Author's response to reviews

Title: ROS generation via NOX4 and its utility in the cytological diagnosis of urothelial carcinoma of the urinary bladder

Authors:

Keiji Shimada (keijishi@naramed-u.ac.jp)
Tomomi Fujii (2byori@naramed-u.ac.jp)
Satoshi Anai (sanai@naramed-u.ac.jp)
Kiyohide Fujiimoto (kiyokun@naramed-u.ac.jp)
Noboru Konishi (nkonishi@naramed-u.ac.jp)

Version: 3 Date: 23 September 2011

Author's response to reviews: see over
Dear Sir

RE: “
’ROS generation via NOX4 and its utility in the cytological diagnosis of urothelial carcinoma of the urinary bladder” by Shimada et al. (MS: 2135278994569578)

Thank you for the review of our manuscript. We would like to resubmit the revised manuscript according to your suggestion.

Responses to the reviewers’ comments are as follows.

Reviewer 1: Benjamin Nisman

Minor revision:

1. The authors indicated 30min incubation of voided urine specimens with 10µM DHE and CM-DCFDA in serum free RPMI medium and after this incubation cells were washed by PBS three times. How do these procedures influence the performance of conventional cytology?

Thank you for the critical comments. The size of tumor cell was slightly decreased after incubation with ROS detecting reagents in cell culture medium for 30 min at 37°C. However, cellular atypia including hyperchromasia, enlarged nucleoli, irregular shape etc was not significantly modified.

We have described in red the following description in the section of Materials and Methods in the revised manuscript.

The size of tumor cells was slightly decreased after incubation with ROS detecting reagents in the cell culture medium for 30 min at 37°C. However, cellular atypia, including hyperchromasia, enlarged nucleoli, irregular shape and so on, was not significantly modified; therefore, there was no problem with diagnosis by conventional cytology.

(Page 10, line 14 to 18 in the revised manuscript)

2. The test requires the use of a fluorescence microscope by a trained operator. Have the problematics of "interobserver variability" been considered?
In the present examination, two urologic cytopathologists (K.S and N.K.) throughout, and interobserver and intraobserver differences were out of the questions. However, inter/intra observer variability is worth considering for cytologists lack experience in handling urine samples and/or fluorescence microscopy. We are trying to construct much more objective rules to make a decision whether ROS positive cells are malignant or not, for examples, setting threshold of fluorescence intensity.

We have described in red the following description in the section of Discussion in the revised manuscript.

Inter- and intra-observer variabilities are worth considering for cytologists who lack experience in handling urine samples and/or fluorescence microscopy. We are trying to construct more objective rules to help decide whether ROS positive cells are malignant—for example, by setting a threshold of fluorescence intensity.

3. The group with malignancy included patients with 50 primary and 28 recurrent bladder cancer of different grades and stages, which may be appropriate for this “proof-of-principle” study. However, the fact that the sample size remains relatively small may lead to estimation byes. The data presented in the tables demonstrate zero sensitivity of urine cytology in low grade recurrent tumors. Is this the result of a small sample size? More data with adequately sized and representative samples are needed to reach definite conclusions. The authors should indicate these limitations.

We agree the referee’s comments. We added the following comments in red in the section of Discussion in the revised manuscript.

The present data demonstrating zero sensitivity of urine cytology in low-grade urothelial carcinomas partly resulted from the small sample size. More data using adequate samples should be collected for definite conclusions.

4. The authors reported that inflammatory cells also produced ROS and these cells were morphologically excluded by Papanicolaou staining. Furthermore, it follows from this study that ROS labeling should be instantly correlated with cytologic findings to avoid false-positive results. This close correlation could lead to an overestimation of the specificity of the new assay. Considering the ROS
labeling as an adjunct to cytological examination the question arises as to whether it might be possible to evaluate and present performance characteristics of this new assay independently from cytology. The authors should shortly discuss these points.

Thank you for the critical comments. We do not consider ROS labeling cytology is a new assay independent of conventional urine cytology. Conventional urine cytology exhibits high specificity but unfortunately low sensitivity to detect bladder cancer. In contrast, ROS labeling methods alone could not exhibit high specificity. Then, we added ROS labeling method to conventional cytology and construct a new assay system with high specificity and sensitivity.

We have added the following description in red in the section of Discussion in the revised manuscript.

Conventional urine cytology exhibits high specificity, but unfortunately, low sensitivity, to detect bladder cancer. In contrast, ROS labeling methods alone could exhibit high sensitivity but not high specificity. In this study, we added ROS labeling methods to conventional cytology and created a new assay system with high specificity and sensitivity.

(Page 20, line 6 to 10)

Reviewer2: Christian Schwentner

Minor-essential reviews: Pls do a final check for typos and spelling errors and proof-reading by native speaker.

According to the comments, manuscript was checked by native speaker.

EDITORIAL REQUESTS:
- Copy-editing

We recommend that you ask a native English speaking colleague to help you copyedit the paper. Please pay special attention to the revised sentences you have inserted during the revisions process.

According to the comments, we asked native speaker to correct our revised manuscript.

-Ethical approval for animal studies

Experimental research that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Any experimental research on animals
must follow internationally recognized guidelines. A statement to this effect must appear in the Methods section of the manuscript, including the name of the body which gave approval, with a reference number where appropriate.

We added the following sentences in red in the section of M&M in the revised manuscript.

Animal experiments were approved by the institutional animal care and use committee at Nara Medical University. Eight week-old female nude mice were maintained on a daily 12-h cycle of light and dark and were fed standard diet and water ad libidum (14, 18).
(PAGE10, line 20 to 22)

Authors' contributions
Authors' contributions - Please include an Authors' contributions section before the Acknowledgements and Reference list.

We added the authors’ contribution in red in the revised manuscript.

Author’s contribution:
KS and TF carried out cell biological analyses using human urothelial carcinoma cell lines and drafted the manuscript. SA and KF carried out the animal experiments. KS and NK participated in cytological diagnosis/analysis using human urine samples. KS and SA participated in the design of the study and performed the statistical analysis. NK conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.
(PAGE21, line 1 to 6)

We hope that these changes are acceptable and meet your expectations.

Yours sincerely,

Keiji Shimada (1st author)
Department of Pathology
Nara Medical University School of Medicine
840 Shijocho, Kashihara, Nara, 634-8521, Japan
Tel: +81-744-22-3051: Fax: +81-744-23-5687
E-mail: keijishi@naramed-u.ac.jp