A Prostate-specific Antigen-like Protein Associated with Renal Cell Carcinoma in Women

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ABSTRACT

Renal cell carcinoma (RCC) is the most common form of cancer affecting the kidney. There is currently no biochemical marker for this disease. We have shown that serum-immunoreactive prostate-specific antigen (PSA) levels, as measured by the two-site Ciba-Corning ACS:180 immunoluminometric assay, are elevated in women with RCC. Although the levels were low (0.13–0.89 μg/l), serum PSA was clearly measurable prior to surgery in 13 of 17 women (76%) with RCC. Significantly, the PSA levels fell to undetectable after nephrectomy. Seventeen normal women also had undetectable (<0.1 μg/l) PSA levels. Two women, who had several serum PSA measurements performed postoperatively, showed a t½ of 2–3 days equivalent to that observed for PSA in men following radical prostatectomy for prostate cancer. The 17 RCCs evaluated in this study consisted of 10 stage A, 4 stage B, and 3 stage C tumors. There was no relationship between tumor size, stage, or serum-immunoreactive PSA level, although the majority of these tumors are low grade. We have shown by reverse transcription-PCR, using PCR primers directed to the NH2-terminal coding region of the KLK3 (PSA) gene and the closely related KLK1 and KLK2 genes, that these genes are not expressed in these tumors. Our findings show, however, that elevated levels of a circulating PSA-like protein are present in women with RCC.

INTRODUCTION

RCC1 arises from the proximal convoluted tubules of the kidneys and is the most common form of cancer affecting the kidney. This highly vascularized tumor accounts for 2% of all malignancies as well as 2% of all cancer deaths (1). There is currently no biochemical marker for this disease. Diagnosis relies on clinical symptoms and radiological procedures, and prognosis is closely linked to clinical stage. Small tumors confined to the kidney are associated with 5-year survival rates in excess of 75% following radical nephrectomy (2). Unfortunately, many patients initially present with metastatic disease, not amenable to curative treatment and associated with a mean survival of less than 12 months.

The widespread use and availability of abdominal ultrasound and computed tomography scans has resulted in an increase in the detection of small renal masses (3, 4). These present an additional clinical problem because it is often difficult to determine the significance of small lesions or cysts detected on ultrasound or computed tomography scan. In many instances, these may be benign lesions that do not require treatment (5). Clearly, there is a need for accurate molecular markers to assist in the diagnosis of RCC, in the monitoring of the disease, and to discriminate between benign and malignant renal lesions detected on other investigations.

PSA, a member of the tissue kallikrein (KLK) family of enzymes, is expressed at high levels in the male prostate and is currently the preferred serum marker used in the detection of prostate cancer and tumor recurrence (6, 7). Several recent studies, however, have shown that PSA is not “prostate-specific,” as was originally thought, but is also expressed in a variety of other tissues including the breast, ovary, and uterus in the female (8–14). One such study (15) reported the detection of ir-PSA levels in the sera of some women (27%) with RCC. This ir-PSA was detected in a RIA using a polyclonal antibody. A two-site immunometric PSA assay, using monoclonal antibodies and used for comparison, did not detect any ir-PSA in the serum of these women (15). These authors dismissed this finding as a false-positive result and suggested that the detected ir-PSA levels represented cross-reactivity with renal kallikrein or KLK1, another member of the tissue kallikrein family. Although this interpretation was not proven, these authors cautioned against the use of less specific PSA assays and the danger of such interference in the interpretation of PSA levels in the diagnosis of prostate cancer in men.

The purpose of this study was to reevaluate the potential use of the measurement of serum PSA as a diagnostic marker for the detection of RCC, particularly in women. We report here that 13 of 17 women (76%) had measurable serum PSA levels that were reduced to undetectable following radical nephrectomy. The human KLK gene family consists of three closely related genes: KLK1 or renal tissue kallikrein, KLK2 and KLK3, or PSA (6). We have also demonstrated that neither PSA nor the KLK1 or KLK2 genes are expressed in tumor tissue taken from these women, as detected by RT-PCR with specific primers to regions encoding the NH2-terminal sequences of these proteins. Thus, we suggest that this ir-PSA-like species, detected in association with RCC, is not encoded by these genes.
MATERIALS AND METHODS

Clinical Samples. Blood was obtained from 17 women (ages 27–83 years) with suspected RCC, 24 h prior to surgery and 2–4 days following radical nephrectomy for serum PSA estimations. Two had serial samples taken postoperatively (at 2, 3, and 5 days) to determine the half-life of serum PSA disappearance. Blood was also obtained from 17 age-matched control females with no history of malignancy. Samples of tumor and normal renal parenchyma were collected at the time of surgical resection from each patient and stored at −80°C for subsequent RNA extraction. All 17 women had histologically confirmed RCC on pathological examination. Tumors were staged according to the Robson system (16) with 10 assessed as stage I, 4 stage II, and 3 stage III tumors. Prostate tumors were staged according to the Robson system (16) with 10 assessed as stage I, 4 stage II, and 3 stage III tumors. Prostate tumors were staged according to the Robson system (16) with 10 assessed as stage I, 4 stage II, and 3 stage III tumors.

PSA Immunoassay. PSA levels in serum were measured using the ACS:180 (Ciba-Corning Corporation, Melbourne, Australia) two-site immunoluminometric assay (ICMA). This assay uses an affinity-purified sheep polyclonal antiserum raised against a purified biological preparation of PSA as the signal antibody and a mouse monoclonal antibody prepared against recombinant PSA as the capture antibody (17). The lower limit of detection is 0.1 µg/L with an interassay coefficient of variation of 20%. The Mann-Whitney test was used to determine statistical differences in the serum PSA levels between the normal and RCC patient groups.

RT-PCR Analysis. Total RNA extraction and RT-PCR was performed essentially as reported previously (10, 14) with minor modifications. Total RNA was isolated from frozen tissue specimens using the acid/guanidinium isothiocyanate method (18). cDNA synthesis was initiated with 2–3 µg of total RNA, 200 ng of random primers (Boehringer Mannheim, Sydney, Australia), and Superscript reverse transcriptase (BRL Life Technologies, Sydney, Australia) in a total volume of 20 µL. PCR was performed with 2 µL of the generated cDNA, using specific primers for PSA (KLK3), renal kallikrein (KLK1) and KLK2, or 1 µL of cDNA with β₂-microglobulin primers as a control for RNA integrity. 1.5 mM Mg²⁺, and Taq polymerase (Amplitaq; Perkin-Elmer, Melbourne Australia) in a total volume of 20 µL. Negative controls included RNA samples minus RT and a PCR control of a prostate RT reaction minus Taq polymerase. PCR was performed for 30–35 cycles at 94°C for 60 s, 45°C (PSA, KLK1/renal kallikrein, KLK2) or 50°C (β₂-microglobulin) for 90 s, and 72°C for 120 s, followed by a 7-min extension period in a Hybaid Omnimgene thermocycler (Integrated Sciences, Sydney, Australia).

The PSA primers were designed from regions in exons 1 (5'UT, 5'-GCACCCCGAGAGCTGTTG-3') and 4 (codons 161–167, 5'-TTTCTCAGATTAGCTTACG-3') to generate a 600-bp PCR product (19–21). Each of these primers had 6–10 differences between each of the three KLK genes to ensure specificity. The β₂-microglobulin primers were designed from exons 2 (codons 23–30, 5'-TGAATGCTATTGTGCTTGG-3') and 3 (3'UT, 5'-CTCCCATGATGCTGCTTACAT-3') to generate a 250-bp product (22).

An aliquot (10 µL) was analyzed by electrophoresis in a 1.0% agarose gel followed by Southern blot analysis with specific oligonucleotide probes for each gene, designed to regions internal to the PCR primers (PSA: codons 44–49, 5'-GATCCAGGCTTCTGCTATGAT-3', KLK2: codons 44–49, 5'-GACCTGGATTCTTCTTATTAGGC-3', and β₂-microglobulin: codons 63–71, 5'-GTGAACTCATGTAGTACAAGAGATA-3'). For the KLK probes, there were again 8 nucleotide differences between the KLK2 and KLK3 probes and 8–10 differences between these and the KLK1 sequence over this region. The probes were end-labeled with digoxigenin-dUTP and terminal transferase (Boehringer Mannheim, Melbourne, Australia).

Hybridization was performed with 10 ng/ml probe in Easyhyb buffer (Boehringer Mannheim) in a total volume of 6 ml for 16 h at 40°C, followed by washes in 2X SSC (1X SSC is 0.15 M sodium chloride-0.001 M sodium citrate, pH 7.0) at room temperature and 0.1X SSC at 42°C. Stringency of hybridization and washes for PSA was monitored by the inclusion of cDNA dot blots for the three KLK genes to ensure that no cross-hybridization occurred (10). Hybridization was detected using the chemiluminescent detection method (Boehringer Mannheim) on Kodak X-AR film (Integrated Sciences, Sydney, Australia).

RESULTS

PSA Immunoreactivity. Serum ir-PSA levels were measured on 17 women with suspected RCC, which was confirmed both at surgery and following pathological examination of the tumors, and 20 age-matched controls. Fig. 1 shows the serum PSA levels of the women with RCC before radical
nephrectomy compared with the controls. Thirteen of the 17 women (76%) had detectable PSA levels ranging from 0.13 to 0.89 µg/liter prior to surgery. Significantly, these levels fell to undetectable (<0.1 µg/l) following removal of the tumor, suggesting that the source of the ir-PSA originated from the RCC. Serum PSA levels were undetectable (<0.1 µg/l) for the 17 normal controls. There was a clear significant difference in the mean serum PSA levels of women with RCC compared with normal controls (RCC, 0.24 ± 0.22 µg/l; normals, 0.05 ± 0.05 µg/l; mean ± SD, P < 0.0001). Two women had sequential serum PSA measurements performed, over a period of 2–5 days following surgery, to assess the time taken for serum PSA disappearance. The t1/2 was 2–3 days (data not shown), similar to that observed for men with prostate cancer following radical prostatectomy (23). There was no relationship between tumor stage, size, or grade and serum PSA levels, although clearly the majority of these tumors are low grade.

**RT-PCR Analysis.** RT-PCR was performed to determine whether the KLK3 gene, which encodes PSA, was expressed in the RCC or, indeed, the adjacent normal kidney tissue. Fig. 2 depicts the RT-PCR/Southern blot analysis for tissue samples from six patients with the highest serum PSA levels. Tissue samples from the remaining seven patients with lower serum PSA levels were also analyzed and gave similar results (data not shown). As shown in both the ethidium bromide-stained gel (upper PSA panel) and Southern blot (lower PSA panel), PSA expression was not detected in any of the RCC tumors or adjacent normal parenchymal tissue from any of these six patients or the normal kidney control. The PCR product apparent on the ethidium-stained gel for patient tumor sample (#3T) was not detected on Southern blot analysis. In contrast, the prostate tumor controls at either end of the gel are clearly positive with a PSA RT-PCR product of the appropriate size (600 bp). The RT-PCR control in lane (–) is negative, as expected. The β2-microglobulin PCR (Fig. 2, lower panels) of the same RT samples indicates that both the purity and concentration of the total RNA used in these RT-PCR reactions was adequate. The RNA sample concentrations used from patient 5 were slightly lower, as depicted by the ethidium bromide stain, but still exhibited a reasonable signal on Southern blot analysis. Because renal kallikrein, another member of the tissue kallikrein (KLK) family of enzymes, is known to be expressed in the normal human kidney (24, 25), we also examined whether this gene was expressed in RCC tumors. RT-PCR for both tissue kallikrein and β2-microglobulin was performed on six different patients’ samples. All gave an identical pattern; the results for four of these tumors are shown in Fig. 3. A renal kallikrein gene PCR product of the appropriate size (242 bp) was observed for each of these normal kidney tissue samples as well as a prostate control known to be positive for tissue kallikrein (Fig. 3, Lane P), but the RCC tumor itself was negative. The β2-microglobulin profile (Fig. 3, lower panels) again indicated the integrity of the samples used. Although the RNA concentration of patient 4’s samples were again low as indicated by the β2-microglobulin profile, there was clearly sufficient RNA to generate a positive tissue kallikrein signal in the adjacent normal kidney sample.
Fig. 4  RT-PCR and Southern blot analysis for KLK2 on paired normal renal parenchyma (N) and RCC tumor (T) for six female patients with RCC (#1–#6). Patient #3 has two different tumor RNA samples instead of the normal tissue RNA, which had all been used. The prostate positive control (P) and negative RT-PCR control (−) are indicated. The upper panel is the ethidium bromide-stained pattern obtained following electrophoresis of the RT-PCR products in a 1% agarose gel. The lower panel is the Southern blot hybridized with a gene-specific oligonucleotide probe for KLK2. Right, the sizes (bp) of the DNA molecular weight marker X (Boehringer Mannheim) fragments. Arrow, the expected size of the KLK2 (600 bp) PCR product.

Finally, we examined whether the third human KLK gene, KLK2, was expressed in these RCC tumors. RT-PCR was performed with specific KLK2 primers, similarly designed to the same NH2 terminal coding region as that for PSA and KLK1. The same RT samples used for PSA and β2-microglobulin amplification, as shown in Fig. 2, were used with the exception of #3N, for which no further sample was available, and which was substituted with another tumor tissue RNA sample form this patient (#3T). As seen in Fig. 4, although the prostate control tissues are positive as expected (26), none of the normal kidney or tumor tissues from these RCC patients are positive. The β2-microglobulin profile in Fig. 2 indicates that sufficient RNA was present in these samples.

DISCUSSION

We have shown that detectable levels of a PSA-like protein are elevated in women with RCC. Two of our findings suggest that this ir-PSA species is derived from the RCC tumor: (a) there were clearly measurable levels of serum PSA, prior to surgery, in 76% of the women with RCC studied, which fell to undetectable following nephrectomy and removal of the tumor; (b) the sequential blood sampling of two of these women following surgery, to assess the time course of PSA disappearance, demonstrated a serum half-life of 2–3 days. This is similar to that reported previously for the t1/2 of PSA in men (2.2 days) following radical prostatectomy for prostate cancer (23).

What was not clear was whether this ir-PSA was indeed PSA or a similar but unrelated protein, cross-reacting in the immunoassay. Pummer et al. (15), in their study in which they reported false-positive serum PSA levels in six women with RCC, used an immunoassay (Pros-Check; Yang Laboratories) that used a rabbit polyclonal antibody to PSA. Similarly, a sheep polyclonal antibody, raised against a purified biological preparation of PSA and which has been purified by high-performance liquid chromatography, is the signal antibody used in the ACS: 180 assay. To verify the authenticity of the PSA measured in their study, Pummer et al. (15) also used a two-site monoclonal-based PSA assay (Tandem-R; Hybritech, Inc.), which then gave undetectable readings for these patients (8). Their conclusion was that it was clearly not PSA being measured but a cross-reacting species detected by the polyclonal antibody. We would agree with this conclusion. Paradoxically, the high-performance liquid chromatography purification of the polyclonal antibody may have generated a less specific antibody for this PSA-like protein because the levels we measured were considerably lower (0.13–0.89 µg/liter) than that reported by Pummer et al. (Ref. 15; 0.5–27.0 µg/liter). The greater number of positive serum ir-PSA values in our study, 13 of 17 women with RCC compared to 6 of 22 patients in the Pummer study (15), may also reflect different threshold levels for the respective assays [this study, 0.1 µg/liter; Pummer et al. (15), 0.25 µg/liter] and our use of a more sensitive assay.

Our approach to determine the authenticity of this PSA immunoreactivity was to examine whether PSA was expressed in the RCC or indeed the normal renal parenchyma of these women. We have shown that the PSA gene is not expressed in these tissues, as detected by the sensitive mRNA detection method of RT-PCR and using primers spanning exons 1 to 4, which encode the NH2 terminal region of this enzyme (19). In contrast, the prostate control tissues were clearly positive, indicating that the primers worked in this tissue, the primary source of PSA expression in humans. Moreover, the β2-microglobulin expression profile indicated the integrity of all of the RCC tumor and adjacent normal kidney parenchyma samples. PSA expression, as detected by RT-PCR, has been reported recently in several tissues (8–14), including the normal endometrium and in breast and ovarian tumors (9–11). In these latter studies, the authors have also confirmed the identity of their RT-PCR products by DNA sequencing and shown that the PSA mRNAs detected in these female tissues are identical to that expressed in the prostate, at least over the regions sequenced. Thus, PSA is expressed in several organs in the female, albeit at considerably lower levels than that of the male prostate, but was not detected in the female kidney or RCC tumors in this study.

PSA is a member of the tissue kallikrein (KLK) gene family of enzymes (6). Renal tissue kallikrein, another member of this family, is expressed in the human kidney (24, 25). To determine whether the ir-PSA in the sera of women with RCC may be due to cross-reactivity with renal tissue kallikrein, we repeated the RT-PCR assay with specific primers to this gene. The normal kidney control and the normal renal parenchymal tissue from the RCC patients showed measurable levels of renal kallikrein mRNA, as expected. The RCC tumors, however, were negative, indicating that renal kallikrein was not the source of the cross-reacting ir-PSA-like species measured in the serum of these women. Renal kallikrein is expressed primarily in the distal tubule of the nephron (25); therefore, it is perhaps not surprising that it is not expressed in these tumors that are derived from epithelial cells of the proximal tubules.

The human tissue kallikrein gene family consists of three structurally related genes. In addition to KLK1 or renal tissue kallikrein and KLK3 or PSA, KLK2 is also a member of this family (6, 27). Like PSA, KLK2 is predominantly expressed in the prostate, although it has also been recently shown to be
expressed in the human endometrium and pituitary, albeit at much lower levels (10, 14). KLK2 expression, however, has not been demonstrated in the kidney. It is interesting to note, though, that PSA and KLK2 are more related to each other than KLK1 or renal kallikrein; it is 80% identical at the protein sequence level compared with 52–60% identity with KLK1 (19–21). This has led to the recent revelation that some PSA antisera cross-react with KLK2 and the suggestion that KLK2 may be an important marker for prostate cancer in its own right (27). The possibility that the PSA antisera in this study may be detecting the closely related KLK2 gene product has not been confirmed because expression of this gene could not be detected in any of the RCC tumors or normal renal tissue from these women.

In summary, we have confirmed that ir-PSA levels can be detected in women with RCC and demonstrated that this immunoreactivity is derived from the RCC tumor. We have extended these findings to show, using the sensitive RT-PCR mRNA detection assay and primers to regions encoding the NH2 terminal regions, that PSA and the closely related renal tissue kallikrein (KLK1) and KLK2 genes are not expressed in these tumors. Thus, we believe that we are measuring a yet-to-be-identified protein that is antigenically similar to PSA. The identification and characterization of this PSA-like antigen may have important implications for women, and possibly men, with renal tumors.

REFERENCES


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