**Reviewer's report**

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

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**Reviewer:** Benjamin Nisman

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In this study the authors investigated the role of the ROS generation via NOX4 in urothelial carcinogenesis and analyzed whether ROS labeling could be used as an adjunct to conventional cytology, improving its diagnostic performance. They found an increased NOX4 expression in all phases of urothelial transformation, from precancerous lesions to high grade/invasive carcinoma, but not in normal urothelium. On mouse models they showed that silencing NOX4 significantly reduced cancer cell growth. The cytological analysis of ROS producing cells in urine of patients at risk for urothelial carcinoma demonstrated a significant improvement of sensitivity for the detection of low and high grade primary and recurrent BC, compared to conventional cytology, with no loss of specificity.

This is an original paper on the pathobiological role of ROS in urothelial carcinoma in which the authors demonstrated the diagnostic value of ROS labeling as adjunct to conventional urine cytology. For these reasons it could represent a significant contribution to urological daily clinical practice. The manuscript theory sounds good. The paper is clear, well written, logical and understandable.

**Minor criticism**

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?

2. The test requires the use of a fluorescence microscope by a trained operator. Have the problematics of "interobserver variability" been considered?

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.
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.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.