Quantification of urinary exosomal PSA for the diagnosis of prostate cancer: a study protocol with emphasis on application of clinical laboratory-based techniques

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Abstract

Background: Prostate cancer is the most common cancer in men and represents a major problem of public health. The current method of diagnosing/screening for prostate cancer is invasive and costly. There have been renewed and innovative studies on searching urinary biomarker for prostate cancer diagnosis, especially with the technologies of urinary exosomes. However, the technologies of urine exosomes usually need expensive machines such as ultracentrifuge and they are difficult to standardization, which hinder their application in clinical laboratory.

Objective: In this study, our objective is to detect urinary exosomes from prostate cancer for the development of a test to aid in the diagnosis of prostate cancer.

Methods: The methods include the collection of first-void urine by using Colli-Pee device, the isolation of urine exosome by optimized precipitation method and the detection of exosomal PSA by Elecsys® total PSA. We have modified and optimized the isolation of urinary exosomes with precipitation method.

Results: We have found the urinary exosomal PSA can be quantified by automatic technique of Elecsys® total PSA. We have received the ethic approval. It will be a 2.5-year study. We shall start to include the patients and controls in July 15, 2024. We expect to start the data analysis in July of 2025.

Conclusions: To our knowledge, it is the first study to detect the urinary exosomal PSA by the technique of Elecsys® total PSA for the diagnosis of prostate cancer. This study emphasizes on the techniques suitable for the implementation in clinical laboratory, which will facilitate application of urinary exosomes to simplify and improve the diagnosis/screening of prostate cancer.

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Abstract

Background:

Prostate cancer is the most common cancer in men and represents a major problem of public health. The current method of diagnosing/screening for prostate cancer is invasive and costly. There have been renewed and innovative studies on searching urinary biomarker for prostate cancer diagnosis, especially with the technologies of urinary exosomes. However, the technologies of urine exosomes usually need expensive machines such as ultracentrifuge and they are difficult to standardization, which hinder their application in clinical laboratory.

Objective:

In this study, our objective is to detect urinary exosomes from prostate cancer for the development of a test to aid in the diagnosis of prostate cancer.

Methods:

The exosomes from a prostate cancer cell line LNCaP was used to set up the techniques. For the analysis of urine samples of patients, the methods include the collection of first-void urine by using Colli-Pee device, the isolation of urine exosome by optimized precipitation method and the detection of exosomal PSA by Elecsys® total PSA. We have modified and optimized the isolation of urinary exosomes with precipitation method.

Results:

By using the exosomes from the prostate cancer cell line, we have found the urinary exosomal PSA can be quantified by automatic technique of Elecsys® total PSA. It will be a 2-year study. We shall start to include the patients and controls in the summer of 2024. We expect the results to be published in the last quarter of 2026.

Conclusions:

This is the first study to detect the urinary exosomal PSA by the technique of Elecsys® total PSA for the diagnosis of prostate cancer. This study emphasizes on the techniques suitable for the implementation in clinical laboratory, which will facilitate application of urinary exosomes to simplify and improve the diagnosis/screening of prostate cancer.

Key words: Liquid biopsy; urinary exosome; diagnosis; prostate cancer.

Introduction

With 50000 to 70000 new cases per year, prostate cancer is the most common cancer in men [1]. The current method of diagnosing/screening for prostate cancer is based on a biopsy of prostate tissue following an elevated blood concentration of PSA (> 4.0 ng/ml). This method has some drawbacks. Prostate tissue biopsy is invasive and painful with possible side effects for patients. PSA is not a specific marker for prostate cancer. In addition to prostate cancer, other factors can lead to an elevated PSA, including age, prostate infection, and benign prostatic hyperplasia. According to the PSA threshold (usually 4.0 ng/ml), its sensitivity is estimated at 67.5 to 80% and its specificity at 60 to 70% [2-4]. Consequently, 20 to 30% of cancers go under the radar (undetected) when only the PSA blood test is considered, and it is useless to perform a prostate biopsy on a large number of patients. It is more relevant to seek to improve the diagnosis/screening of prostate cancer.

Extracellular vesicles are characterized by a lipid bilayer membrane and are subcategorized into small extracellular vesicles (exosomes) and large extracellular vesicles [5]. The size of the exosomes is between 30 to 150 nm. Exosomes form in multivesicular bodies and are then released into the extracellular medium by exocytosis [5]. Almost all cell types can secrete exosomes. Exosomes are involved in multiple processes of tumorigenesis and development, including promotion of angiogenesis, differentiation and infiltration, regulation of immunity, and response to treatment [6,7]. They are widely present in body fluids, including blood, saliva, urine, breast milk, pleural fluid, and ascites [6,7]. Since exosomes carry tumor markers such as nucleic acids, proteins, and lipids, they can serve as a liquid biopsy to aid in the diagnosis of tumors.

Urine has the advantage of containing an abundance of exosomes. Because the prostate and urine are in close proximity, prostatic exosomes can be secreted into urine. The characterization of exosomes

released by tumor cells and collected in urine could be a new tool to improve the diagnosis of prostate cancer. Therefore, our objective is to detect urinary exosomes from prostate cancer for the development of a test to aid in the diagnosis of prostate cancer. We hypothesize that urinary exosomal PSA may be a new tool for the diagnosis of prostate cancer.

Methods

Overview and study design

This is a case-control single center study. The study team is composed of urologists, oncologists and biologists from CHU Saint-Etienne, which is a public and univeristy medical center for patient care and medical research. CHU Saint-Etienne favorizes the medical research projects and provides a complet administrative guidance and methological aid to implement medical research. Before the project began, many meetings were held to discuss the methological issues as well as administrative issues such as informed consent. The research assistants will be engaged in document collection and sample collection.

The study scheme is presented in Figure 1.

Primary objective

The primary objective of this study is to quantify the urinary exosomal PSA from prostate cancer with clinical laboratory-based techniques.

Secondary objective

The secondary objective of this study is to compare TEM, FCM and RT-qPCR for the detection of tumor urinary exosomes from patients with prostate cancer.

Primary Endpoint

The primary endpoint is to quantify urinary exosomal PSA.

Secondary Endpoint

The secondary endpoint is to compare the percentage of detection according to the different techniques (TEM, FCM and RT-qPCR).

Inclusion of patients with a prostate cancer

Patients who meet the following criteria are eligible for inclusion of patients with a prostate cancer: (1) patient over 50 years old; (2) patient with a positive biopsy for prostate cancer; (3) patients without urinary tract infection; (4) patients benefiting from social security; and (5) patient having accepted and signed the consent.

Inclusion of patients with a BPH

Patients who meet the following criteria are eligible for inclusion of patients with a benign prostate hypertrophy: (1) patient over 50 years old; (2) patient with histologically confirmed diagnosis of BHP; (3) patients without urinary tract infection; (4) Patients benefiting from social security; and (5) patient having accepted and signed the consent.

Inclusion of controls

Patients who meet the following criteria are eligible for inclusion of controls: (1) patient over 50 years old; (2) patient with a normal PSA; (3) patient with no clinical evidence of BPH or prostate cancer; (4) prostate volume less than 30 cc; (4) patients without urinary tract infection; (5) patient having accepted and signed the consent; and (6) patients benefiting from social security.

Exclusion of patients and controls

Patients meeting any of the following criteria are excluded from the study: (1) patients under 50 years old; (2) patients taking treatment or hormones known to modify the level of serum PSA in the 3 to 6 months preceding inclusion; (3) patients with a urinary tract infection History of prostate cancer; (4) history of invasive therapeutic procedures for benign prostatic hypertrophy or for symptoms of urinary tract disorders within 6 months prior to inclusion; (5) patient with a history of concurrent bladder or renal tumors in the 6 months preceding inclusion; and (6) no signature of consent.

Procedure for a patient

After checking the inclusion and non-inclusion criteria, the study information leaflet will be given to patients. Patients will have an one-hour reflection period. The 20 ml first void urine sample will be

collected by using Colli-Pee device in the department before oncological treatment. The end of a patient's study corresponds to urine collection.

Urine sample processing

Urine samples are kept in refrigerator and processed within 24 hours after collection. Urine is centrifuged at 3000 g for 15 minutes at 4°C to remove the exfoliated urinary cells. Supernatant is aliquoted and used for the isolation of urinary exosomes.

Isolation of urinary exosomes

5 ml urine supernatant will be used for the isolation of urinary exosomes. Urinary exosomes are isolated by using PEG-based precipitation method. We have optimized the PEG-based technique for the isolation of urinary exosomes. After the adjustment of urine PH, a solution of PEG is mixed with urine. .The mixture is incubated at 4°C for 2 hours. The mixture is centrifuged at 4500 g at 4°C to collect urinary exosomes. The exosome pellet is suspended in 200 µl PBS solution for the quantification of PSA.

Quantification of urinary exosomal PSA

In both exosomes isolated from a prostate cancer cell line LNCaP and isolated from urine samples, the concentrations of total PSA is determined in the automatic analyzer Cobas 8000 (Roche Diagnostics). In the Cobas 8000, total PSA is measured in a c602 module using Elecsys® total PSA reagent kits based on electrochemiluminiscent immunoassays in a “sandwich” configuration. The concentration of total PSA is expressed in µg/ml.

Urinary exosome characterization

We are going to characterize urinary urinary exosomes by using TEM, FCM and NTA.

TEM is performed on the exosome pellets, which are suspended in PBS. 10 µl urinary exosome suspension is placed on 200 mesh copper grids (AGS138; Oxford Instruments; Oxford, UK) and allowed it to adhere for 20 minutes. The excess sample is wicked off by using a piece of filter paper. The grids are washed three times with distilled water. The grids are incubated with a drop of staining

solution Uranyless (Delta Microscopy, Auressac, France) for 20 seconds and blotted with a filter paper. The urinary exosomes are observed at an accelerating voltage value of 100 kV using a Hitachi electron microscopy (H800; Hitachi, Tokyo, Japan).

For FCM analysis, the technique of Aldehyde/sulphate latex beads (A37304, ThermoFisher) is used. 1 µl of Aldehyde/sulphate latex beads is added to 100 µl of exosome suspension, and mixed well. 900 ul of PBS is then added, and the mixture is incubated for 2 hours at room temperature on a rotary wheel. 100 µl of 100 mM glycine is then added and the mixture is incubated at room temperature for 30 minutes. The mixture is then pelleted by centrifugation at 2650 g for 5 minutes, and the supernatant is removed. The pellet is washed by the addition of 1ml PBS+0.5% FBS solution. The mixture is centrifuged at 2650 g for 5 minutes. Finally, the pellet is resuspended in PBS and the antibody of anti-human CD63-FITC (IM1165U, Beckman Coulter), CD81-FITC (B25329, Beckman Coulter) or CD9-FITC (IM1755U, Beckman Coulter) or the antibody of isotype control (A07795, Beckman Coulter) is added. The incubation with antibody is performed for 30 minute at dark. After twice washing with PBS, the staining is observed by using a conventional FCM.

NTA for urinary exosomes is performed using NanoSight LM10 fitted with a 488 nm laser and 500nm long-pass filter (Malvern Instruments, Malvern, UK). The urinary exosome sample is diluted in PBS to a final volume of 1 ml. The diluted sample is then injected into the laser chamber. Particle counts/mL and size distribution of particles in solution are collected and calculated.

Sample size

To calculate the necessary number for inclusion of prostate cancer patients, our hypotheses are as follows: a percentage of 75% detection being considered insufficient (P0=0.75), and a percentage of 90% being considered sufficient (P1=0.90), unilateral alpha = 5% and power = 95%. Based on these assumptions, using the one-step Fleming method, 65 subjects are required.

In order to verify the specificity, we shall also include 65 BPH and 65 controls.

Statistical analysis

For quantitative analysis, number of observations available, mean, standard deviation, median, minimum and maximum will be calculated and compared. For qualitative analysis, absolute and relative frequencies (expressed in %) will be calculated and compared. Sensitivity and specificity will be calculated. The area under the ROC curve will be determined.

Data sources

The sponsor (via the research technicians or the investigators) undertakes to respond to any request for access to data within a maximum of one month. Furthermore, only personnel authorized by the sponsor (investigators, research engineers, research technicians) and representatives of the health authorities will have access to this information.

Study data will be collected directly in the observation notebooks. These data will be validated by the investigator who will sign (electronically) the observation notebooks.

The information collected as part of this study is as follows: (1) demographic data (age, gender, weight, height); (2) medical history (stage and grade of cancer); and (3) results of different analysis techniques.

Missing data must be justified. Any correction made in the Case Report Form must be traceable.

Ethical approval

This study has received an ethical approval by the ethics committee of CHU Saint-Etienne **(**IRBN262024/CHUSTE).

Results

We have found the urinary exosomal PSA can be quantified by automatic technique of Elecsys® total PSA. It will be a 2-year study. We shall start to include the patients and controls in the summer of 2024. We expect the results to be published in the last quarter of 2026.

Discussion

The study of urinary markers for the diagnosis of prostate cancer is a renewed and innovative research area in contemporary era [8,9]. The liquid biopsy by urinary exosomes is promising for the

diagnosis of prostate cancer. According to recent studies, tumor can discharge more exosomes into body fluid. We expect the quantity of urinary exosomes from patients with a prostate cancer is significantly increased than that of controls. Therefore, the exosomal PSA levels in prostate cancers are significantly increased in the patients with a prostate cancer. The increased exosomal PSA level may be a new tool for the diagnosis of prostate cancer. A recent study supports that level of urinary exosomal PSA is increased compare with that of patient with a benign prostate hypertrophy or normal controls [10]. However, to implement the urinary exosomal PSA into clinical laboratory, improvement or optimization is necessary for every step such as urine collection, isolation of urinary and quantification of PSA. This study is the first one to optimize the precipitation method to isolate the urinary exosome.

This study presents some unique characteristics: the collection of first-void urine by using standardized Colli-Pee device, the isolation of urine exosome by optimized precipitation method and the detection of exosomal PSA by Elecsys® total PSA. The first-void urine is important because it contain more exosomes from prostate. In this study, the first-void urine is to be collected without digital prostate message while the published study needed a digital prostate message before the collection of urine [10]. At present, there has been no consensus on the isolation method. The method suitable for clinical laboratory is preferable. In this study, we decide to adapt the precipitation method. The precipitation technique by using PEG for the isolation of exosomes is considered suitable for clinical laboratory [11]. Many commercial kits such as the commercial ExoQuick kit use this technology. We have modified and optimized the precipitation method to isolate urinary exosomes. We recently found that urinary exosomal PSA could be detected by automate assay Elecsys® total PSA in clinical laboratory. To our best knowledge, it is the first study to detect urinary exosomal PSA form prostate cancer by the technique of Elecsys® total PSA.

Urinary exosomes from prostate cancer express many tissue markers. The contents in urinary exosomes are relatively stable because of the protection by a lipid bilayer membrane. Many tumor

markers including PSA, PSMA and PCA3 have been identified in urinary exosomes as potentially interesting [12-14]. Recent results show that urinary exosomes are particularly useful for those who need a prostate biopsy in men with a PSA between 2-10 ng/ml [15,16]. It is considered as a major advance in the diagnosis of prostate cancer by avoiding unnecessary tissue biopsy [15,16]. To characterize urinary exosomes from prostate cancer, we shall employ the techniques of RT-qPCR, FCM and TEM to study the tumor marker. Conventional FCM seems to be not suitable for the detection of exosomes because of the detection limit of size. For FCM, we will use the technique of FACS-Beads in latex [17,18] and the classic technique of detection by flow cytometry on a DXFLEX cytometer. The technique of FACS-Beads is interesting. The RT-qPCR technique is clinical laboratory routine. We shall perform RT-qPCR according to the TaqMan technique [17,18]. We have worked on cancerous exosomes in biological fluids for several years [17-22]. Recently we have developed an immunostaining and transmission electron microscope observation test to identify exosomes from clear cell kidney cancer [19]. We wish to use this technique to characterize tumor exosomes in the urine of patients with prostate cancer. In this project, we will use immunolabeling of exosomal and tumor biomarkers and observation of exosomes under a transmission electron microscope to further confirm our objective.

In conclusion, this study emphasizes on using the clinical laboratory-based techniques to detect the urinary exosomes from prostate cancer. Our perspective is to develop a test based on the analysis of urinary exosomes to improve the diagnosis of prostate cancer.

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approval from the ethical committee of CHU Saint-Etienne (IRBN262024/CHUSTE). We thank the association AIRE (Aide à la Recherche Médicale de Proximité) for providing financial aid to this project.

Data Availability

The data sets generated and analyzed during this study are not publicly available to maintain participant confidentiality, but will be available from the corresponding author upon reasonable request.

Conflict of Interest

None declared.

Author Contributions

GL, BB, LB and PC conceptualized the study and were involved in the methodology for the project; YT, NM, LW and PF were involved in the techniques of sample analysis; GL and PC wrote the original draft of the manuscript; BB and LB performed the review of the manuscript; BB and LB supervised the project; LB and CP were responsible for the project administration.

Abbreviations

PSA: Prostate specific antigen.

FCM: Flow cytometry.

RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

BPH: Benign prostate hypertrophy.

TEM: Transmission electron microscopy.

NTA: Nanoparticle Tracking Analysis.

ROC: Receiver Operating Characteristic.

PEG: polyethylene-glycol.

PSMA: Prostate-specific membrane antigen.

PCA3: Prostate Cancer Antigen 3.

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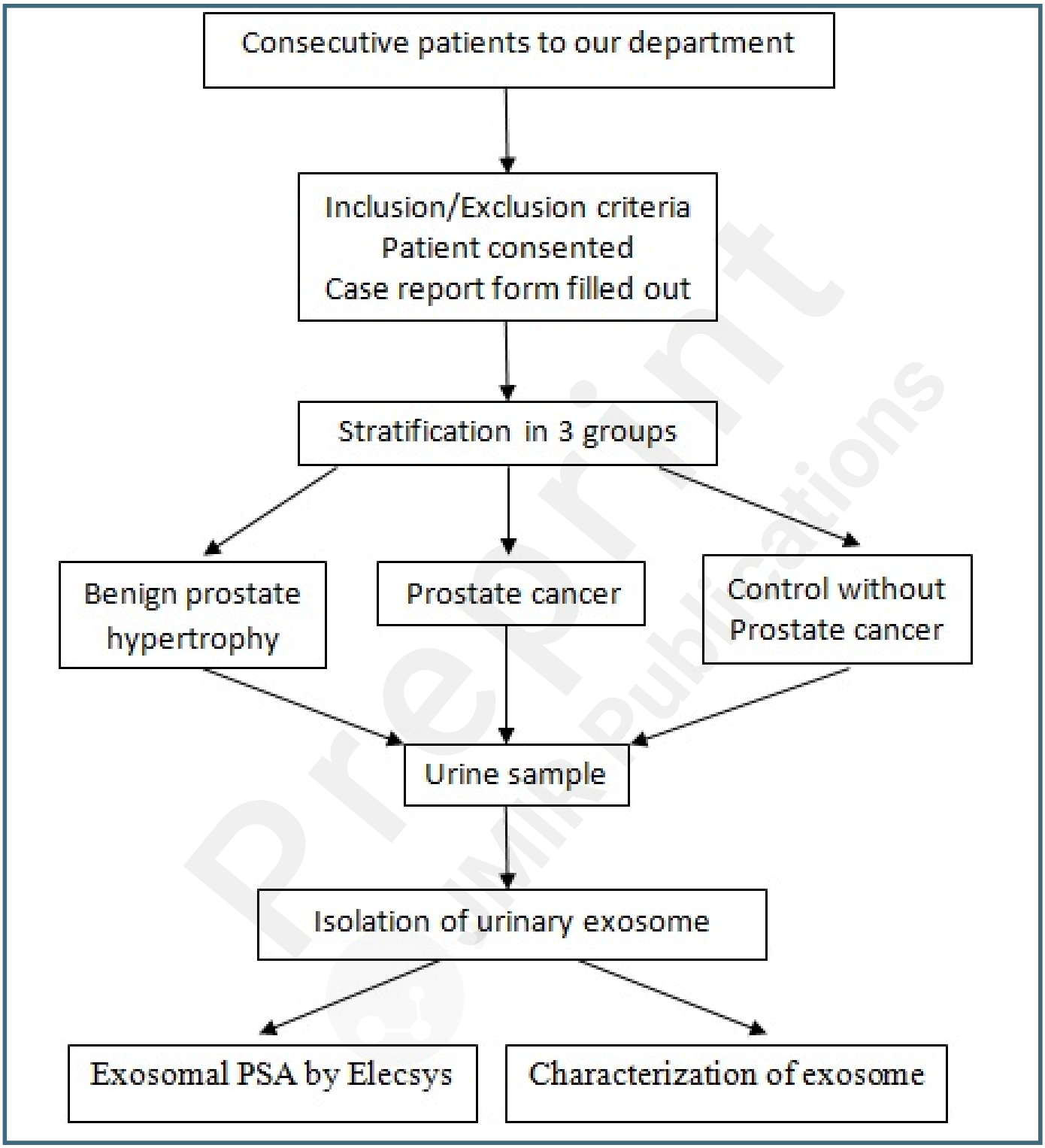
Supplementary Files

Figures

The study scheme.



Multimedia Appendixes

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