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: 2 Date: 12 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.

Reviewer1: 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℃. However, cellular atypia including hyperchromasia, enlarged nucleoli, irregular shape etc was not significantly modified. We have described the following description in the section of Materials and Methods in the revised manuscript.

   The size of tumor cell was slightly decreased after incubation with ROS detecting reagents in cell culture medium for 30 min at 37℃. However, cellular atypia including hyperchromasia, enlarged nucleoli, irregular shape etc was not significantly modified, therefore, no trouble occurred in diagnosis by conventional cytology.

   (Page 10, line 10 to 13 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 the following description in the section of Discussion in the revised manuscript.

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.

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 the section of Discussion in the revised manuscript.

The present data demonstrating zero sensitivity of urine cytology in low grade urothelial carcinomas is partly caused by a small sample size. More data using adequate samples should be accumulated 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 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 the present research, we added ROS labeling method to conventional cytology and constructed a new assay system with high specificity and sensitivity.

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.

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