunits in cancer patients compared to healthy individuals.” Response: A “peer” comparison with studies in the literature would be difficult to interpret. In fact, to our knowledge, all these studies refer to the evaluation of hTR mRNA levels in urine samples, and the only study evaluating the expression of this molecule in washing fluids is not methodologically comparable. The discussion on this matter has been accordingly completed (please, see pag. 11, last paragraph).

REFEREE 2 (Susanne Fuessel):

Major Compulsory Revisions:

1) Comment: “The authors measured transcript levels of the housekeeping gene beta-actin for normalization purposes. But all results presented in the tables and figures seem to represent the pure transcript levels of hTR, hTERT and CKS2. Furthermore, it remains unclear what the unit “ng/µl” in tables 1 and 2 does represent. If the authors had used normalized transcript levels the variables would be dimensionless. The use of non-normalized data for diagnostic purposes appears doubtful since the total volume of bladder washings and the numbers of exfoliated cells are varying between the different patients. This must be clarified. Response: As correctly pointed out, normalization is essential for biological as well diagnostic purposes, for the reasons well outlined by the referee and also for sample-to-sample differences in RNA input, RNA quality, and reverse transcription efficiency. Authors are fully
aware of the importance of normalization and can reassure the reviewer that the transcript levels of hTR, hTERT and CKS2 were normalized against beta-actin. The unit “ng/µl” was added to point out that both the target gene and the housekeeping gene were measured by an absolute quantitative method of detection expressing them in “ng/µl”. However, the reviewer is right when she points out that the variables are dimensionless. Tables 1 and 2 have been accordingly changed and normalization better described in the revised manuscript (please, see pag. 7 lines 19-21).

2) **Comment:** “The use of non-normalized data can also be the reason for the conflicting result that hTR transcript levels decrease in tumor patients in comparison to the controls. Other studies have shown increased transcript levels of hTR in urine specimens from bladder cancer patients and a correlation between hTR expression and telomerase activity which is specific for tumor cells”. **Response:** As above clarified, the transcript levels of hTR, hTERT and CKS2 were normalized against beta-actin. Other studies in the literature have shown increased transcript levels of hTR in urine specimens from bladder cancer patients. However, a “peer” comparison between studies and ours, would be difficult to interpret. In fact, to our knowledge, all these studies refer to the evaluation of hTR mRNA levels in urine samples, and the only study evaluating the expression of this molecule in washing fluids is not methodologically comparable. The discussion on this matter has been accordingly completed (please, see pag. 11, last paragraph).

3) **Comment:** “In this context, correct citation of reference 25 in the discussion chapter should be verified”. **Response:** Reference 25 has been verified and accordingly corrected.

4) **Comment:** “The authors should also check the specificity of the primers and probe used for hTR detection”. **Response:** The specificity of the primers and probe used for hTR, as well as for hTERT and CKS2 detection, has been carefully checked. In particular, all oligo sequences were designed by Beacon Designer 4 software (Stratagene, La Jolla, CA), as
already stated in the manuscript (please, see pag. 6, lines 14-15), and further checked by NCBI BLAST search.

5) Comment: “Additionally, the authors should explain the logistic regression model in more detail to make the differences between the ROC analyses of the single markers presented in the third and the fourth paragraph clear.” Response: We added a sentence to the methods section to clarify that ROC curves can be calculated after fitting a logistic regression model (please, see pag. 8, lines 5-7). The estimated coefficients and the individual values of study variables allow the calculation of model based probabilities of a positive outcome for each study case. Disease status and model based predicted probabilities can be used to calculate AUCs for each single marker or a combination of predictors included in the logistic regression model.

Minor Essential Revisions:

6) Comment: “Did the authors use a LightCycler instrument from Roche or the MX3005P System from Stratagene? This should be revised”. Response: The authors used the MX3005P Real-Time PCR System from Stratagene (please, see pag.7, lines 5-6).

Discretionary Revisions:

7) “The numbers of subjects in each group could be already mentioned in the abstract”.  Response: The abstract has been accordingly modified (please, see pag.2, line 6).

References have been checked and accordingly modified (Ref. number 34).

EDITORIAL REQUESTS:

1) “Please, clarify ethical approval” and 2) “Please, clarify informed consent”. I confirm that this study received ethical approval and is in compliance with the Helsinki Declaration. In
addition, all patients gave their informed consent to participate in the study. A statement to each of these effects has been reported in the Methods section of the revised manuscript (see pag. 5, lines 4-7).

I hope that our manuscript is now acceptable for publication.

Thank you for your attention.

Yours sincerely

Letizia Mezzasoma

(on behalf of all authors)