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Advances in Brief

Detection of Prostate Cancer Cells Circulating in Peripheral Blood by Reverse Transcription-PCR for *hKLK2*

Masako Kawakami, Toshikazu Okaneya, Kenichi Furihata,[1] Osamu Nishizawa, and Tsutomu Katsuyama

Department of Urology [M. K., O. N.] and Laboratory Medicine [M. K., K. F., T. K.], Shinshu University School of Medicine, Matsumoto, and Department of Urology, Nagano Municipal Hospital, Nagano [T. O.], Japan

Abstract

Two of the human tissue kallikrein family, hK2 and hK3 (prostate-specific antigen), are primarily produced by the prostatic epithelium under the regulation of androgens. In this study, we detected prostate cancer cells that expressed *hKLK2* or *hKLK3* mRNA in the peripheral blood of patients with prostate cancer using reverse transcription-PCR (RT-PCR). We then demonstrated some differences in characteristics, such as differentiation of cancer cells and response to antiandrogen therapy, between *hKLK2* and *hKLK3* mRNA-expressing prostate cancer cells. Total RNA was isolated from 41 patients with known prostate cancer, 7 patients with benign prostatic hyperplasia, and 20 normal volunteers. By RT-PCR, *hKLK2* mRNA was detected in 7 patients (33%), and *hKLK3* mRNA was detected in 17 (81%) of 21 stage D prostate cancer patients. In contrast, all patients with benign prostatic hyperplasia and healthy volunteers were negative. From comparison of the background of the patients positive for *hKLK2* and/or *hKLK3* mRNA, it became evident that the response to antiandrogen therapy and the expression of *hKLK2* mRNA were reciprocally correlated, in contrast with the expression of *hKLK3* mRNA. Additionally, our study clearly demonstrated that the detection of *hKLK2* mRNA in the peripheral blood was useful for screening patients with certain prostate cancers that did not express hK3. We conclude that taking advantage of the difference between *hKLK2* mRNA and *hKLK3* mRNA expression is clinically useful for following up prostate cancer patients.

Introduction

Prostate cancer is becoming one of the most prevalent life-threatening disorders among elderly Japanese men as it is in Western countries. Several biomarkers, such as PSA,[2] now designed as hK3, and prostatic acid phosphatase have been developed for screening, diagnosis, and monitoring of prostate cancer. Some recent research has suggested that novel prostatic tumor markers, such as hK2 (1, 2) and prostate-specific membrane antigen (3, 4), are worth investigating, but they are not available for clinical laboratory tests yet.

The gene encoding hK2 (*hKLK2*) is a member of the human tissue kallikrein gene family to which *hKLK1* and *hKLK3* also belong (5). Both hK2 and hK3 are primarily produced by the prostatic epithelium, whereas expression of hK1 is widely observed in the pancreas, kidney, submandibular gland, and other nonprostatic tissues (6–9). Although the amino acid sequences of hK2 and hK3 (PSA) are 78% homologous (7, 10) and their expression is regulated by androgens (8, 9), each one is thought to have a distinct physiological function. For instance, hK3 (PSA) is a chymotrypsin-like protease (11), whereas hK2 is a trypsin-like protease (10). In addition, the expression of *hKLK2* mRNA is about 10–50% that of *hKLK3* mRNA in human prostate tissue (12). We hypothesized that there might be different characteristics, such as the level of differentiation and the response to antiandrogen therapy, between hK2- and hK3-producing prostate cancer cells. We planned to investigate our hypothesis by immunological method using antibodies developed recently (2, 13), but they are not commercially available yet. We, therefore, developed a procedure for detection of prostate cancer cells expressing *hKLK2* and/or *hKLK3* mRNA in the peripheral blood of clinical D stage prostate cancer patients. Subsequently, we analyzed the characteristics of *hKLK2* and/or *hKLK3* mRNA-expressing prostate cancers.

Materials and Methods

Cell Line. A human prostate cancer cell line, LNCaP (14), was obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% FCS at 37°C in an atmosphere containing 5% CO_2. LNCaP is a cell line established from a prostate cancer patient and is known to synthesize hK2 (9).

Patients. Forty-one patients known to have prostate cancer were enrolled in this study after obtaining their informed consent. This study was approved by the human research committee of Shinshu University. Clinical staging was done according to the guidelines of the National Prostatic Cancer Project. They were clinically and/or surgically staged as follows: 7 stage A; 5 stage B; 8 stage C; and 21 stage D (4 stage D_1 and 17 stage D_2). We defined antiandrogen refractory patients as those who showed a significant increase in the size or number of metastatic lesions or a markedly elevated serum PSA level after antiandrogen therapy. Ten healthy female and 10 healthy male volunteers were also enrolled in this study as controls, as well as 7 patients with BPH diagnosed by biopsy or transurethral resection.

Preparation of Total Cellular RNA. Five ml of whole blood anticoagulated with EDTA was collected and layered on top of 5 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden) in a 15-ml polypropylene tube. The tube was centrifuged at 400 × *g* for 30 min at 20°C. The leukocyte layer was carefully transferred to a new tube, resuspended in 10 ml PBS, and then centrifuged at 400 × *g* for 10 min at 4°C. Total RNA was isolated from the pellet after it was solubilized in 1 ml of ISOGEN-LS (Nippon Gene, Tokyo, Japan), which contains 50% phenol, according to the manufacturer's instructions. The RNA pellet obtained was dried briefly under a vacuum and then dissolved in 10 μl of RNase-free H_2O. Total RNA was similarly isolated from LNCaP cells that were harvested near confluence. Serially diluted suspensions of LNCaP cells were mixed with 10^7 lymphocytes isolated from a normal control. Subsequently, a total RNA mixture of LNCaP and lymphocytes was prepared similarly. The concentration of total RNA was determined by spectrophotometry.

RT-PCR. An aliquot containing 1 μg of total RNA was mixed with 0.5 μg of oligo$(dT)_{15}$ primer (Promega Corp., Madison, WI) and made up to a final volume of 10 μl with RNase-free H_2O. The sample was incubated at 65°C for 10 min and then rapidly chilled on ice. The mixture was added to 10 μl of the master reaction solution to give the final concentrations of the following components: 200 μM each of deoxynucleotide triphosphates (Idaho Technology, Idaho Falls, ID), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 10 units per reaction of placental RNase inhibitor (Promega), and 100 units per reaction of Moloney murine leukemia virus reverse transcriptase

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[1] To whom requests for reprints should be addressed, at Department of Laboratory Medicine, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto 390, Japan. Phone: 81-263-37-2801; Fax: 81-263-34-5316.

[2] The abbreviations used are: PSA, prostate-specific antigen; RT-PCR, reverse transcription-PCR; BPH, benign prostatic hyperplasia.

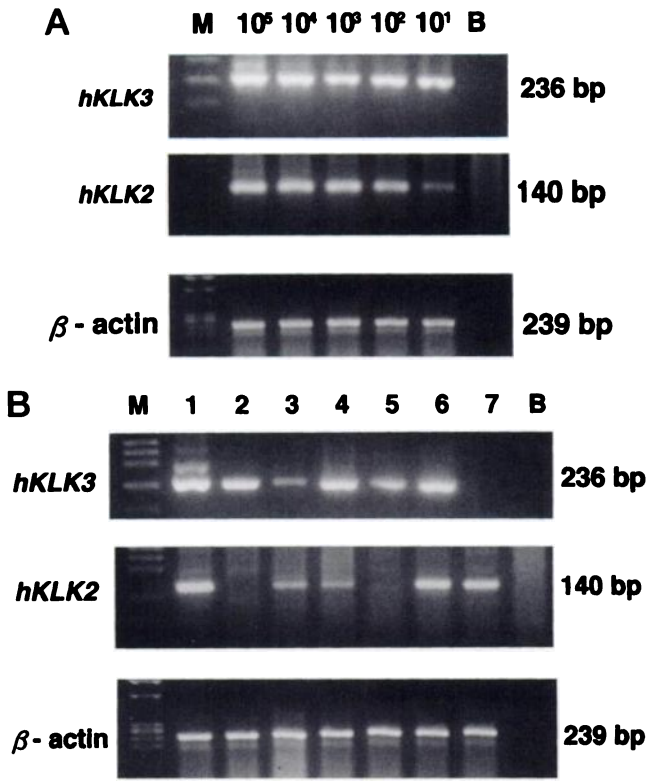

Fig. 1. *A*, sensitivity of the RT-PCR in detecting *hKLK2* mRNA isolated from LNCaP cells serially diluted and mixed with 10^7 lymphocytes. The 236-bp *hKLK3* fragment was only amplified from specimens containing LNCaP cells (*top*). The 140-bp *hKLK2* fragment was only amplified from specimens containing LNCaP cells (*middle*). The 239-bp fragment of β-actin mRNA was amplified by RT-PCR from all specimens (*bottom*). *Lane M*, size marker. *Lane B*, negative control without cDNA. *B*, ethidium bromide-stained gel showing the results of the RT-PCR for *hKLK2* and *hKLK3* from patients with prostate cancer. *Lanes 1, 3, 4, 6,* and *7* correspond to patients nos. 1, 3, 4, 6, and 7 (Table 3). *Lanes 2* and *5*, specimens from patients nos. 2 and 5 after initiation of antiandrogen therapy. *Lane M*, size marker. *Lane B*, negative control without cDNA.

(Life Technologies, Inc., Gaithersburg, MD). The reaction mixture was incubated at 42°C for 1 h.

One μl of the synthesized cDNA was added to 24 μl of reaction solution containing 200 μM each of deoxynucleotide triphosphates (Idaho Technology), 1 μM each of the primers OKA5 (forward, 5′-GGT GGC TGT GTA CAG TCA TGG AT-3′ corresponding to nucleotides 159–181) and OKA3 (reverse, 5′-ACA CAC TGA AGA CTC CTG GGG CG-3′ corresponding to nucleotides 541–563), and 1 unit of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). The PCR was performed on a Perkin-Elmer 9600 DNA thermal cycler under the following conditions: 1 cycle of denaturation at 98°C for 1 min, followed by 2 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Then 28 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min were followed by a final extension at 72°C for 5 min. Subsequently, 1 μl of the PCR product was further amplified under the same conditions except that the reverse primer OKA3 was replaced by the nested primer HKI3 (5′-TGT CTT CAG GCT CAA ACA GGT TG-3′ corresponding to nucleotides 276–298). Then 10–15 μl of the nested PCR product were electrophoresed on 3% agarose gel and stained with ethidium bromide. A 140-bp fragment of *hKLK2* cDNA was amplified and visualized under UV light. We detected *hKLK3* mRNA according to the method of Israeli *et al.* (3). As an internal control, a 239-bp fragment of β-actin mRNA was coamplified by RT-PCR using primers β5 (5′-TGA CTG ACT ACC TCA TGA AG-3′ corresponding to nucleotides 595–614) and β3 (5′-AAG GCT GGA AGA GTG CCT CA-3′ corresponding to nucleotides 815–834).

Nucleotide Sequencing. The amplified 140-bp fragment of *hKLK2* cDNA was first electrophoresed on 3% agarose gel and then recovered from the excised portion of the gel using GENCLEAN II KIT (BIO 101, Inc., Vista, CA) according to the manufacturer's instructions. The purified PCR product was directly sequenced on a ABI model 373A DNA sequencer (Applied Biosystems, Foster, CA) using an ABI prism Dye Terminator Cycle Sequence Ready Reaction kit.

Results and Discussion

Sensitivity and Specificity of the RT-PCR for *hKLK2* and *hKLK3*. The *hKLK2* gene is located about 12 kilobase pairs downstream from the *hKLK3* gene in a head-to-tail fashion on chromosome 19 (15), and both hK2 and hK3 are only produced in the human prostatic epithelium (6–9). We designed oligonucleotide primers specific for *hKLK2* mRNA after comparing its nucleotide sequence with that of *hKLK3*. The pair of primers did not yield amplified products of similar length from a coding region of *hKLK3* subcloned into the pCR3 vector (Invitrogen, San Diego, CA). We detected amplified DNA corresponding to the 140-bp fragment of *hKLK2* mRNA from as few as 10 LNCaP cells mixed in 10^7 normal lymphocytes (Fig. 1*A*). The nucleotide sequence of the 140-bp PCR product was identical to the corresponding region of the *hKLK2* mRNA sequence registered in GenBank (accession no. S39329). A fragment of β-actin mRNA was also amplified by RT-PCR from the same RNA to serve as an internal control. All of the RT-PCR reactions yielded detectable β-actin mRNA fragments of 239 bp, as expected (Fig. 1*A*). The RT-PCR for *hKLK3* mRNA had the same sensitivity as that for *hKLK2* mRNA, although the expression of *hKLK3* mRNA was influenced by various conditions such as the culture medium and the degree of cellular confluency.

We demonstrated that the RT-PCR was highly specific for detection of *hKLK2* or *hKLK3* mRNA by showing no amplification from peripheral blood leukocyte RNA samples of 20 normal volunteers and 7 BPH patients. A 239-bp fragment of β-actin mRNA was uniformly amplified from all of the samples, but *hKLK2* and *hKLK3* mRNA were not detected (data not shown). These data clearly indicate that *hKLK2* and *hKLK3* mRNA were only amplified from prostatic cells and not from leukocytes.

Detection of *hKLK2* mRNA and *hKLK3* mRNA in Patients with Prostate Cancer. Total RNA extracted from the peripheral blood leukocytes of 41 patients with prostate cancer was examined for *hKLK2* mRNA expression by the RT-PCR. Seven patients (17%) were positive for *hKLK2* mRNA (Table 1), and an additional seven were among the 17 patients in clinical stage D_2. On the other hand, 12 patients in clinical stages other than D (6 stage A, 2 stage B, and 4 stage C) were positive for *hKLK3* mRNA in the peripheral blood. They might have been understaged because prostate cancer cells were already circulating in their blood (Table 1). *hKLK3* mRNA was

Table 1 *Clinical stage and results of the hKLK2 and hKLK3 RT-PCR assay*

		No. of patients positive by RT-PCR	
Clinical stage	No. of patients	*hKLK2* (%)	*hKLK3* (%)
A	7	0 (0)	6 (86)
B	5	0 (0)	2 (40)
C	8	0 (0)	4 (50)
D_1	4	0 (0)	3 (75)
D_2	17	7 (41)	14 (82)
Total	41	7 (17)	29 (70)

Table 2 *Pathological diagnosis and results of the hKLK2 and hKLK3 RT-PCR assay*

		No. of patients positive by RT-PCR	
Differentiation	No. of stage D patients	*hKLK2*	*hKLK3*
Well	3	0 (0%)	3 (100%)
Moderately	13	5 (38%)	10 (77%)
Poorly	5	2 (40%)	4 (80%)
Total	21	7 (33%)	17 (81%)

Table 3 *Clinical profile of the patients with positive hKLK2 RT-PCR assays*

Patient no.	Clinical stage	Grade[a]	Serum PSA(ng/ml)	RT-PCR *hKLK2*	RT-PCR *hKLK3*	Therapy	Hormone responsiveness[b]
1	D_2	MD	180	+	+	Antiandrogen	ID
2	D_2	MD	61	+	+		
			<0.5[c]	−	+	Antiandrogen	D
3	D_2	PD	2.2	+	+	Antiandrogen	D
			85.8[d]	+	+		ID
4	D_2	PD	121	+	+	Radical prostatectomy + radiation + antiandrogen	ID
5	D_2	MD	94.4	+	+		
			<0.5[c]	−	+	Antiandrogen	D
6	D_2	MD	1216	+	+		
			<0.5[c]	−	+	Antiandrogen	D
7	D_2	MD	<0.5	+	−	Antiandrogen	ID

[a] Grade, pathological grade; MD, moderately differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma.
[b] D, responded to antiandrogen therapy; ID, refractory to antiandrogen therapy.
[c] These samples were collected after initiation of antiandrogen therapy.
[d] This sample was collected after 3 months of therapy.

detected in a total of 29 patients, and 6 (21%) of them were also positive for *hKLK2* mRNA (Table 3). This finding agreed with the previous report (16) that the expression of *hKLK3* mRNA was predominant over that of *hKLK2* mRNA in human prostate tissue. In addition, the levels of *hKLK2* and *hKLK3* mRNA expression were different in prostate cancer cells, although both hK2 and hK3 expression are controlled by androgens.

Pathological Diagnosis and Expression of *hKLK2* mRNA and *hKLK3* mRNA. The pathological diagnoses of the stage D patients were as follows: 3 had well-differentiated adenocarcinoma, 13 had moderately differentiated adenocarcinoma, and 5 had poorly differentiated adenocarcinoma (Table 2). Among them, 5 of the 13 patients with moderately differentiated adenocarcinoma (38%) and 2 of the 5 with poorly differentiated adenocarcinoma (40%) were positive for *hKLK2* mRNA expression in the peripheral blood. On the other hand, none of the stage D patients with well-differentiated adenocarcinoma were positive for *hKLK2* mRNA expression in the peripheral blood. Among the three patients with well-differentiated adenocarcinoma, one was in clinical stage D_1, and two were in stage D_2. All of the patients with well-differentiated adenocarcinoma, 77% of the patients with moderately differentiated adenocarcinoma, and 80% of the patients with poorly differentiated adenocarcinoma were positive for *hKLK3* mRNA expression (Table 2), although Tremblay *et al.* reported previously that grade I tumors (M. D. Anderson Hospital grading system) presented a rather uniform pattern of staining for both hK2 and hK3 (2). These results indicated that expression of *hKLK2* mRNA was closely associated with pathological differentiation of the cancer rather than with the clinical stage. Taken together, our findings show that *hKLK2* mRNA was only expressed in undifferentiated cancer cells of stage D_2 patients, suggesting that its detection was an indicator of a poor prognosis.

Clinical Course and Detection of *hKLK2* mRNA. Three patients (nos. 2, 5, and 6), who were positive for *hKLK2* mRNA expression in the peripheral blood before antiandrogen therapy, responded well to the therapy. After 3 months of antiandrogen therapy, *hKLK2* mRNA was no longer detected in their peripheral blood, and serum PSA levels fell into the reference range. In contrast, they were still positive for *hKLK3* mRNA, as they were before therapy (Table 3). Patient no. 3 was noteworthy because he was initially diagnosed as hormone responsive due to normal serum PSA levels, but PSA increased after 3 months of the antiandrogen therapy to reveal that he was actually hormone resistant. He was the only patient positive for *hKLK2* mRNA among the antiandrogen "responsive" patients. These findings suggested that responsiveness of prostate cancer to antiandrogen therapy and the expression of *hKLK2* mRNA by the cancer were reciprocally correlated. In contrast, *hKLK3* mRNA was more ubiquitously detected in patients with advanced prostate cancer regardless of their responsiveness to antiandrogen therapy. It may be useful to know the differences between *hKLK2* and *hKLK3* mRNA expression in prostate cancers when following up patients on antiandrogen therapy.

Patients nos. 1, 4, and 7 were positive for *hKLK2* mRNA in the peripheral blood and were refractory to antiandrogen therapy. Among them, patient no.7 was negative for *hKLK3* mRNA, and the serum PSA level was within the reference range (Table 3). Several studies have also indicated that 20–25% of prostate cancers express no or very low levels of PSA (17, 18). These lines of evidence clearly indicate that detection of *hKLK2* mRNA may be important for screening and follow-up of patients with prostate cancer that does not express PSA. Immunological methods to detect serum hK2 are expected to be developed, but our RT-PCR would be the method of choice for this purpose until antibodies become commercially available.

In conclusion, we clearly demonstrated that the expression of *hKLK2* mRNA was associated with the progression of prostate cancer in pathological differentiation or in refractoriness to antiandrogen therapy. Our RT-PCR for *hKLK2* may be useful to complement the measurement of PSA for screening, staging, and monitoring of patients as an indicator of advanced prostate cancer.

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References

1. Young, C. Y. F., Seay, T., Hogen, K., Charlesworth, M. C., Roche, P. C., Klee, G. G., and Tindall, D. J. Prostate-specific human kallikrein (hK2) as a novel marker for prostate cancer. Prostate, *31* (Suppl. 7)*:* 7–24, 1996.
2. Tremblay, R. R., Deperthes, D., Tetu, B., and Dube, J. Y. Immunohistochemical study suggesting a complementary role of kallikreins hK2 and hK3 (prostate-specific antigen) in the functional analysis of human prostate tumors. Am. J Pathol., *150:* 455–459, 1997.
3. Israeli, R. S., Miller, W. H., Su, J. S. L., Powell, C. T., Fair, W. R., Samadi, D. S., Huryk, R. F., DeBlasio, A., Edwards, E. T., Wise, G. J., and Heston, W. D. W. Sensitive nested reverse transcription polymerase chain reaction detection of circulating prostatic tumor cells: comparison of prostate-specific membrane antigen and prostate-specific antigen-bases assays. Cancer Res., *54:* 6306–6310, 1994.
4. Kawakami, M., and Nakayama, J. Enhanced expression of prostate-specific membrane antigen gene in prostate cancer as revealed by *in situ* hybridization. Cancer Res., *57:* 2321–2324, 1997.
5. Berb, T., Bradshaw, R. A., Carretero, O. A., Chao, J., Chao, L., Clemints, J. A., Fahnestoch, M., Fritz, H., Gauthier, F., and MacDonald, R. J. A common nomenclature for members of the tissue (glandular) kallikrein gene families. Recent progress of kinins. Agents Actions Suppl., *38* (Pt. 1)*:* 19–25, 1992.
6. Chapdelaine, P., Paradis, G., Tremblay, R. R., and Dube, J. Y. High level of expression in the prostate of a human glandular kallikrein mRNA related to prostate-specific antigen. FEBS Lett., *236:* 205–208, 1988.

7. Morris, B. J. hGK-1: a kallikrein gene expressed in human prostate. Clin. Exp. Pharmacol. Physiol., *16:* 345–351, 1989.
8. Young, C. Y. F., Montgomery, B. T., Andrews, P. E., Qiu, S., Bilhartz, D. L., and Tindall, D. J. Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. Cancer Res., *51:* 3748–3752, 1991.
9. Young, C. Y. F., Andrews, P. E., Montgomery, B. T., and Tindall, D. J. Tissue-specific and hormonal regulation of human prostate-specific glandular kallikrein. Biochemistry, *31:* 818–824, 1992.
10. Schedlich, L. J., Bennetts, B. H., and Morris, B. J. Primary structure of a human glandular kallikrein gene. DNA, *6:* 429–437, 1987.
11. Watt, K. W. K., Lee, P. J., M'Timkulu, T., Chan, W. P., and Loor, R. Human prostate-specific antigen: structural and functional similarity with serine proteases. Proc. Natl. Acad. Sci. USA, *83:* 3166–3170, 1986.
12. McCormack, R. T., Rittenhouse, H. G., Finlay, J. A., Sokoloff, R. L., Wang, T. J., Wolfert, R. L., Lilja, H., and Oesterling, J. E. Molecular forms of prostate-specific antigen and the human kallikrein gene family: a new era. Urology, *45:* 729–744, 1995.
13. Kumar, A. K., Goel, A. S., Hill, T. M., Mikolajczyk, S. D., Millar, L. S., Kuus-Reichel, K., and Saedi, M. S. Expression of human glandular kallikrein, hK2, in mammalian cells. Cancer Res., *56:* 5397–5402, 1996.
14. Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, T. M., Mirand, E. A., and Murphy, G. P. LNCaP model of human prostatic carcinoma. Cancer Res., *43:* 1809–1818, 1983.
15. Riegman, P. H., Vlietstra, R. J., Klaasen, P., van der Korput, J. A., Geurts van Kessel, A., Romijn, J. C., and Trapman, J. The prostate-specific antigen gene and human glandular kallikrein-1 gene are tandemly located on chromosome 19. FEBS Lett., *247:* 123–126, 1989.
16. Henttu, P., Lukkarinen, O., and Vihko, P. Expression of the gene coding for human prostate-specific antigen and related hGK-1 in benign and malignant tumors of the human prostate. Int. J. Cancer, *45:* 654–660, 1990.
17. Allhoff, B. P., Proppe, K. H., Chapman, C. M., Lin, C. W., and Prout, G. R., Jr. Evaluation of prostate-specific acid phosphatase and prostate specific antigen in identification of prostatic cancer. J. Urol., *129:* 315–318, 1983.
18. Stain, B. S., Vangore, S., and Petersen, R. O. Immunoperoxidase localization of prostatic antigens: comparison of primary and metastatic sites. Urology, *24:* 146–152, 1984.

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