Peer Review File

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Reviewer Comments: According to the cancer statistics in 2016, bladder cancer, commonly referred to the bladder urothelial carcinoma, was the fourth common new diagnosed malignant tumor in male, with about 76,960 new cases every year in both sexes in United States. In the manuscript "The Diagnostic and Prognostic Value of Nuclear Matrix Protein 22 in Bladder Cancer", authors evaluate the diagnostic and prognostic value of Urine Nuclear Matrix Protein 22 (NMP22) for bladder cancer. Couple questions are required to be answered before accepted.

Comment 1: There were similar reports (Curr Opin Urol. 2019 May;29(3):203-209) and (Urol Int. 2001;66(2):72-7) in the PubMed. What is the novel idea in the paper? Please elaborate in the introduction.

Reply 1: The first report (Curr Opin Urol. 2019 may; 29 (3): 203-209) is a review rather than an original study. This review summarized the biomarkers which have been used in the detection of bladder cancer in recent years, including NMP22. It is mentioned that NMP22 alone has limited sensitivity and specificity, and should be combined with cystoscopy to improve the detection rate of bladder cancer. In this report, the relationship between NMP22 and pathology of bladder cancer was not analyzed in detail.

The second report (Urol Int. 2001; 66 (2): 72-7) is similar to our study. It also analyzed the relationship between NMP22 and bladder cancer with different pathological grades and stages. There are two main differences between our study and this study. The first is the different conclusions. This study found that NMP22 was more sensitive to muscle invasive bladder cancer than superficial bladder cancer, while our conclusion was the opposite. This inconsistency reflects the limitations of NMP22 detection (see question 6). Secondly, we studied the predictive value of NMP22 for bladder cancer staging and grading, that is, if NMP22 is positive, whether the probability of diagnosis of bladder cancer with a certain grade and stage will increase. This was not mentioned in that study.

Comment 2: What is the meaning of "Ta, T1, T2" in the abstract?

Reply 2: Based on the American Joint Committee TNM staging system for bladder cancer (Seventh Edition, 2010), Ta refers to noninvasive papillary carcinoma, T1 refers to tumor invades subepithelial connective tissue, T2 refers to tumor invades muscularis propria.

Changes in the text: We have modified our text as advised (see Page 3, Line 14-15, and Page 8, Line 9-10).

Comment 3: In the introduction, please enrich the progress of the treatment for bladder urothelial carcinoma. Please supplement the progress of the molecular diagnostic biomarkers for bladder cancer in the introduction.

Reply 3: In the field of bladder cancer treatment, Trans-urethral Resection of Bladder Tumor (TURBT), total and partial cystectomy are still the most important treatments for resectable disease. For unresectable bladder cancer, chemotherapy and radiotherapy are classical treatments, and the emergence of immunotherapy and targeted therapy in recent years provides more treatment options for bladder cancer that cannot be controlled by radiotherapy and chemotherapy. Considering that the prognosis of unresectable bladder cancer is significantly worse than that of resectable bladder cancer, the early diagnosis of bladder cancer and finding proper prognostic factors were important topics in clinical.

In the early diagnosis of bladder cancer, in addition to the traditional cystoscopy and urine cytology, molecular biomarkers are also used more and more because of its noninvasive and easy to implement. The molecular biomarkers of bladder cancer, that is, the components with diagnostic value in the urine of patients with bladder cancer, include exfoliated tumor cells, proteins, genes and tumor metabolites. The detection of these biomarkers can provide valuable information for the diagnosis and follow-up of bladder cancer. At present, there are many biomarkers for molecular diagnosis of bladder cancer. Some detect specific proteins in urine, such as Bladder Tumor Antigen (BTA) test, Nuclear Matrix Proteins 22 (NMP22) test, Cytokeratin 8 and 18 fragments test; some detect DNA in urine, such as AssureMDx test; some detect mRNA in urine, such as Xpert Bladder Cancer test and CxBladder Detect test; some detect tumor associated cellular antigens or aneuploidy for chromsomes in urine sediment, such as ImmunoCyt test and UroVysion test. Among these examinations, NMP22 is one of the most widely used in clinical practice.

Changes in the text: We have modified our text as advised (see Page 5, Line 4-11,

13-22, and Page 6, Line 1-3).

Comment 4: How about the expressions of NMP22 in other cancer? Why not to test the expression of NMP22 in tissues?

Reply 4: NMP22 is one of many nuclear matrix proteins, which specifically exists in urothelial cells. The content of NMP22 in cancerous urothelial cells is 80 times higher than that in normal cells (Asian PAC J cancer prev. 2010; 11 (5): 1279-82). Therefore, NMP22 is designed for the detection of bladder cancer, and the research on NMP22 is almost all concentrated in the field of bladder cancer. We searched the relevant reports and found only two studies in other cancer (Urology. 2000 Feb; 55 (2): 227-30, Urology. 2002 Oct; 60 (4): 593-7). These two studies analyzed the value of NMP22 in the diagnosis of renal cell carcinoma, and found that NMP22 might have diagnostic value for renal cell carcinoma, but there is no follow-up study.

NMP22 assay is designed for noninvasive detection of bladder cancer, including initial diagnosis of bladder cancer or postoperative follow-up for recurrence of bladder cancer. NMP22 is highly expressed in bladder cancer cells, which can be released into the urine as soluble complex or fragment after cell apoptosis. Therefore, the detection of NMP22 content in urine can provide valuable information for the diagnosis of bladder cancer. If NMP22 is detected in tissues, the sensitivity and specificity might be higher, but this will lose the significance of NMP22 as a noninvasive test. In addition, if the tissue has been obtained, the diagnosis of bladder cancer can be made directly by pathological analysis, and NMP22 detection is not needed.

Changes in the text: We have modified our text as advised (see Page 14, Line 1-8, and Page 13, Line 18-22).

Comment 5: What are the exclusion criteria for enrolled patients?

Reply 5: In this study, the following patients will be excluded: a) complicated with other urogenital diseases, including acute or chronic inflammation of the urinary system; b) combined with tumors in other sites; c) complete pathological reports are not available.

Changes in the text: We have modified our text as advised (see Page 7, Line 7-10).

Comment 6: Please supplement the analysis of high rate of false negative in the discussion.

Reply 6: NMPs are the non-chromatin network framework of the nucleus, which determine the morphology of the nucleus and organize the DNA into three-dimensional structure. They play an important role in the process of DNA replication, transcription, RNA processing, gene expression regulation and so on. NMPs are a kind of insoluble proteins, but they can be decomposed in the process of apoptosis and released into the surrounding environment. More than ten kinds of NMPs have been identified, some of which are tissue-specific and tumor specific. NMP22 is one of many nuclear matrix proteins, which is specific in urothelial cells. The content of NMP22 in cancerous urothelial cells is 80 times higher than that in normal cells. NMP22 in bladder cancer cells can be released into the urine in the form of cleavage fragments or complexes during cell apoptosis. The detection of these components in urine can help to determine whether there is bladder cancer or not. However, the disadvantages of this detection are also obvious. Due to the inconsistent rate of apoptosis and exfoliation of bladder cancer cells, the concentration of NMP22 released into urine will also change constantly. Therefore, the concentration of NMP22 in urine is not stable, but will change with time. For the same bladder cancer patient, the NMP22 concentration may be quite different between the first urination in the morning and the urine excreted after drinking a lot of water in the afternoon. Repeated tests may improve the detection rate, but the cost may be unacceptable to patients; at the same time, there is no relevant research on the relationship between specific detection times and detection rate. In addition, the test urine is taken by the patients themselves, so whether the patients keep the urine according to the doctor's instructions is uncertain, which may affect the experimental results as well. Current clinical studies have also found this phenomenon. In different studies, the sensitivity and specificity of NMP22 are quite different, which is also the reason why we believe that there is a risk of using NMP22 alone. However, this does not mean that NMP22 is worthless. During cystoscopy, some early bladder cancer or carcinoma in situ may be difficult to detect by naked eyes, but these tumor cells can release NMP22 into the urine. If combined with cystoscopy and NMP22 detection, the detection rate of bladder cancer could be significantly increased. Relevant studies have confirmed that, combined with NMP22 and cystoscopy, the detection rate of bladder cancer can be as high as 99% (JAMA. 2006 Jan 18; 295 (3): 299-305). Therefore, it is the most important to fully understand the advantages and disadvantages of NMP22 test and reasonably apply it in clinical.

Changes in the text: We have modified our text as advised (see from Page 11, Line 15 to Page 13, Line 2).

Comment 7: Are there any prognostic factors for bladder cancer?

Reply 7: Prognostic factors refer to the factors that can help to predict the prognosis of patients, usually predict the survival and recurrence of patients. In this study, we analyzed the predictive value of NMP22 in the pathological grading and staging of bladder cancer. That is to say, if NMP22 is positive, will the patient be more likely to be diagnosed with bladder cancer of a certain grade and stage.

There are many prognostic factors in bladder cancer, including oncogene and tumor suppressor gene (Ras, ErbB, Rb, TP53, p21), cell proliferation and apoptosis related indicators (Ki-67, Fas, FasL), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Transforming Growth Factor (TGF), etc (Adv Anat Pathol. 2015 Mar;22(2):102-12). These factors have certain value in predicting the survival and recurrence of bladder cancer

Changes in the text: We have modified our text as advised (see Page 14, Line 9-17).

Comment 8: Why not to analyze the specificity of NMP22?

Reply 8: In this study, we did not analyze the specificity of NMP22 test in detecting bladder cancer, for only patients confirmed with bladder cancer were included. In this retrospective study, all patients were diagnosed with bladder cancer in outpatient, admitted to hospital for surgery, and confirmed as bladder cancer by postoperative pathology. These patients routinely completed NMP22 examination after admission. The sensitivity of NMP22 for bladder cancer was calculated by the formula: number of NMP22 positive bladder cancer patients / (number of NMP22 positive bladder cancer patients + number of NMP22 negative bladder cancer patients) × 100%. The specificity formula was: number of NMP22 positive bladder cancer patients / (number of NMP22 positive bladder cancer patients + number of NMP22 positive healthy patients) × 100%. Since we did not include any patients without diagnosis of bladder cancer, we did not analyze the specificity of NMP22 detection.