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PROSTATE-SPECIFIC ANTIGEN, ITS MOLECULAR FORMS, AND OTHER KALLIKREIN MARKERS FOR DETECTION OF PROSTATE CANCER

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Prostate-specific antigen (PSA) is a serine protease produced by the prostate gland at very high concentrations. PSA is secreted into the seminal plasma in high concentrations (0.5 to 5 g/L), where it plays a role in semen liquefaction. The retrograde release of PSA into the bloodstream is a rare event in young, healthy men. It occurs with a frequency of less than one PSA molecule per million secreted PSA molecules, leading to a concentration of less than 4 ng/mL PSA in the serum. The perturbation of the prostate gland architecture often results in excessive escape of PSA into the circulation. Prostate cancer, benign prostatic diseases, and physical trauma to the prostate can result in significant increases in serum PSA. Thus, an elevated serum PSA level is a sensitive marker of prostate gland abnormalities, including prostate cancer and many other conditions affecting the integrity of the prostate gland. These noncancerous alterations have severely challenged the usefulness of PSA as a tumor marker for the early detection of prostate cancer. This disease is the most common malignancy in men, with an estimated 198,100 new cases and 31,500 deaths in 2001.[1] Several calculated parameters, such as PSA density, PSA transition zone density, PSA velocity, and age and race-specific PSA ranges, have been only partially successful in enhancing the specificity of PSA and thus reducing somewhat the number of unnecessary prostate biopsies.[2] The discovery of different PSA forms, such as free PSA (fPSA) and PSA bound to alpha$_1$-antichymotrypsin (PSA-ACT) in the early 1990s renewed the clinical research on this biomarker.[3,4]

This review summarizes the current clinical use of fPSA and focuses on new developments regarding the molecular forms of PSA and the promising role of additional kallikreins (especially hK2) for prostate cancer detection.

NEW KALLIKREIN GENE FAMILY

Until recently, only three human kallikrein genes were identified: the pancreatic/renal kallikrein (KLK1, encoding for hK1), the human glandular kallikrein 2 (KLK2, encoding for hK2), and PSA (KLK3, encoding for hK3, widely known as PSA).[5] Recently, 12 new members of the human kallikrein family were characterized.[6] This family of proteases now consists of 15 members (Fig. 1), which are classified with a new nomenclature.[7] The kallikrein genes (named KLK1 to KLK15; encoding for hK1 to hK15) share significant homologies, genomic motifs, and other similarities, and all cluster within a 300-kb region on human chromosome 19q13.4. All genes have five coding exons and significant sequence homologies at the DNA and amino acid levels (40% to 80%) and at least 12 of the kallikreins are regulated by steroid hormones.[6] In addition to PSA, hK2 has already shown, in preliminary clinical studies, to add significant information for detecting prostate cancer, especially at low PSA values.[8–10] Additionally, it has been shown that hK2 can activate the conversion of the proPSA to active PSA[11] and that these two kallikreins may act in concert in extraprostatic locations.[6] Interestingly, a current investigation by Denmeade *et al.*[12] could not confirm that hK2 activates proPSA to PSA. Very recent immunoassay studies for the kallikreins hK6 and hK10 have indicated possible roles as serum biomarkers for nonprostatic diseases, especially ovarian cancer.[6,13] It is quite possible that several kallikreins,

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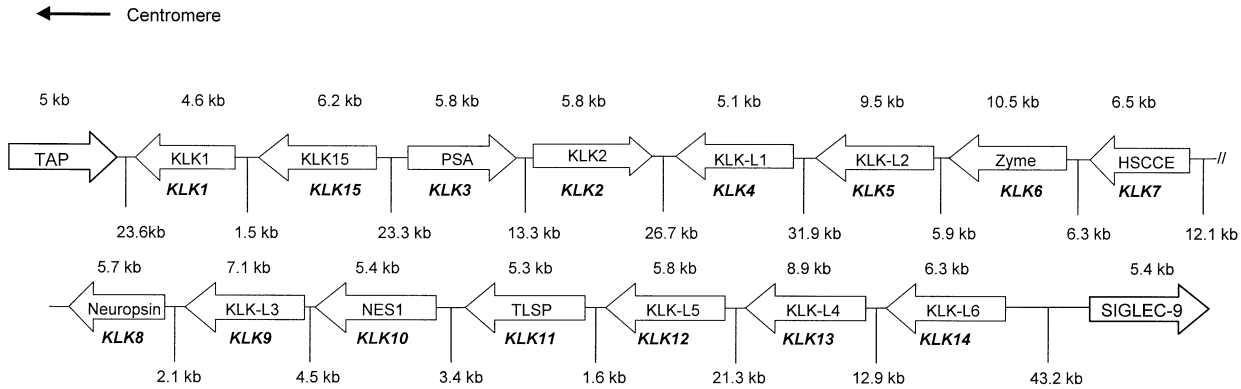

FIGURE 1. *New gene locus of the kallikrein family around chromosome 19q13.4 at an approximate 300-kb region. Direction of transcription illustrated by arrows. Boxes represent the former gene names; below the boxes, the new nomenclature (KLK = kallikrein) is indicated. The genomic length of each gene, in base pairs, is designated above the related boxes. Distances between genes, in base pairs, are shown between boxes. The Siglec and ACPT (testicular acid phosphatase) genes frame the kallikrein gene family but do not belong to this family. This figure is not drawn to scale. For full gene names and details, see Yousef and Diamandis.*[6]

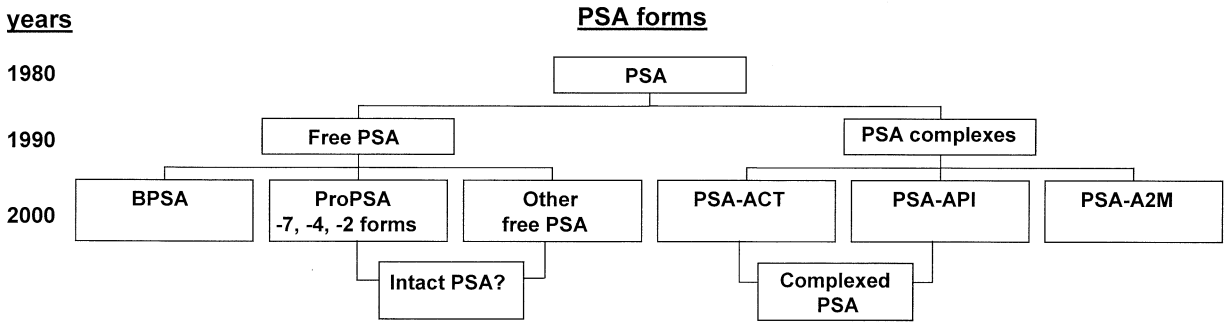

FIGURE 2. *Survey of the research development of the molecular forms of PSA. Approximate years of discovery are indicated on the left. Each box represents a different molecular form of PSA. The Bayer cPSA measures PSA-ACT and PSA-API. BPSA = BPH-associated fPSA; proPSA = precursor form of fPSA; "intact" PSA (fPSA-I) = other inactive and intact PSA, which also detects proPSA; PSA-ACT = PSA bound to alpha$_1$-antichymotrypsin; PSA-API = PSA bound to alpha$_1$-protease inhibitor; PSA-A2M = PSA bound alpha$_2$-macroglobulin.*

in addition to PSA, may add clinical information for various cancers, including prostate cancer.

MOLECULAR FORMS OF PSA (hK3)

PSA circulates in the serum in free (unbound) and complexed (bound to protease inhibitors) forms.[3,4] Approximately 65% to 95% of the PSA is bound to ACT (PSA-ACT); fPSA represents, on average, only 5% to 35% of the total PSA (tPSA) concentration. The relative amount of fPSA tends to be increased in benign disease compared with prostate cancer. The fPSA/tPSA ratio is now routinely used to increase the specificity for prostate cancer and reduce unnecessary biopsies. Other PSA complexes with alpha$_2$-macroglobulin (PSA-A2M) and alpha$_1$-protease inhibitor (PSA-API) are now measurable in serum as well.[14,15] Complexed PSA (cPSA) is being evaluated in numerous studies to determine its clinical utility. The Bayer cPSA assay measures both PSA-ACT and PSA-API and has been proposed as a single assay, as an alternative to the fPSA and tPSA assays.[16,17] Other existing PSA complexes are so far not measurable in serum.[18] fPSA has recently been shown to exist in three molecular forms; proPSA,[19] BPSA,[20] a special clipped form of fPSA, and inactive "intact" PSA (fPSA-I).[21] Figure 2 gives an overview of the molecular forms of PSA.

The reason for the differences between benign prostatic hyperplasia (BPH) and prostate cancer regarding the molecular forms of PSA and, especially, the higher PSA-ACT amounts in the serum of patients with cancer, are not yet completely understood. It is assumed that because of the loss of tissue architecture in prostate cancer the intracellular active PSA gains quicker access to the circu-

lation and the protease inhibitors like ACT and A2M can complex to PSA more easily.[22] If PSA reaches the circulation from normal or BPH cells, it first has to leak backward into the extracellular space, where it is susceptible to proteolytic degradation.[22] This may explain the decreased capability of PSA to form complexes with ACT in patients with BPH.

The earlier hypothesis that higher intracellular ACT production in the prostatic epithelium leads to higher serum PSA-ACT concentrations in patients with prostate cancer compared with those with BPH could not be confirmed, because prostatic tissue PSA is present almost exclusively as uncomplexed PSA.[20,23] On the other hand, the recent studies demonstrating distinct molecular forms of fPSA in the transition zone and prostate tumor tissue may provide a rationale for the source of fPSA in the blood.[20,24]

CLINICAL USE OF PERCENT fPSA

The use of percent fPSA (%fPSA), which is the fPSA/tPSA ratio, has been established as a clinical routine parameter since the mid-1990s. Various retrospective studies[2,18] and prospective studies[25–27] have demonstrated a significant improvement in the specificity for the 4 to 10 ng/mL PSA range and for lower PSA values (less than 4 ng/mL). Generally, with %fPSA cutoffs ranging from 17% to 30%, to obtain 90% to 95% sensitivities, the number of unnecessary biopsies could be reduced by approximately 20%, with a minimum loss in the sensitivity to detect prostate cancer. However, for a more efficient interpretation of the %fPSA values, possible influencing factors such as prostate volume, tPSA, cancer stage and grade, prostatic intraepithelial neoplasia (PIN), race, sample stability, prostatic manipulations, and drug treatment history should be considered. These factors have been comprehensively discussed.[2,18] We briefly summarize some of these factors.

In two recent studies of 1709 patients[28] and 1622 patients,[29] the investigators confirmed the earlier findings of a positive correlation of the %fPSA to the prostate volume. In the relevant tPSA range of 4 to 10 ng/mL, this relationship seemed to be stronger for higher PSA levels.[29]

The %fPSA tends to inversely correlate with tPSA. A significant downward trend of %fPSA for the tPSA ranges less than 4 ng/mL, 4 to 10 ng/mL, and greater than 10 ng/mL was shown.[27,28] Regarding the histologic grade and Gleason grade, it appears that low %fPSA values are associated more with higher grades. A prospective multicenter clinical trial demonstrated that %fPSA, followed by the Gleason sum, was the strongest predictor for the pathologic outcome.[30] Moreover, aggressive cancer may be detected much earlier using %fPSA instead of tPSA.[31]

Several new investigations have focussed on the impact of isolated PIN as precursor of prostate cancer on the %fPSA value. The release of PSA into the blood may be different because of the integrity of the basal cell layer in PIN tissue contrary to cancer tissue. Significant differences in the mean %fPSA levels between patients with exclusively PIN lesions (15.0%) and patients with prostate cancer (12.1%) were found.[32] Therefore, a decrease of the %fPSA values in patients with PIN should be considered possible concomitant evidence of prostate cancer.

It has been proposed to use %fPSA as a priority decision tool for first-time biopsy in men with unsuspicious digital rectal examination findings and a tPSA value within the range of 4 to 10 ng/mL, as well as for lower PSA values.[33] This will further enhance the number of detected cancers per biopsy. Artificial neural networks that include clinical relevant data can add substantial information, and there is a need to evaluate these artificial neural networks for clinical routine use to save more biopsies in the future.[34] However, only limited data on the %fPSA cutoff recommendations for tPSA values less than 4 ng/mL are available.[26] With specificity cutoffs of 90% to 95%, the number of unnecessary biopsies can be reduced using %fPSA at low tPSA concentrations, but many cancers would be undetected. The most recent data from Catalona *et al.*[35] on 841 biopsied men with PSA concentrations ranging from 2.6 to 4.0 ng/mL and a high cancer detection rate of 29% argue for a high %fPSA sensitivity or even a general biopsy within this tPSA range. More studies need to be done to find an appropriate conclusion for this low PSA range. However, it is at least clearly seen that expanding the range of additional fPSA measurements from 4 to 10 ng/mL to 2 to 10 ng/mL or to 2.5 to 10 ng/mL could be beneficial for detecting significant cancer at the lower PSA values.

USE OF OTHER MOLECULAR FORMS OF PSA

PSA-ACT and cPSA

For the measurement of different PSA isoforms, only assays for PSA-ACT (Roche Diagnostics, Mannheim, Germany) and cPSA (Bayer Diagnostics, Tarrytown, NY) have been commercially available. It is known that the complex of PSA-ACT is the predominant form of PSA in patients with prostate cancer.[3,4] Early analytical problems regarding an overestimation because of the nonspecific binding of the ACT-cathepsin G-complex have now been solved. To date, no study has shown a clear advantage to measuring the PSA-

ACT alone or calculating the PSA-ACT/tPSA ratio compared with %fPSA to enhance the specificity of prostate cancer detection.[36]

The cPSA assay (Bayer Immuno 1) uses a blocking antibody to fPSA and detects PSA-ACT and PSA-API but not PSA-A2M.[37] Proposals to use the cPSA test alone have been debated, but, in general, the use of the cPSA/tPSA ratio has resulted in similar sensitivity and specificity data as that found with %fPSA. In studies with an overrepresentation of patients with BPH at lower PSA levels and patients with prostate cancer at higher PSA levels, cPSA enhanced the specificity of tPSA to levels equal or even better than that of %fPSA.[16,17] However, other studies using more equal tPSA distributions in patients with BPH and prostate cancer could only show a slightly better diagnostic performance of cPSA alone compared with tPSA.[36,38,39] Only the cPSA/tPSA ratio could reach specificity levels comparable to %fPSA.[18,40] The preliminary data of a cPSA multicenter study has revealed a better performance of the cPSA/tPSA ratio in the 4 to 10 ng/mL tPSA range compared with cPSA alone, but in the lower range of 2 to 6 ng/mL, cPSA outperformed the cPSA/tPSA ratio.[41] Studies on the use of the PSA complexes (PSA-ACT and cPSA) for low PSA values less than 4 ng/mL are rare, but these results indicate a possible use and warrant further prospective evaluations.[42,43]

PSA-A2M and PSA-API

The measurement of the PSA-A2M complex in serum has been demonstrated using PSA immunoadsorption followed by pH manipulations to release the encapsulated PSA from the 25-fold larger molecule A2M.[15] PSA-A2M represents a considerable proportion of tPSA in the serum and the PSA-A2M/PSA ratio is higher in patients with BPH (12%) than in patients with prostate cancer (8%). However, the previous report of a high proportion of about 60% of PSA-A2M could not be confirmed. It has also been shown that the sum of %fPSA and PSA-A2M could further enhance the specificity of tPSA and %fPSA.[15]

A method to analyze PSA-API has also recently been reported by the same investigators.[14] In a study of patients with and without cancer, the amount of PSA-API was 1.6% of tPSA in patients with BPH and 0.9% of tPSA in patients with prostate cancer.[14]

Both complexes, PSA-A2M and PSA-API, reveal, like %fPSA, higher amounts in patients with BPH, but the levels did not correlate with fPSA, in contrast to the PSA-ACT complex, which is higher in patients with cancer.

BPSA

The BPSA is a specifically clipped subform of fPSA that is highly associated with the transition zone containing BPH nodules in prostatic tissue.[20] A dual monoclonal assay with a detection limit of 0.06 ng/mL was tested in serum of patients with BPH, with symptomatic BPH, and without clinical BPH but referred to a urologist, as well as healthy control subjects.[44] In the control group, BPSA was almost undetectable. The median BPSA values for the clinical BPH group were significantly elevated and BPSA discriminated clinical patients with BPH from patients without BPH better than did %fPSA.

ProPSA Forms

Recently, proPSA forms were isolated in the serum and tissue of patients with prostate cancer.[19,24] The completely natural proPSA protein contains 244 amino acids (−7) compared with fPSA (237 amino acids). The proPSA in serum and prostatic tissue exists as a mixture of different designated forms, including the (−7), (−5), and (−4) forms, and partially as the (−2) and (−1) forms.[45] Interestingly, Mikolajczyk and coworkers (personal communication) found the (−4) and (−2) forms to be the predominant form in serum samples from patients with prostate cancer with tPSA values less than 20 ng/mL. The combined measurements of these different cancer-associated proPSA forms and BPH-associated BPSA could further enhance both the sensitivity and the specificity of PSA.

Very recently, a newly developed assay has been reported with a detection limit of 0.035 ng/mL for nonclipped fPSA called "intact" PSA.[21] The "intact" fPSA (fPSA-I) assay detects both proPSA and other inactive nonclipped fPSA. Although the absolute concentrations of fPSA-I did not differ in 383 patients with negative or cancer-positive biopsies, the fPSA-I/fPSA ratio was significantly higher in patients with cancer.[21]

Taken together, a new area may be emerging for fPSA molecular forms that may substantially help improve the differentiation between BPH and prostate cancer.

hK2 FOR EARLY DETECTION OF PROSTATE CANCER

PSA and hK2 share the highest homology, with 78% and 80% identity at the amino acid and DNA level in the human kallikrein family, which is composed of 15 distinct genes.[6] Both kallikreins have been detected in relatively small quantities in nonprostatic tissues and biologic fluids.[6,18] The hK2 mRNA amounts to 10% to 50% of the PSA mRNA in prostatic tissue, but in serum and seminal plasma, hK2 has a concentration of only 1% to 3% of PSA. The picogram per milliliter levels in serum

posed analytical challenges for hK2 immunoassays, but reliable prototype assays are now available in several research laboratories.

In 1998, Kwiatkowski *et al.*[8] reported first that the hK2/fPSA ratio enhances the discrimination of patients with prostate cancer and patients with BPH. In a larger study with 937 serum samples, Partin *et al.*[10] described an enhanced prostate cancer detection rate using both ratios, %fPSA and hK2/fPSA, within the PSA ranges of 2 to 4 ng/mL and 4 to 10 ng/mL. Other studies confirmed the advantage of the additional use of hK2 and its ratios to fPSA and %fPSA, especially at low PSA concentrations, for detection of prostate cancer.[9] The higher expression of hK2 in malignant than in benign tissue, as revealed by immunohistochemistry, was hypothesized to be responsible for the higher hK2/fPSA ratios in patients with cancer than in patients with BPH. Using quantitative measurements of hK2 in matched prostate cancer samples, Magklara *et al.*[46] found that hK2 is decreased in malignant versus benign tissue, but the degree of down regulation is somewhat lower than for PSA. In preliminary studies, hK2 was found to discriminate between high and low-grade tumors and between Stage 2 and Stage 3 tumors.

In two very recent reports, hK2 was shown to be the most accurate marker for deciding to perform a repeated biopsy[47] and describing the amount of Gleason grade 4/5 cancer in patients with prostate cancer.[48] Until now, the Gleason grade 4/5 cancer volume has been the only independent predictor of biochemical failure after radical prostatectomy, and therefore, a serum marker is critically needed.[49]

Analogous to PSA, hK2 was also found in different molecular forms in the serum, but, contrary to PSA, free hK2 is the predominant form and hK2-ACT represents only 4% to 19% of the total hK2.[50] The proform of hK2 (prohK2) is also present in the serum and is increased in prostatic diseases.[51] A novel complex of hK2, hK2-PI6 has been shown to be highly associated with prostate tumor tissue relative to the transition zone and peripheral zone normal cells.[52] Apparently, hK2-PI6 is formed as a consequence of tumor necrosis.[52]

OTHER KALLIKREINS AND KALLIKREIN-RELATED PROSTATE CANCER MARKERS

A differential regulation of kallikrein genes or other genes in cancerous and noncancerous prostatic tissue might indicate potential new serum markers. Among all kallikreins, at least eight (KLK2 to 4, KLK 10 to 13, and KLK 15) are expressed in relatively high amounts in prostatic tissue.[6] Despite a high expression of KLK4 in prostatic tissue,[53] no reports are available to date about the usefulness of this kallikrein for prostatic diseases. The newly developed hK10 immunoassay shows elevated serum concentrations for ovarian cancer (median 1.9 μg/L) but lower and similar concentrations in patients with prostate cancer compared with healthy males.[13] KLK11, KLK12, and KLK13 are also highly expressed in prostatic tissue, but no further studies are yet available.[6] KLK14 is, like PSA and hK2, down regulated in prostate cancer tissue compared with noncancerous tissue of the same gland.[54] The regulation of KLK15 was analyzed in matched prostate tissue samples from 29 patients.[55] Almost one half of the patients had higher KLK15 levels in the cancerous tissue and only three showed a down regulation. A KLK15-positive expression was found in all pT3 staged patients and all patients with grade 3 tumors, but only two thirds of the low-grade and low-stage patients showed this up regulation.[55]

In addition to these kallikreins, five novel prostate-specific genes have recently been cloned, including Prostin,[56] which has subsequently shown to be identical to hK15, Prostein,[57] PSGR,[58] a new member of the G protein-coupled olfactory receptors, Trp-p8,[59] and PART-1.[60] Prostin (hK15) is more active than hK2 in the proteolytic conversion of proPSA to active PSA.[56] Prostein showed an exclusive expression in the prostate, but no difference was found between normal versus cancerous tissue.[57] The PSGR gene was analyzed in matched prostate cancer tissue from 52 patients and overexpression was observed in 62% of the tumor specimens, no change in 27% and down regulation in 11.5% of the specimens.[58] A comparison of the grade or stage of the cancers was not performed. The Trp-p859 and PART-1 genes[60] were overexpressed in cancerous tissue compared with noncancerous prostatic tissue. These newly cloned genes, which are localized to the prostate and/or are members of the kallikrein family, will be intensively studied in the future as potential new markers for prostate cancer detection.

CONCLUSIONS

As the first fully clinically evaluated molecular form of PSA, %fPSA has shown its effectiveness in improving specificity over tPSA alone. Artificial neural networks may further predict the need for a biopsy, but the number of unnecessary biopsies is still very high. The evaluation of the new promising molecular forms of PSA, such as proPSA, BPSA, "intact" PSA, PSA-A2M, and PSA-API, as well as the hK2 and its molecular forms (eg, hK2-PI6 and prohK2) is needed. The development of antibodies for various kallikreins and new prostate-specific proteins will be of great interest. For the future, one of the most important goals for prostate cancer

serum marker development is the search for a marker that can predict Gleason grade 4/5, which, at present, is the only independent prognostic factor for biochemical failure after radical prostatectomy. None of the hitherto existing serum markers fulfill this most important clinical need. The molecular forms of fPSA and hK2 show a potential to clarify this issue. The other kallikreins and new prostate-specific genes and their related proteins are now available for research studies to find a good serum marker for predicting Gleason grade 4/5 prostate cancer.

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