# Urinary exosomal CA9 as a liquid biopsy for diagnosis of bladder cancer

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# Abstract

Background

The purpose of this study was to assess the possibility of using urinary exosomal CA9 as a new liquid biopsy for the diagnosis of bladder cancer.

Methods

158 bladder cancer patients and 88 healthy subjects were included in the study. An in-house technique was developed to isolate urinary exosomes. The expression level of CA9 was detected by RT-qPCR. The diagnostic performance of urinary urinary exosomal CA9 was evaluated.

Results

The level of urinary exosomal CA9 was significantly higher in bladder cancer group than in healthy control group ($p < 0.001$). The area under the ROC curve was 0.806 (95% CI: 0.753–0.858). With an optimal cutoff of 0.433, the sensitivity was 68.35% and the specificity was 85.23% for the diagnosis of bladder cancer.

Conclusions

The urinary exosomes were abundant in the urine of cancer patients. CA9 could be detectable in urinary exosomes. The urinary exosmal CA9 may present a new liquid biopsy for the diagnosis of bladder cancer.


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# Background

Despite of the technique advances, bladder cancer remains a severe healthy problem. Bladder cancer is the ninth common malignant disease worldwide with estimated 430000 new cases per year (1). About 75% of newly diagnosed cases are non-muscle-invasive bladder cancer (NMIBC) (2). The treatment of NMIBC involves transurethral resection of bladder tumor. These tumors frequently recur. Approximately 30% of bladder cancer patients have muscle-invasive disease (MIBC) at diagnosis (2, 3). Of the patients with MIBC, 30% were initially diagnosed with NMIBC. The high rate of recurrence and high risk of disease progression impose the regular surveillance to bladder cancer patients (4). All these facts indicate that search for new and non-invasive methods to detect and monitor bladder cancer are of maximum interest.

Exosomes are membrane-bound nanoparticles secreted by cells into the extracellular space (5).

It has been proved that exosomes play a significant role in physiological and pathologicl processes (6). Numerous studies have shown that exosomes participate in cancer onogenesis and cancer progression (7, 8).Exosomes have become recently a hot top in the research area of biomarkers because exosomes carry specific proteins, lipids and nucleic acids of their origins. More importantly, exosomes provide a

convenient source of potential disease biomarkers because exosomes are abundant in accessible body fluids (9, 10, 11). In fact, exosome is generally considered as a major source of liquid biopsy. Urine contains a large number of exosomes. Carbonic anhydrase 9 (CA9) is a transmembrane member of the carbonic anhydrase family. It catalyses the reversible hydration of carbon dioxide into bicarbonate and a proton, thus enabling tumour cells to maintain a neutral pH despite an acidic microenvironment. CA9 is overexpressed in urinary blander cancer and is considered as a new promising marker for urinary bladder cancer (12). We hypothesize that urine from bladder cancer patients may contain tumoral exosomes that carry CA9 marker. These exosomes may serve as a new source of liquid biopsy for the detection of bladder cancer.

## Methods

# Patients

Inclusion criteria comprised patients with the suspicion of bladder cancer due to the occurrence of hematuria and/or on the basis of initial findings during cystoscopy or during imaging examinations. In total, 158 patients undergoing surgical resection were included. Histopathological examination of the resected bladder specimens served as the final diagnosis. The patients' characteristics were summarized in table 1. 58 urine samples from healthy subjects were used as control. There was no significant difference in average ages between the two groups of cancer patients ad healthy subjects. The urine was collected before surgery. All participants got necessary information and provided a consent agreement. This study was reviewed and approved by Peking Union Medical College Hospital ethics committee.

# Isolation of urinary exosomes

Urine samples were processed immediately after collection. Centrifugation was performed at 3000 g to remove cells and large debris. 20 ml urine was used to isolate the exosomes by using a urinary exosome isolation solution (Hope Tech Biotechnology Co. Ltd, Guangzhou). Briefly, 4 ml isolation solution was added into urine. The mixture of solution was vortexed and put in the refrigerator overnight. The mixture was centrifuged at 3000 g at 4 °C for 30 minutes to recover the urinary exosomes.

# Extraction of RNA

Total RNA extraction was performed by using miRNeasy Micro Kit according to the manufacture indications with some modifications (Qiagen S.A.). Briefly, 500 µl Qiazol solution (GITC-containing buffer) was used to dissolve the urinary exosomes. 100 µl Chloroform was added. The misture was vortexed and centrifuged at 14000 rmp at 4 °C for 15 minutes. The supernatant was ontained and 1.5 volume of 100% ethanol was added. The mixture was centrifuged by the extraction column (Qiagen S.A.). The column was washed respectively by RWT buffer and by REP buffer. The RNA was eluted in 20 µl RNase free water and then quantified by using Nanodrop. The RNA specimens were stored in − 80 °C until RT-PCR.

# RT-qPCR

Complementary DNA (cDNA) was synthesized from total RNA using Taqman Reverse Transcription Kit (Invitrogen). For RT reactions, 100–500 ng of RNA extract, 4 µl of RT Buffer and 1 µl of reverse transcription mix were used. Reaction mixture was incubated at 37 °C for 60 minutes, at 95 °C for 5 minutes and then held at 4 °C. The cDNA specimens were stored at -20 °C until PCR. For PCR detection, we used the Taqman technique. PCR was performed in 25 µl reaction mixture containing 2 µl cDNA product, 1 µl of sense and antisense primers and 12.5 µl PCR Master MIX (GIAGEN). After a denaturing temperature at 95 °C for 10 minutes, 40 cycles were performed with denaturing temperature at 95 °C for 15 seconds, annealing temperature at 60 °C for 20 seconds and extension at 72 °C for 34 seconds. A final dissotion cycle was performed at 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds. A positive and negative (PCR mix without cDNA) controls were included in each round of PCR. To check the quality of extracted RNA, we examined the GAPDH gene expression. We designed GAPDH primers as follows: sense primer 5'- CACATGGCCTCCAAGGAGTAA – 3' and antisense primer 5'- TGAGGGTCTCTCTCTTCCTCTTGT – 3'. The Taqman probe of GAPDH was 5'-FAM- CTGGACCACCAGCCCCAGCAAG-3'-TAMRA. The primers for CA9 were as follows: sense primer 5'- CATCCTAGCCCTGGTTTTTGG – 3' and antisense primer 5'- CCTTCTGTGCTGCCTTCTCAT – 3'. The Taqman probe of CA9 was 5'-FAM- CTGTCACCAGCGTCGCGTTCCTT-3'-TAMRA The primers were designed to span the exons to avoid the genomic contamination. The cycle threshold (Ct) value was established by using the detection software SDS v2.0.1 (Applied Biosystem). GAPDH was used as the housekeeping gene and the relative quantification of urinary exosomal CA9 was calculated by comparative Ct method ($2^{-\Delta ct}$).

# Statistical analysis:

Statistical analyses were based on SPSS v13 software package. The t-test was used to check the difference of urinary exosomal CA9 levels between the bladder cancer group and healthy control group. Significant difference was established when $p < 0.05$. ROC curve and the area under the ROC curve (AUC) were used to evaluate the diagnostic value.

## Results

The in-house technique was effective for the isolation of urinary exosomes. Figure 1 shows the distribution of relative expression levels of urinary exosomal CA9. The level of urinary exosomal CA9 was significantly higher in bladder cancer group (8.32, 0.04–186.3) than in healthy control group (0.32, 0.03–3.46) ($p < 0.001$).

To evaluate the diagnostic value of urinary exosomal CA9, we performed the ROC curve analysis (Fig. 2). The AUC of ROC curve was 0.806 (95% CI: 0.753–0.858). With an optimal cutoff of 0.433, the sensitivity was 68.35% and the specificity was 85.23% for the diagnosis of bladder cancer.

# Discussion

Currently, cystoscopy and cytology are considered as the standards for the diagnosis and surveillance of bladder cancer (2, 3). Cystoscopy is an invasive examination which can cause patient discomfort and possible complications such as haematuria or urinary tract infections. Cytology is a non-invasive with a high specificity (13), but the major problem with cytology is its low sensitivity because urine samples often lack the tumoral cells. Therefore, the search for urine markers has always been a research subject (14).

It is now believed that exosomes play important roles in normal and pathological conditions and can serve as useful biomarkers for cancer diagnosis (15). Exosomes from bladder tumor cells accumulate in the urine where they are stable and easily accessible. Urine can be non-invasively accessible, making it the most attractive source of liquid biopsy. Many mRNA markers have been found to be useful (16, 17, 18, 19). However, the urine markers depend on urinary tumor cells. This hinders the sensitivity of urine markers. The supernatant of urine is usually discarded. In this study, we found that abundant exosomal RNA existed in urinary supernatant.

We developed an in-house method that is easily implanted into clinical laboratory. Recently, the mRNA markers in urinary EVs are successfully used as a source of liquid biopsy of prostate cancer (20). In this study, we tested if CA9 mRNA markers can be detected in urinary exosomes to serve as a new source of liquid biopsy for bladder cancer. Compared to healthy groups, urinary exosomal CA9 was significantly elevated in bladder cancer patients. Urinary cellular CA9 was recently studied as a urine marker for the diagnosis of bladder cancer (12). In that study, the sensitivity and specificity for bladder cancer detection were respectively 86.2% and 95.1%. However, the informative urine specimen rate was 85% in that study (20). About 15% urine samples were without urinary cells and were excluded from that study when urinary cellular CA9 was studied (12). In the present study, we showed the feasibility of detection of tumoral CA9 in urinary exosomes. The urinary exosomal CA9 produced an acceptable sensitivity and specificity for the detection of bladder cancer. To our knowledge, it is the first article to deal with urinary exosomal CA9 for the diagnosis of bladder cancer. This strategy can enlarge the use of urinary mRNA markers such as CA9 for the detection of bladder cancer, which depends solely on urinary cells. We anticipate that urinary exosomes from the tumor cells may be abundant even at the early stage. We believe that urinary exosomes will become a new source of liquid biopsy for bladder cancer. We admit that it is a pilot study. The next step is to evaluate its clinical use. It will be interesting to study how this technique can improve the urinary cytology as a diagnostic and surveillance method for bladder cancer.

In conclusion, our in-house method of isolation of urinary exosomes is easily performed in clinical laboratory. Tumoral CA9 can be detectable in urinary exosomes. Urinary exosomal CA9 may provide a new liquid biopsy for the detection of bladder cancer.

# Conclusions

The urinary exosomes were abundant in the urine of cancer patients. CA9 could be detectable in urinary exosomes. The urinary exosmal CA9 may present a new liquid biopsy for the diagnosis of bladder cancer.

# Abbreviation

NMIBC, non-muscle-invasive bladder cancer

CA9, Carbonic anhydrase 9

# Declarations

Acknowlegements
We appreciate the hard work of our colleagues who helped us reserve and collect the relative date in the database.

Availability of data and materials
The data in the present study are available from the first author or corresponding author via rational requests.

Author's contributions
Jin Wen designed the study and was responsible for writing, analysis, interpretation and revision. All of the authors participated in collecting the data and explaining the results. Han Z Li and Guo R Li supervised the study and revised the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate
This study was approved by the ethics committee of Peking Union Medical College Hospital and written informed consent from each of the patients were acquired.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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# Figures

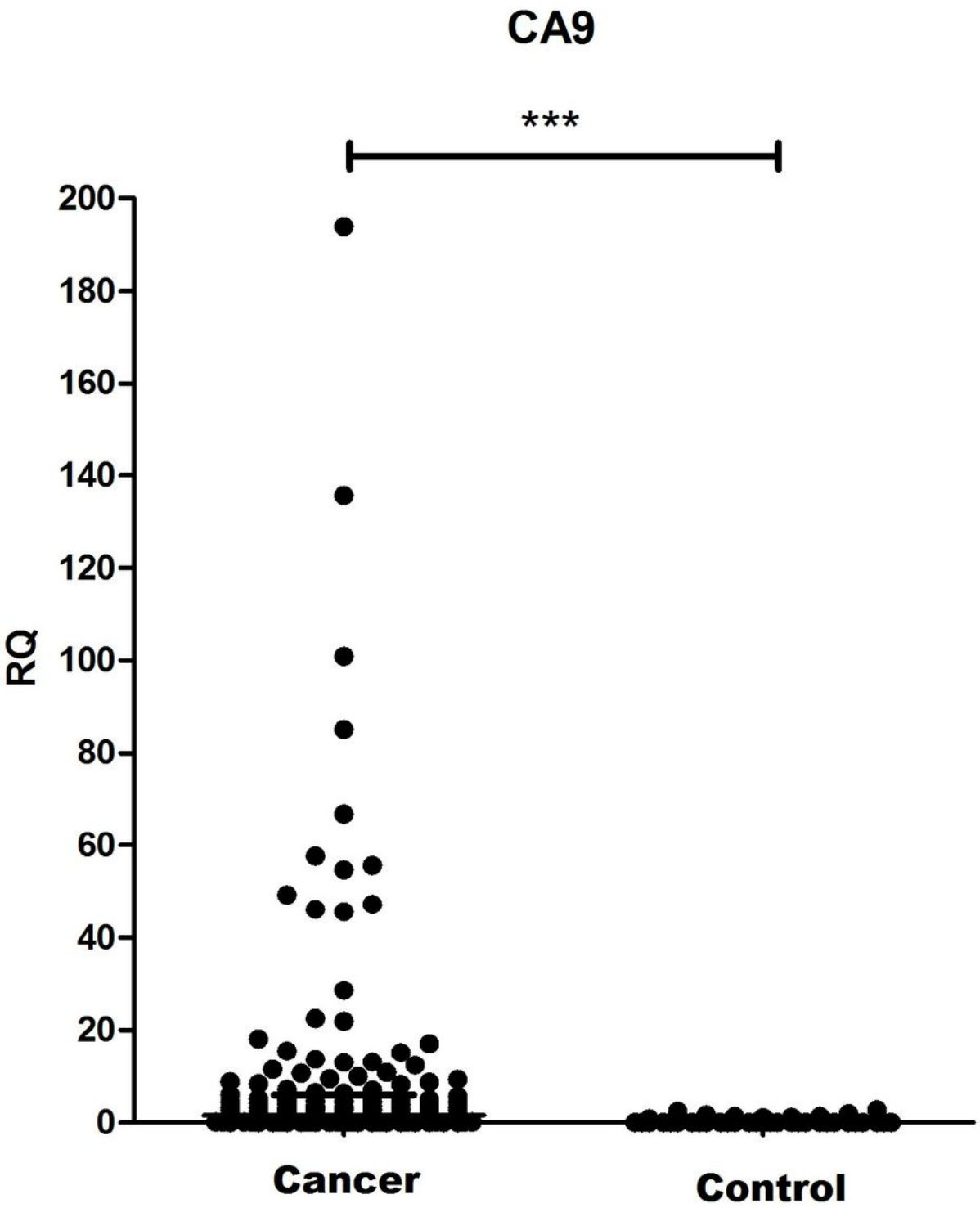
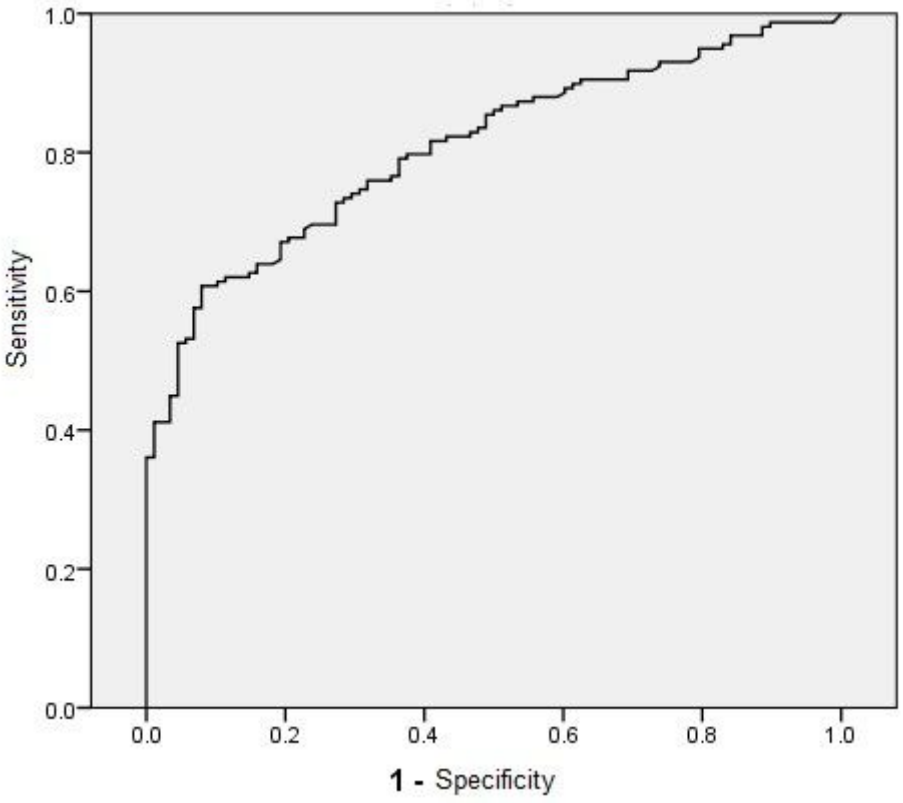



**Figure 1**

The expression levels of urinary exosomal CA9. The level of urinary exosomal CA9 was significantly higher in cancer group than in healthy control group ($p<0.001$).




## Figure 2

The ROC curve analysis of urinary exosomal CA9. The AUC of ROC curve was 0.806 (95% CI: 0.753 - 0.858). With an optimal cutoff of 0.433, the sensitivity and the specificity were respectively 68.35% and 85.23%.