

Frequency of Expression of Prostate-Specific Antigen mRNA in Lung Tumors

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The presence of prostate-specific antigen (PSA) protein and messenger RNA (mRNA) was studied in 52 primary lung tumor tissues. The PSA protein was detected more frequently and at higher levels in lung tumor extracts from men. The levels of PSA protein in tumor extracts correlated with preoperative and postoperative serum PSA levels, suggesting a possible contamination of the tumor extracts with PSA from residual blood in the tumor vasculature. The PSA mRNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot hybridization in 24 (68%) of 35 tumors from men, in 9 (53%) of 17 tumors from women, and in 5 (71%) of 7 adjacent normal lung tissue specimens. The levels of PSA protein did not associate with patient age, the

tumor stage, grade, or histologic type, or the nodal status. Similarly, PSA mRNA was not associated with any clinicopathologic variables, but squamous cell carcinomas, especially in men, were more frequently positive. A by-product of the RT-PCR procedure was cloned and sequenced and found to be a 450-base pair sequence not previously deposited in the data bank. We conclude that PSA mRNA and protein frequently can be detected in lung tumors and normal tissues from men and women but at levels much lower than those seen in breast carcinomas in women. The significance of the new 450-base pair sequence remains to be determined. (Key words: Lung cancer; Prostate-specific antigen; Prognostic markers; Polymerase chain reaction) *Am J Clin Pathol* 1997;108:184-190.

Prostate-specific antigen (PSA) is a 33-kd serine protease encoded by a gene localized on chromosome 19 and has 80% sequence similarity with the human glandular kallikrein gene.¹ Prostate-specific antigen is generally believed to be expressed only by prostatic epithelial cells.² It is currently used as a marker for screening, diagnosis, and monitoring of prostate cancer.^{2,3} Immunohistochemical detection of PSA in tumors of nonprostatic origin has been reported as a rare event, but convincing evidence now exists that the PSA gene is expressed in many human tissues.⁴ The highest levels of PSA are found in the prostate and in seminal plasma.⁵ Significant levels are also found in female breast discharge fluid,⁶ in breast milk,⁷ and in extracts of normal, hyperplastic, and cancerous breast

tissues.⁸⁻¹⁴ Prostate-specific antigen was also found in some ovarian tumor extracts,¹⁵ in endometrium,¹⁶ salivary glands,¹⁷ and in many other tumor types.¹⁸

Bilgrami et al¹⁹ described a man with nonprostate cancer—lung adenocarcinoma—and a high serum PSA concentration. They postulated that the PSA may have arisen from the lung cancer tissue. However, the tumor stained positive for PSA by polyclonal but not monoclonal anti-PSA antibodies. We also recently reported the presence of immunoreactive PSA protein in some lung tumors.²⁰

Recently, polymerase chain reaction (PCR)-based methods for PSA messenger RNA (mRNA) have been applied to the detection of prostate cancer micrometastases or minimal residual malignant disease.²¹⁻²⁴ Smith et al²⁵ detected PSA mRNA expression in circulating blood cells from normal males and females and in several nonprostatic cell lines, including lung, ovarian, and myeloid leukemia cells.

Traces of PSA protein have been detected in lung tumor extracts.²⁰ However, PSA is also present in serum, and it is possible that PSA in lung tumor extracts may have arisen from traces of blood entrapped into tumor vessels, especially in male patients. We have studied, by reverse transcription-PCR (RT-PCR), the frequency of PSA mRNA expression in a series of 52 primary lung tumors.

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MATERIALS AND METHODS

Patients

Fifty-two tumor tissue specimens were obtained from patients undergoing surgery for primary lung cancer. Seven specimens of histologically normal lung tissue were recovered from adjacent tissues of the lung tumors. Diagnosis was histologically confirmed, and tumors were staged using the TNM classification system. Tumor tissues and adjacent normal tissues, excised during surgery, were "snap frozen" in liquid nitrogen and subsequently stored at -80°C until use.

Detailed clinicopathologic features of our patient population are summarized in Table 1.

Preparation of Cytosolic Extracts

Approximately 0.2 g of tissue were pulverized to a fine powder at -80°C , and the cells were then lysed for 30 minutes on ice with 1 mL of lysis buffer (50 mmol/L tromethamine [Tris] buffer, pH 8.0, containing, per liter, 150 mmol sodium chloride, 5 mmol EDTA, 10 g NP40 surfactant, and 1 mmol phenylmethylsulfonyl fluoride). The lysate was centrifuged

at 15,000g at 4°C for 30 minutes, after which the supernatant was immediately assayed for PSA immunoreactivity and total protein content.

PSA Measurements

The PSA in the cytosolic lung extracts was measured with a highly sensitive and specific immunofluorometric technique. In short, our PSA assay is based on a monoclonal coating antibody, in microtiter wells, a monoclonal biotinylated antibody for detection, and alkaline phosphatase-conjugated streptavidin. We used the alkaline phosphatase substrate diflunisal phosphate; when dephosphorylated, it forms highly fluorescent complexes with Tb^{3+} and EDTA at alkaline pH. The method has a detection limit of 1 ng/L and is precise and sensitive. A full description of the method and its analytical capabilities have been described elsewhere.²⁶ Total protein in the tumor extracts was measured with a commercial kit based on the bicinchoninic acid method (Pierce Chemical Co, Rockford, Ill).

Isolation of Total RNA

For total RNA isolation, we used a commercial reagent (TRIZOL, Gibco BRL, Gaithersburg, Md) and followed the instructions of the manufacturer. The integrity of the RNA was confirmed electrophoretically, and its amount and purity were established by spectrophotometry at 260 and 280 nm. Absorbance ratios at 260 and 280 nm were 1.6 or higher.

Reverse Transcription-Polymerase Chain Reaction

We synthesized copy DNA (cDNA) from the isolated total RNA with a first-strand cDNA synthesis kit and SuperScript II reverse transcriptase (Gibco BRL) as described previously.²⁷ We used two oligonucleotide primers to amplify a 754-base pair (bp) region of PSA cDNA. The primers, originally described by Deguchi et al,²¹ have the following sequences: PSA-A1, 5'-TGCGCAAGTTCACCCTCA-3' and PSA-A2, 5'-CCCTCTCCTTACTTCATCC-3'.

For actin cDNA amplification, we used the following primers, previously published²¹: 5'-ACAATGAGCT-GCGTGTGGCT-3' and 5'-TCTCCTTAATGTCACG-CACG-3'. Actin primers amplify a 372-bp fragment.

The PCR was performed in 0.2-mL thin-walled reaction tubes on a gene amplification system (GeneAmp 2400, Perkin-Elmer, Palo Alto, Calif). The total reaction volume was 50 μL . The reaction mixture

TABLE 1. CLINICOPATHOLOGIC FINDINGS IN 52 PATIENTS WITH PRIMARY LUNG TUMORS

Variable*	No. (%) of Patients
Men	35 (67)
Women	17 (33)
Stage	
I	36 (69)
II	7 (13)
IIIA	8 (15)
IIIB	1 (2)
IV	0 (0)
Grade [†]	
1	3 (6)
2	33 (70)
3	10 (21)
4	1 (2)
Histologic type	
Squamous	25 (48)
Adenocarcinoma	21 (40)
Small cell	2 (4)
Carcinoid tumor	3 (6)
Large cell	1 (2)
Nodal status	
Negative	38 (73)
Positive	14 (27)

*Age range, 42 to 89 years (median 68 years).

[†]Grade unknown for 5 patients.

contained PCR buffer (50 mmol/L potassium chloride, 10 mmol/L Tris buffer, pH 8.3, 1.5 mmol/L magnesium chloride, 10 mg/L gelatin), 200 μ mol/L of deoxyribonucleotide triphosphates, 1 μ mol/L PCR primers, 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind) and 5 μ L of cDNA target (added last). The PCR for PSA and actin was performed with 1 cycle at 94°C for 5 minutes, 30 cycles with denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds, and one cycle at 72°C for 7 minutes. Portions (20 μ L) of the PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide staining. Negative controls did not contain template DNA.

Cloning of PCR Products

We cloned the 754-bp PCR product of PSA cDNA and another five PCR products with molecular weights between 250 and 500 bp using a cloning kit (TA, Invitrogen, San Diego, Calif). We followed the protocol recommended by the manufacturer. Large quantities of the recombinant plasmids were prepared by culturing transformed *Escherichia coli* cells in Luria Bertani-ampicillin media and extracting the plasmids with a plasmid purification kit (Qiagen Midi, Qiagen, Chatsworth, Calif).

RNA Labeling by In Vitro Transcription

The recombinant pCR 2.1 transcription vector contains promoters for SP6 and T7 RNA polymerases. After linearization of the vector with Hind III restriction endonuclease, the T7 RNA polymerase was used to create "run-off" transcripts using an RNA labeling kit (DIG, Boehringer Mannheim, Indianapolis, Ind). The DIG-uridine triphosphate was used as a substrate and was incorporated into the transcript. The DIG-labeled RNA was used as a nonradioactive probe in Southern blots.

Hybridization

Aliquots of PCR products (20 μ L) were electrophoresed on 2% agarose minigels containing ethidium bromide. The gels were then transferred by Southern blot onto positively charged nylon membranes (Boehringer Mannheim, Indianapolis, Ind) by overnight alkali capillary blotting with use of 0.4 mol/L sodium hydroxide. The membranes were then baked for 15 to 30 minutes at 120°C. For hybridization, the membranes were placed in tubes with 20 mL of

hybridization buffer per 100 cm² of membrane. We used a commercial hybridization solution (DIG Easy Hyb, Boehringer Mannheim). Prehybridization was at 68°C for 1 hour. The solution was then replaced with 5 mL per 100 cm² of membrane of hybridization buffer containing 200 ng/mL of DIG-labeled PSA RNA probe. Hybridization was performed for 12 to 16 hours at 42°C. Filters were subsequently washed twice with 2 \times SSC (saline-sodium citrate) containing 1 g/L sodium dodecyl sulfate (SDS) at room temperature (5 minutes per wash) and twice with 0.1 \times SSC containing 1 g/L SDS at 68°C (15 minutes per wash). Detection was accomplished by using an antibody against digoxigenin, labeled with alkaline phosphatase and a chemoluminogenic substrate (CDP-Star, Tropix, Bedford, Mass). We followed the instructions of the manufacturer and captured the chemoluminescence on x-ray film using exposure times of 10 to 60 seconds.

DNA Sequencing

The PCR products were directly sequenced using the Thermo Sequenase kit (Amersham International, Buckinghamshire, England). The protocol recommended by the manufacturer was used throughout. Sequencing primers, labeled at the 5'-end with Cy5 fluorescent dye (Amersham), were as follows: PSA-S1, 5'-AAGGTGACCAAGTTCATG-3' (binds 19 nucleotides internally from PCR primer PSA-A1) and PSA-S2, 5'-CCATCCCATGCCAAAGGA-3' (binds 19 nucleotides internally from PCR primer PSA-A2). The cloned PCR products were sequenced using vector-specific sequencing primers labeled with Cy5. All sequencing reactions were loaded on an automatic sequencer (ALF Express, Pharmacia Biotech, Uppsala, Sweden). Sequence comparisons were performed with BLAST and DNASIS software (Hellixx, Toronto, Canada).

Statistical Analysis

The distribution of demographic and clinicopathologic variables, including age, stage, histologic grade and type, and nodal status were compared between PSA-positive and PSA-negative patients with the use of contingency tables analyzed by the χ^2 test or Fisher's Exact Test as appropriate. All analyses were performed using SAS software (SAS Institute, Cary, NC).

RESULTS

Because of the marked differences between serum PSA concentrations in men and women (men have

approximately 500-fold higher levels), all data were analyzed separately between men ($n = 35$) and women ($n = 17$). The PSA levels in tumor cytosols were expressed as nanograms of PSA per gram of total protein to normalize for the amount of tissue extracted. The average total protein concentration of these extracts was ~ 1 g/L. Any level of PSA less than 1 ng/g or less than 1 ng/L was considered as nondetectable because it is below the detection limit of the PSA assay used.²⁶

The distribution of lung tumor PSA content between men and women is shown in Table 2. In the same Table, we summarize data of serum PSA in the patients. Preoperative (~ 5 days before operation) and postoperative (~ 5 days after operation), PSA levels were measured. We found the following: (1) Levels of PSA in tissue extracts from men are higher than those in women. (2) We found no consistent changes between preoperative and postoperative levels of serum PSA in men or women. (3) Analysis of normal lung tissue extracts from three men and four women revealed that PSA was also detectable at median levels of 1.9 ng/g for men and 2.9 ng/g for women.

We studied whether the PSA levels in the tumor cytosols were associated with the serum levels in men and women. The Spearman correlation coefficient between tumor cytosol PSA and preoperative serum PSA levels in men was 0.80 ($P = .0001$) and in women was 0.53 ($P = .05$). The Spearman correlation coefficient between tumor cytosol PSA and postoperative serum PSA levels in men was 0.51 ($P = .01$) and in women was 0.37 ($P = .18$). These data suggest a significant correlation between tumor levels of PSA and serum levels of PSA, at least in men.

In our series, only four patients died during the follow-up of 6 to 12 months. For this reason, and because of the small number of patients, no survival analysis in relation to PSA levels in the tumors was performed.

The PSA mRNA was detected in lung tumors with RT-PCR and Southern blot hybridization. From the 35

lung tumors from men, 24 (68%) were positive for PSA mRNA. From the 17 lung tumors from women, 9 (53%) were positive for PSA mRNA. Of the 7 adjacent normal lung tissues, five (71%) were also positive for PSA mRNA. Examples of PSA mRNA detection in lung tumors are shown in Figures 1 and 2. In addition to the expected 754-bp PCR product, we also detected bands of molecular weights between 250 and 500 bp in six tumors (see Fig 2). The identity of these bands is discussed in the final paragraph of this section. Sequencing of the 754-bp PCR product from one woman with lung cancer confirmed the 100% identity with the published sequence of PSA cDNA²⁸ (data not shown).

The association between PSA protein and mRNA expression in lung tumors and other clinicopathologic variables was analyzed by contingency tables and χ^2 . In men, using the median PSA level in lung tumors (expressed as ng/g of total protein) as the cutoff, we found no association between PSA protein and the presence or absence of mRNA ($P = .33$), patient age ($P = .88$), tumor stage ($P = .24$), tumor grade ($P = .36$), histologic type ($P = .67$) or nodal status ($P = .23$). The only statistically significant association was with preoperative ($P = .003$) and postoperative ($P = .006$) serum PSA, an association also revealed by Spearman correlation analysis (already described). In women, no significant association was found between tumor PSA levels and any of the clinicopathologic variables (data not shown).

We further studied whether the presence of PSA mRNA in lung tumors was associated with any of the clinicopathologic variables. In men and women, the presence of PSA mRNA was not associated with patient age, tumor stage, tumor grade, preoperative or postoperative serum PSA levels, or nodal status. We found some evidence that the presence of mRNA in men is associated with squamous cell carcinomas. From the 11 tumors that were PSA mRNA-negative, four (36%) were squamous cell, six (54%) were adenocarcinoma, and one (9%) was of other histologic type. From the 24

TABLE 2. DISTRIBUTION OF PROSTATE-SPECIFIC ANTIGEN IN LUNG TUMOR CYTOSOLS AND IN PREOPERATIVE AND POSTOPERATIVE SERUM SAMPLES FROM MEN (M) AND WOMEN (F)

	Percentile									
	0		25		50		75		100	
	M	F	M	F	M	F	M	F	M	F
Tumor cytosols (ng/g)	<1	<1	1.03	<1	3.96	<1	6.2	2.9	180	208
Preoperative serum sample (ng/L)	58	0	316	0	950	0	1,799	3.1	10,000	4
Postoperative serum sample (ng/L)	7	0	641	0	1,005	0	1,695	2.1	10,000	5.1

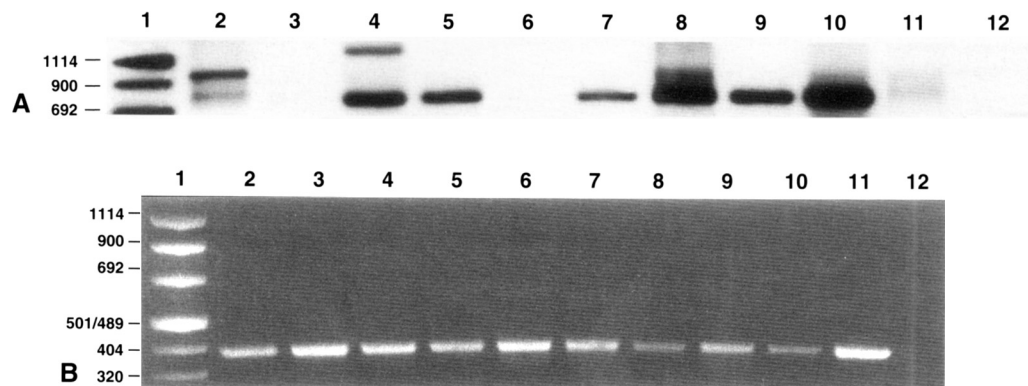


FIG 1. A, Reverse transcription–polymerase chain reaction (RT-PCR) for prostate-specific antigen (PSA) messenger RNA (mRNA) extracted from lung tumors. B, RT-PCR for actin mRNA extracted from lung tumors. Lane 1, Molecular weight markers. Sizes in base pairs (bp) are shown in left column. Lanes 2 through 11, Lung tumors. Lane 12, PCR-negative control (no template added). In A, the PCR products were detected by Southern hybridization and chemiluminescence. In B, the PCR products were detected by ethidium bromide staining. The 754-bp PCR product between molecular weight markers 900 and 692 bp corresponds to PSA. The 372 bp PCR product (in panel B) corresponds to actin. Tumors in lanes 2, 4, 5, 7, 8, 9, and 10 are positive for PSA mRNA.

PSA mRNA-positive tumors, 17 (71%) were squamous cell, 4 (17%) were adenocarcinoma, and 3 (12%) were of other histologic type ($P=.07$).

Figure 2 shows that we also detected aberrant PCR products of molecular weights between 250 and 500 bp. We have isolated and cloned these six products and subsequently sequenced all of them using vector-specific sequencing primers. Three of these products proved to be primer-dimer artifacts that hybridized to the probe because of sequence complementarity between the PCR primers and our riboprobe that contains the primers in its sequence. The other three aberrant PCR products represented the same sequence consisting of 450 bp (Fig 3). This PCR product was created only by primer PSA-A2 because the complete sequence of this PCR product had in one end the primer PSA-A2 and at the other end of the same strand, a sequence complementary to PSA-A2. The 450-bp sequence, verified by sequencing of both strands, had no sequence homology to PSA cDNA, kallikrein cDNA, or any other sequence deposited in GenBank as revealed by BLAST or DNASIS software analysis.

DISCUSSION

Prostate-specific antigen is produced at very high levels by prostatic epithelial cells, and it is secreted into the seminal plasma during ejaculation. It is believed that PSA is one of the proteases that facilitates semen liquefaction by digesting high molecular-weight proteins of seminal vesicle origin.²⁹ Other substrates for PSA have been proposed, including insulinlike growth factor binding protein 3,³⁰ parathyroid hormone

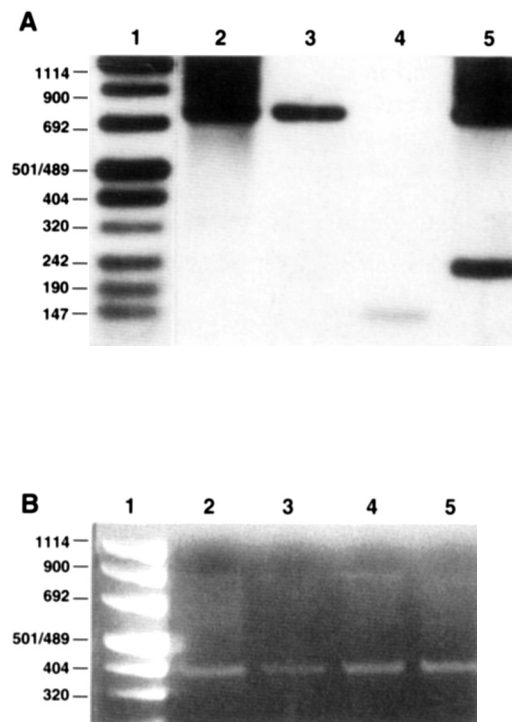


FIG 2. A, Reverse transcription–polymerase chain reaction (RT-PCR) for prostate-specific antigen (PSA) messenger RNA (mRNA) extracted from lung tumors. B, RT-PCR for actin mRNA extracted from lung tumors. Lane 1, Molecular weight markers. Sizes in base pairs (bp) are shown in left column. Lanes 2 through 5, Lung tumors. Other comments as in Figure 1. Aberrant PCR bands appear in lanes 4 and 5 of panel A. Other aberrant bands appeared at molecular weights of 250 to 500 bp (not shown).

1	TGCCAATGGG	TTATCCAGCA	AGAAGCAGAT	CAGAATATAG	GAAATATCAC
51	TCTTTTGACA	TGAATGTAGT	TGAAATCCAT	TTTTAAGTTA	GTTAAGGACT
101	AACTTAAAAA	TACCTACAAA	ATCCAGGGAG	ACTGACTTCA	GTATTTGTCA
151	TGGATCTTCA	GAATACTGAA	TCCCAAATTT	CAATACTCGC	TTCTCAAACA
201	GGGAGAACTG	TTCTAAGGTG	ACCCAGGAAT	GCCAGCCATA	CAAATATGCT
251	CAAGACATGG	AAAAAAACAT	TAAAATGGAT	TTGCTGCAAC	TGATATTCTG
301	TGTTCTGCAA	TGCTAGGTGT	GCCCTTGTAG	TCCAAGAACT	AACACTGCAG
351	ACTGCAACTA	GAATTGTGGT	CCCTGGTCAT	GTCACTTCCA	AGAAGTATCA
401	GGTTTAAGAA	CTAAGCAGTA	TGTGTAAGCT	GAAAAGGGAT	TAAGAGTGAG

FIG 3. Sequence of a 450-base pair polymerase chain reaction (PCR) product detected in addition to the 754-bp prostate-specific antigen PCR product in some lung tumors. This sequence has no homology to any other sequence deposited in GenBank.

related peptide,³¹ and a substrate that releases peptides with smooth muscle contraction.³² Others have found that PSA may act as a negative growth regulator in hormone-dependent breast cancers by stimulating the conversion of estradiol to estrone.³³

The PSA in lung tissue was previously found using immunologic assays that measure PSA protein.²⁰ However, because PSA is also present in the blood, the possibility exists for contamination of tissue extracts by residual blood in the tissue. Also, the close sequence homology between PSA and the human glandular kallikrein-1 gene may cause problems of cross-reactivity.³⁴ We have shown that contamination of the tumor extracts by blood PSA is likely, because we found a significant correlation between tumor extract PSA levels and serum PSA levels, especially in men.

We attempted to show evidence for PSA expression in lung tissue by searching for PSA mRNA presence in lung tissue extracts. We found PSA mRNA in 69% of lung tumors from men, 53% of lung tumors from women, and 71% of normal lung tissues. These data support the view that low levels of PSA mRNA are found in the majority of lung tissue extracts. These levels are much lower than those found in female breast tissue because, in this tissue, PSA mRNA could be detected without Southern blot hybridization,²⁷ and PSA protein levels in breast tumor extracts are much higher than those reported in Table 2.¹⁴ As in the case of breast tissue, normal lung tissue contains PSA mRNA as well.

We found no association between the presence of PSA mRNA and the clinicopathologic variables studied, including patient age, tumor stage, tumor grade, histologic type, or nodal status. Consequently, the prognostic value of this marker in lung cancer is questionable.

The regulation of PSA mRNA expression in lung tissue and its possible biologic role are unknown. In breast cancer, we have shown that PSA gene regulation occurs

through the steroid hormone receptor system.^{9,14} Beattie et al³⁵ reported the presence of high-affinity receptors for androgen, estrogen, and glucocorticoids in lung tissues and carcinomas. Beer and Malkinson³⁶ reported similar data in mouse lung tumors.³⁶

Chelly et al³⁷ described the phenomenon of illegitimate transcription, which postulates that cells may express extremely low levels of mRNA of any gene. With the availability of highly sensitive technologies for amplifying mRNA, and especially by RT-PCR, this phenomenon can be studied in detail. We do not know whether the presence of PSA mRNA in lung tumors represents illegitimate transcription or a phenomenon in which the PSA gene is regulated and expressed at relatively low levels in lung tumors and normal tissue. The presence of corticosteroid hormone receptors in lung tissue and tumors suggests that the PSA gene is regulated by corticosteroid hormones.

By cloning and sequencing aberrant PCR products, we were able to isolate a 450-bp unique sequence not previously deposited in GenBank (see Fig 3). Additional study will reveal whether this fragment belongs to a new gene that is overexpressed in lung tumors. Such study is in progress.

Our data support the view that PSA is frequently expressed at low levels in cancerous and normal lung tissue. The role of this molecule in lung physiology and pathobiology is unknown.

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