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The kallikrein family of proteins as urinary biomarkers for the detection of prostate cancer

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Abstract

Background: Several urinary biomarkers have been assessed as showing a discriminatory ability to differentially diagnose prostate cancer, albeit with manipulation of the prostate. Here we examine the clinical utility of multiple members of the kallikrein family of proteins in non-manipulative urinary biomarker testing.

Methods: Forty urine samples were collected from patients admitted for urological examination. Twenty, with a confirmed benign diagnosis and 20 with prostate cancer. The levels of 14 kallikrein proteins were measured in patient's urine and normalized for creatinine.

Results: Ten of the 14 kallikreins tested had detectable levels in urine. However, none showed statistical significance in discriminating patients. Serum PSA was superior to urine PSA and other urinary kallikreins in separating patients with and without prostate cancer.

Conclusions: We were unable to distinguish men with and without prostate cancer using multiple kallikreins as urinary biomarkers. These results highlight the difficulties in diagnosing prostate cancer via urine testing for soluble biomarkers.

Introduction

The routine implementation of PSA testing in the clinic in the early 1990's has had a profound impact on the early diagnosis of prostate cancer (CaP) and has resulted in an increase in CaP incidence [1]. However, the use of this marker is currently being debated since it is not clear if PSA screening has led to a decline in mortality due to CaP [2]. In addition, the large number of unnecessary biopsies due to false-positive PSA results, places a large burden on the healthcare system and leads to patient discomfort [3]. As a result, there is a need for more specific and more sensitive biomarkers for CaP. In this regard, urine testing is considered a promising method of CaP diagnosis but has resulted in only limited success, based on the biomarkers discovered and validated thus far [4]. Here, we evaluate the human kallikrein (KLK) family of proteins as possible novel urine markers for CaP.

The KLKs are a subgroup of the serine protease enzyme family. The KLK gene family consists of 15 members (*KLK1–15*) [5]. The most prominent is *KLK3*, widely known as prostate specific antigen (PSA) or hK3. In addition to PSA, several other members of the KLK family have shown promise as biomarkers for CaP diagnosis. One is human glandular kallikrein 2 (*KLK2*), a relatively new biomarker of prostatic tissue [6]. Other KLKs such as *KLK4*, *11* and *15* have also shown some value for early CaP diagnosis and for determining tumor aggressiveness [7–9]. Human *KLK4*, *5*, *6*, *8*, *9*, *10*, *11*, *13*, *14* and *15* have been shown to be novel biomarkers for ovarian, breast and CaP [10,11]. To determine the clinical utility of the KLKs as CaP urine markers we performed a pilot study using urine from patients with biopsy-confirmed CaP and benign conditions.

Materials and methods

Patients

This study included patients who were presenting with either clinical symptoms, a suspicious digital-rectal examination (DRE)

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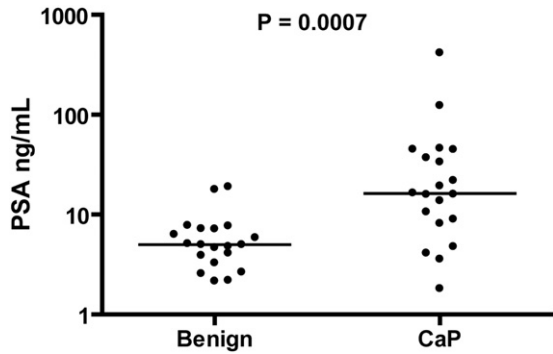*E-mail address:* ediamandis@mtsinai.on.ca (E.P. Diamandis).

Fig. 1. Serum PSA in the benign and CaP groups. Median levels of each group are represented by horizontal lines. Significance was determined by the Mann–Whitney test.

or elevated serum PSA. Each patient underwent prostate needle biopsy to confirm or exclude the presence of cancer by pathological examination. Urine and serum were collected from patients just prior to prostate biopsy and stored at −80 °C until further use and freeze-thawed no more than once before KLK measurements. Twenty benign and 20 CaP patients were selected from the patient cohort. The clinical data on these patients is listed in Supplementary Table 1.

Measurement of KLKs in patient urine

A panel of kallikreins (KLKs 2–15) was assayed in urine samples using ELISA methodologies developed and validated previously. More details for each ELISA method can be found in our previous publication [12]. Briefly, two types of sandwich antibody ELISAs were performed, depending on the kallikrein: monoclonal–monoclonal or monoclonal–polyclonal. A coating antibody was incubated in a flat-bottomed plastic 96 well plate for ~ 12 h. The coating antibody solution was then aspirated and the plate washed 6 times using a wash buffer. The urine samples were then added to each well in duplicate, diluted 1:1 with assay buffer and incubated for 2 h at room temperature with shaking. Afterwards, the plate was washed 6 times and a detection antibody was added and incubated with shaking for an additional 1 h and then washed 6 times. A streptavidin–alkaline phosphatase conjugate was added and the plate was incubated with shaking for 15 min and then washed 6 times again. A substrate solution was then added to the plate and it was incubated with shaking for an additional 10 min. A developing solution was added to the plate and it was incubated with shaking for 2 min. Fluorescence was then measured in each well using a time-resolved fluorometer, as previously described [12].

Creatinine concentrations were measured using an enzymatic colorimetric assay with the Roche Modular system. The assay was conducted according to the Roche reagent kit. Serum PSA was also measured with the same system.

Statistical analysis

To determine if there were significant differences between the median urine levels measured for benign and CaP patient groups tested for the KLKs, the Mann–Whitney non-parametric test was employed using 95% confidence intervals and two-sided *P* values. Statistical significance was defined as $P < 0.05$. Similarly, statistical significance was also determined for age differences between groups and serum PSA levels.

Results

The clinical characteristics of the patient groups are listed in Supplementary Table 1. The Gleason score of CaP patients ranged from 7 to 9. Prostate cancer patients were older, with a median age of 68 versus 61 for BPH patients ($P = 0.0035$). In addition, serum PSA levels were significantly higher in the CaP group, $P = 0.007$ (Fig. 1). However, there were no significant differences in the urine levels of KLKs 2, 3, 4, 5, 6, 8, 10, 11, 13, and 14 between the benign and CaP groups (Mann–Whitney test). KLK levels were normalized for urine creatinine concentrations in each sample. Four KLKs were unmeasurable in urine (KLKs 7, 9, 12, 15).
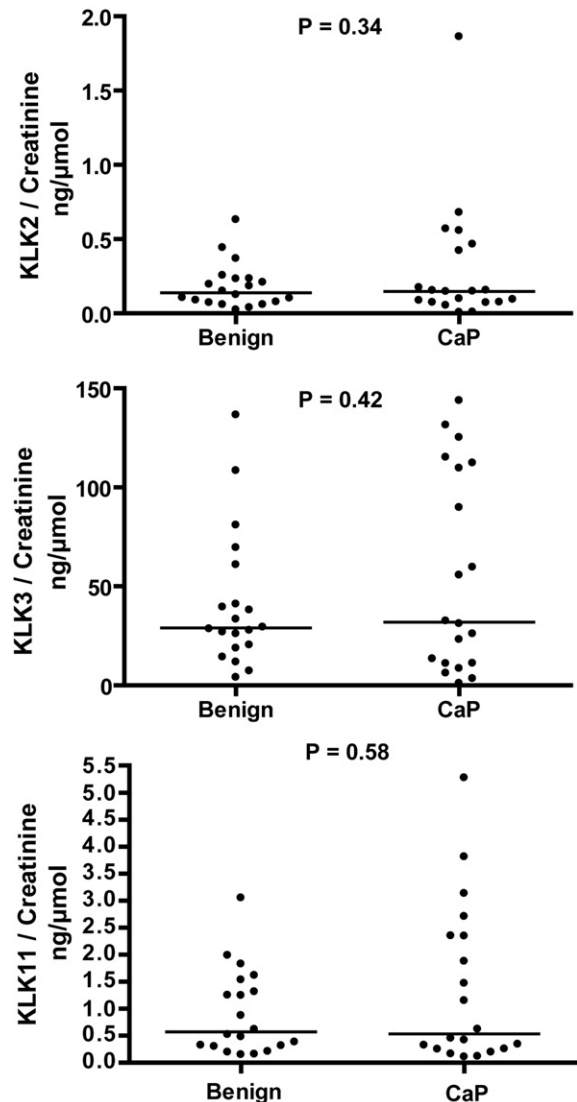

Fig. 2. Urinary KLK2, KLK3 (PSA) and KLK11 in the benign and CaP groups. Median levels of each group are represented by horizontal lines. Significance was determined by the Mann–Whitney test. Values are in ng/μmol of creatinine.

Some data for KLKs that are highly expressed in the prostate (KLKs 2, 3 and 11) are shown in Fig. 2. The rest of the data are summarized in Supplementary Fig. 1.

Discussion

Urine testing has been used for many years as a non-invasive method for the diagnosis of a variety of diseases, including cancer. While urine testing has been shown to be very useful in some diseases, it is currently being investigated with respect to early diagnosis of CaP [4].

Of the urine markers that have shown clinical utility for diagnosis of CaP, very few have been able to compete with serum PSA measurements, and many require some sort of prostate manipulation before urine collection. Some of these markers are complementary to each other, but this effect is incremental and mostly helps in identifying later stage tumors [4,13]. The most widely studied test for CaP in urine, PCA3, relies on detection of upregulated transcripts in cancer cells, and it is not a soluble biomarker test [14,15].

From examining the architecture of the prostate, it would seem reasonable to assume that a cell or protein would be able to traverse the ductal system from a tumor site, enter the urine and then be measured. However, this does not seem to be the case, based on previous studies [4] and data of this study. Prostate massage is required to give an analyte or cell a 'push' in order for it to exit the glandular architecture and enter the prostatic urethra. Unlike during ejaculation, there is no contraction of the smooth muscle in the prostate during urination to empty the prostate gland of its secretions. Therefore, we have ascertained that the current issue with urine markers for CaP is that they require extensive manipulation of the prostate, in order for the marker to be coaxed from its place and released in to the urinary tract. Subsequently, the first void of urine then needs to be collected and the marker, be it either DNA, RNA or protein, should be preferentially measured in that sample to achieve the best possible sensitivity. While urine is considered more stable a biological fluid than serum or seminal plasma, in terms of nucleic acid or protein degradation, the use of urine testing for CaP will inevitably suffer from the differences in duration and intensity of prostate massage between patients. This difference can result in variable amounts of the marker being released into the prostatic urethra and hamper patient comparisons. Issues with compliance are also significant. It is therefore of considerable interest to uncover markers for CaP that can be detected in urine without the need for prostatic massage.

By measuring the KLK family of proteins in urine, of which many have been associated with CaP, we hoped to discern if this would be feasible. However, we were not able to detect any differences in any of the KLKs in urine between benign and CaP patient groups. One might have expected that due to the high grade (Gleason 7–9) nature of the tumors some PSA (or other KLKs) might have leaked into the prostatic urethra as it does in to the bloodstream. Unfortunately, perhaps the PSA produced by the bulbourethral glands masks any differences in PSA leaking from the prostate. Likewise, KLK2, which is also produced in copious amounts in the prostate, but is not produced in the bulbourethral glands, is only detected at trace amounts in urine. The question

that then arises is: are there any secretions of the prostate that enter the urine through the prostatic urethra normally and when there is a tumor? And are these proteins arising from the prostate or from the systemic circulation through kidney clearance? Or some of them arise in urine from accessory sexual glands such as the periurethral glands [16]?

From our data we did not see any changes of prostatic KLKs in urine of CaP patients. Our failure to see such changes in highly expressed and relatively specific prostatic proteins such as KLK2, KLK3 and KLK11 suggests that it is unlikely that other soluble prostatic proteins may work better in this respect. The discriminatory ability of serum PSA in the same patients (Fig. 1) suggests that serum may be a superior fluid for CaP diagnosis than urine, when soluble prostatic proteins are used as biomarkers. Albeit, as can be seen in Fig. 1, serum PSA does suffer from not being able to completely discriminate healthy and BPH groups. A large overlap is seen which highlights the lack of specificity PSA has in screening a population. On the other hand, molecular tests relying on cancer cell identification in urine, such as PCA3 [14,15], may be more appropriate and better performing in urine.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2009.06.015.

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