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Circulating prostate-specific antigen mRNA during radical prostatectomy in patients with localized prostate cancer: with special reference to neoadjuvant hormonal therapy

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Abstract To determine the potential risk of hematogenous dissemination of prostate cancer cells during radical prostatectomy (RP), we investigated the pre- and intraoperative circulating prostate-specific antigen (PSA) mRNA in patients with clinically localized prostate cancer, with special reference to neoadjuvant hormonal therapy (NHT). Using a nested reverse transcriptase (RT) polymerase reaction (PCR) assay, PSA mRNA in the peripheral blood was evaluated pre- and postoperatively in a total of 23 patients, 10 of whom received NHT with antiandrogens. The RT-PCR assay employed detected one LNCaP cell in 10^7 mononuclear blood cells, and showed no positive signal in the blood samples from all 15 healthy controls. Pre- and intraoperative circulating PSA mRNA was positive in 11 (48%) and 18 patients (78%), respectively. All 11 patients with positive preoperative PSA mRNA continued to be positive during RP, and seven (58%) of 12 patients with negative preoperative PSA mRNA had a positive conversion. Although the patients' ages, preoperative serum PSA values and clinical or pathological stages were not associated with the pre- and intraoperative PSA mRNA results, the NHT group showed a significantly lower incidence of preoperative PSA mRNA positivity (2/10) than the group receiving RP alone (9/13) (20% vs 69%, $P = 0.036$). NHT, however, showed no suppressive effect on either intraoperative positivity or positive conversion of circulating PSA mRNA. The present study suggests that a substantial number of patients receiving RP are at risk of hematogenous dissemination, and NHT with antiandrogens has a minimal or no suppressive effect on the circulating PSA mRNA during surgical manipulation of the prostate. Because the clinical significance of circulating cancer cells remains to be determined, long-term follow-up in association with the circulating cancer cells assessed by the RT-PCR is essential in order to establish the role of molecular staging as well as NHT.

Key words Prostate cancer · Circulating PSAmRNA · RT-PCR · Radical prostatectomy · Hematogenous dissemination · Neoadjuvant hormonal therapy

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Introduction

With the widespread use of prostate-specific antigen (PSA) tests for the detection of prostate cancer, the incidence of clinically localized prostate cancer has markedly increased [7]. Although recent reports have indicated that radical prostatectomy (RP) is highly effective in eradicating the cancer [25], it has been shown that clinically localized prostate cancers include a substantial fraction of advanced cancers which are incurable even by radical treatment [32]. In support of this concern, the non-progression rate monitored by the serum PSA was reported to be around 50% at 10 years following RP [34]. The major reason for local progression or distant metastasis after RP is inaccuracy in preoperative staging modalities; serum PSA assays, computed tomography scans, transurethral ultrasonography, or endorectal coil magnetic resonance imaging can detect the organ-confined disease with only 40–50% accuracy before RP [17, 19]. Another cause might be the dissemination of tumor cells during RP. Some patients with pathologically organ-confined prostate cancer show distant metastasis after curative operations [22], which could be explained by the spillage of cancer cells into the systemic blood circulation and/or the surgical field during RP [13].

Reverse transcriptase polymerase reaction (RT-PCR) is a highly sensitive method for detecting a small amount of messenger ribonucleic acid (mRNA). Moreno et al. [18] first reported that circulating prostate cancer cells were detected by using RT-PCR targeting PSA mRNA.

Subsequently, others have confirmed that circulating prostate-specific messages such as PSA and prostate-specific membrane antigen (PSMA) can be detected by RT-PCR assays in patients with prostate cancer even if the tumor is confined to the prostate [7, 9, 12, 14, 26, 28, 30]. These reports suggested that the RT-PCR assay can verify the mechanism of hematogenous dissemination more clearly. Recently, two research groups have shown a high positive rate of circulating prostate-specific messages during or immediately after RP, indicating a possibility of hematogenous dissemination of cancer cells during RP [8, 20].

With the availability of a reversible form of hormonal therapy such as LH-RH agonists or antiandrogens, neoadjuvant hormonal therapy (NHT) has been advocated to improve the curability of RP. Although several randomized trials showed a significantly lower rate of positive surgical margins, normalization of PSA levels, and a decrease in prostate size after NHT [2, 15, 21], there is still controversy regarding whether NHT prolongs the disease-free survival [1]. In the present study we screened the pre- and intraoperative peripheral blood specimens of patients with prostate cancer for the presence of PSA mRNA, and determined the relationship between the positive circulating PSA mRNA and NHT to appreciate the possible benefit of NHT for the prevention of hematogenous dissemination of cancer cells during RP.

Materials and methods

Patient selection and pathological analysis

Between 1996 and 1997, a total of 23 patients with prostate cancer who underwent RP at our institute and affiliated hospitals were entered into the study. All patients were found to have no metastatic lesions by imaging modalities including a pelvic CT scan and a radionuclide bone scan prior to RP. The patients were categorized into two groups. One group comprised 10 patients who received NHT with antiandrogens for 3–6 months before RP. The other consisted of 13 patients who underwent RP without any preoperative treatment. The decision to initiate NHT was made at the physician's discretion. Serum PSA concentration measured by a Tandem PSA assay (Hybriteck, San Diego, Calif.) at the time of diagnosis was under 40 ng/ml in all cases. The clinicopathological stage was evaluated according to the TNM classification of the International Union Against Cancer [3]. Prostatectomy specimens were processed by standard histological methods with systematic step-sections at 3–5 mm intervals. All specimens were examined at the authors' institute, being characterized as having organ-confined status and lymph node or seminal vesicle involvement. The Gleason scoring system was used for tumor grading.

Sample collection and blood preparation

At each blood sampling, 5 ml of peripheral venous blood was collected in heparinized tubes. Preoperative specimens were collected on the day of operation, at least 2 weeks after prostate needle biopsy. In addition, blood specimens were obtained at two to four time points during RP as intraoperative samples. The whole blood was immediately subjected to gradient isolation of nucleated cells using Ficoll. The nucleated cells were washed in 1 × phosphate-buffered saline and stored at −80°C until RNA extraction.

RNA extraction and PSA RT-PCR assay

RNA extraction was performed according to the single-step guanidium thiocyanate method [5]. To improve sensitivity and specificity, a nested RT-PCR was performed on the cDNA made by Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO BRL, Life Technologies, Rockville, Md.) in accordance with the manufacturer's instructions. The PCR reaction was performed in a Perkin Elmer 2400 thermocycler (Perkin Elmer, Norwalk, Conn.). To exclude false-positive signals from contaminated genomic DNA, each set of RT-PCR primers was designed to span introns [12]. The primers used were: P1(on exon 2): 5′-TAC CCA CTG CAT CAG GAA CA-3′, P2 (on exon 5): 5′-CCT TGA AGC ACA CCA TTA CA-3′, P3 (on exon 3): 5′-ACA CAG GCC AGG TAT TTC AG-3′, P4 (on exon 4): 5′-GTC CAG CGT CCA GCA CAC AG-3′. Twenty cycles of the first round of PCR with P1 and P2 primers were carried out with 20 μl of PCR mix as reported previously [12]; each cycle consisted of a denaturation step at 95°C for 1 min, primer annealing at 60°C for 1 min and primer extension at 72°C for 1 min. The second round of PCR was carried out on 1 μl of the first round PCR product as a template, and the PCR conditions were the same as for the first round of PCR apart from 30 cycles of amplification and P3/P4 primers. The size of the final PCR product was 355 bp. Total RNA integrity was tested by control RT-PCR reactions, in which all samples were positive with β2-microglobulin primers [12]. In each experiment, a control sample without a template was prepared to exclude contamination.

Determination of PSA signal

Southern analysis was employed to detect the PSA-specific signal. The nested PCR products were separated by gel electrophoresis using 2% agarose and then transferred to a nylon membrane. The oligonucleotide probe (5′-CAC AGC TCC CCA CAC CCG CTC TAC GAT ATG-3′) designed to hybridize exon 3 of the PSA gene was end-labeled by a radioisotope, and hybridization was carried out in high stringent conditions. The PSA-specific signal was determined by autoradiography with 8 h exposure at room temperature.

Statistical analysis

The associations between PSA mRNA and the clinicopathological parameters were assessed using Student's *t*-test or Fisher's exact test, and a P value of less than 0.05 was considered significant.

Results

Sensitivity of the RT-PCR assay

We evaluated the sensitivity of the RT-PCR assay by a dilution experiment with a PSA-producing cell line (LNCaP) [11]. The RT-PCR assay employed showed no false positive results on any of the 15 healthy controls, including six women (data not shown); thus the detection limit of this assay was established by adding LNCaP cells to a blood sample from a healthy woman. This dilution experiment confirmed that the assay was able to detect one LNCaP cell in 10^7 mononuclear blood cells (Fig. 1). This means that a single LNCaP cell in 5 ml of peripheral blood resulted in a positive RT-PCR signal.

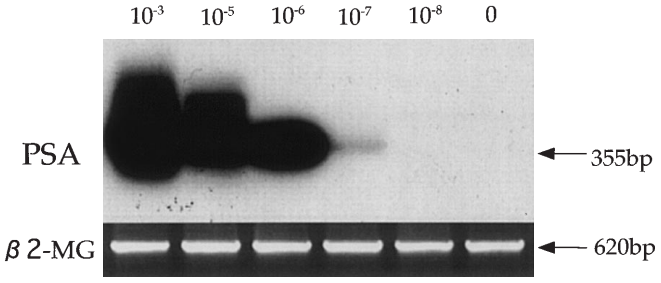

Fig. 1 Reverse transcriptase (RT) polymerase chain reaction (PCR) assay for prostate-specific antigen (PSA). The number by each lane indicate the ratio of LNCaP cells to mononuclear blood cells. A 355 bp PSA-specific signal is detected in lanes 10^{-3} to 10^{-7}, indicating that the assay employed is able to detect one LNCap cell in 10^7 mononuclear blood cells. β2-*MG* control RT-PCR assay with β2-microglobulin primers showing 620 bp signal

Table 1 Clinicopathological characteristics of patients with prostate cancer and the comparison between two groups with or without neoadjuvant hormonal therapy

	Total	Neoadjuvant therapy	
		Yes	No
No. of patients	23	10	13
Mean age (years)	67.0	68.0	66.3
PSA (ng/ml)[a]	15.6 ± 2.4	17.8 ± 4.2 (8.2 ± 3.5)[b]	14.0 ± 2.9
T stage (no.)			
T1	1	0	1
T2	17	6	11
T3	5	4	1
pT stage (no.)			
pT2	12	5	7
pT3	11	5	6
Seminal vesicle (no.)			
+	5	4	1
–	18	6	12
Lymph node (no.)			
+	2	1	1
–	21	9	12
Organ-confined (no.)			
Yes	11	5	6
No	12	5	7
Mean Gleason sum	6.6	6.8	6.5

[a] The serum level of PSA in patients with or without neoadjuvant therapy was determined before neoadjuvant therapies or radical prostatectomy, respectively. Value indicates mean ± SE
[b] PSA value after neoadjuvant therapy

Clinicopathological characteristics of the patients

The clinicopathological characteristics of the 23 patients are summarized in Table 1. Eleven patients (48%) had an organ-confined cancer by pathological examination of the surgical specimens. Ten patients underwent NHT with antiandrogens; a steroidal antiandrogen (chlormadinone acetate) was used in seven patients and flutamide in three. The pretreatment serum level of PSA (mean value 17.8 ng/ml) before NHT in these patients was not significantly different from that (mean value 14.0 ng/ml) in patients undergoing RP alone. There was no significant difference in any of the clinicopathological parameters between the NHT and RP-alone groups.

Preoperative PSA mRNA in peripheral blood

Preoperative PSA mRNA was positive in the peripheral venous blood samples from 11 patients (48%). The relationship between the clinicopathological parameters and the preoperative PSA mRNA status is shown in Table 2. The patient's age, preoperative serum PSA value and clinical or pathological stage were not significantly associated with the positive PSA mRNA results. However, the NHT group showed a significantly lower incidence of PSA mRNA positivity (2/10) than the RP-alone group (9/13) (20% vs 69%, $P = 0.036$).

Positive conversion of PSA mRNA during RP

When at least one blood sample obtained at two to four time points during RP in each patient showed a positive signal for PSA mRNA, the intraoperative PSA mRNA was determined to be positive. In almost all cases with intraoperative positive PSA mRNA, multiple intraoperative samples showed positive signals, thus confirming the reliability of the results. Figure 2 shows representa-

Table 2 Relationship between clinicopathological characteristics and PSA mRNA in the peripheral blood of patients with prostate cancer

	Preoperative PSA mRNA		Intraoperative positive conversion[a]	
	Positive	Negative	Yes	No
No. of patients	11	12	7	5
Mean age (years)	68.0	66.2	66.5	64.8
PSA (ng/ml)	14.2	17.0	13.8	21.5
T stage (no.)				
T1	0	1	1	0
T2	10	7	4	3
T3	1	4	2	2
pT stage (no.)				
pT2	7	5	3	2
pT3	4	7	4	3
Seminal vesicle (no.)				
+	2	3	3	0
–	9	9	4	5
Lymph node (no.)				
+	1	1	1	0
–	10	11	6	5
Organ-confined (no.)				
Yes	6	5	3	2
No	5	7	4	3
Mean Gleason sum	6.2	7.0	6.9	7.3
Neoadjuvant therapy (no.)[b]				
Yes	2	8	5	3
No	9	4	2	2

[a] Intraoperative positive conversion was analyzed for 12 patients with negative preoperative PSA mRNA
[b] Statistically significant in preoperative PSA mRNA status, yes versus no; $P = 0.036$

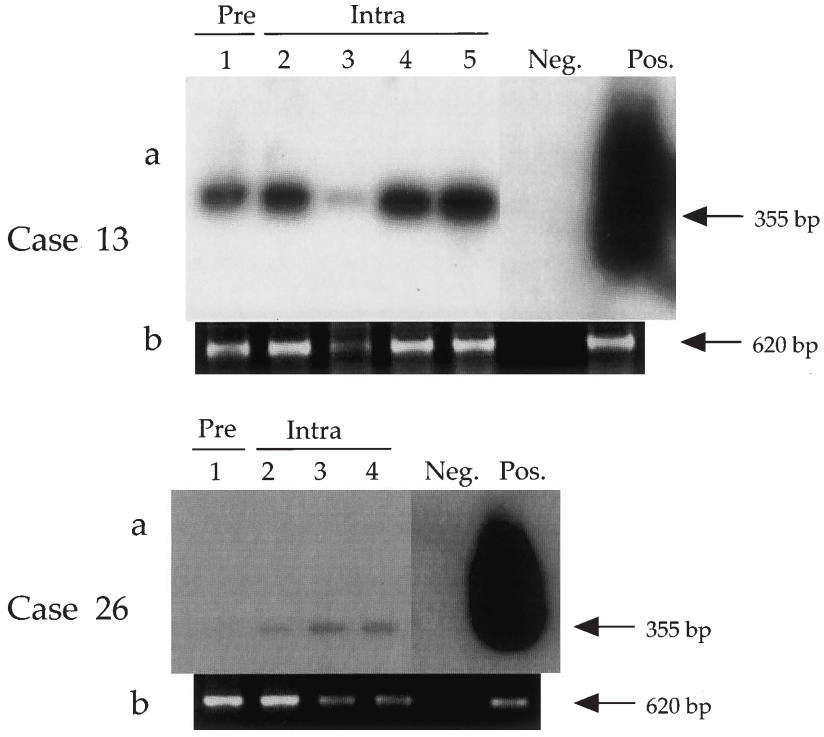

Fig. 2 Representative reverse transcriptase (RT) polymerase chain reaction (PCR) assay of prostate-specific antigen (PSA) in cases 13 and 26. Lane 1 and lanes 2 to 5 show the preoperative (*Pre*) and intraoperative (*Intra*) PSA mRNA signals, respectively. **a** The 355 bp PSA-specific signal is detected in all blood samples (lanes 1 to 5) from case 13. In contrast, intraoperative blood samples (lanes 2 to 4) from case 26 show a positive PSA signal despite a negative preoperative signal (lane 1). **b** Control RT-PCR assay with β2-microglobulin primers shows a 620 bp signal, confirming the total RNA integrity. *Neg* negative control without template, *Pos* positive control using mRNA obtained from LNCaP cell lines

tive autoradiographs demonstrating a PSA mRNA signal in the pre- and intraoperative blood samples from two patients. We found positive PSA mRNA in the peripheral blood from 18 patients (78%) during RP. All 11 patients with positive preoperative PSA mRNA showed positive PSA mRNA during RP (Table 3). Seven (58%) of 12 patients with negative preoperative PSA mRNA showed a positive conversion of intraoperative PSA mRNA; that is, five (63%) of eight patients with NHT and two (50%) of four patients without NHT had a positive conversion, the frequency of intraoperative PSA mRNA positivity between the two groups being insignificant (Table 2). There was no significant association among the clinicopathological parameters, positive intraoperative PSA mRNA (data not shown) and positive conversion during RP.

Table 3 PSA mRNA during radical prostatectomy

Preoperative PSA mRNA	Intraoperative PSA mRNA	
	Negative	Positive
Negative (n = 12)	5 (42%)	7 (58%)
Positive (n = 11)	0	11 (100%)
Total 23	5 (22%)	18 (78%)

Discussion

In the present study a highly sensitive nested RT-PCR assay detected PSA mRNA in the preoperative peripheral blood in 11 (48%) of 23 patients with clinically localized prostate cancer. Several studies have been done to determine the efficacy of PCR-based technology in the "molecular staging of prostate cancer. The detection rates among the individual studies were widely disparate, probably due to the nature of PCR-based assays affecting sensitivity and specificity. Overall detection rates in the previous reports were approximately 30% for patients with pT1/pT2 and 50% for those with pT3 cancers, respectively [24]. These figures are similar to those observed in the present study.

As to the efficacy of molecular staging, we failed to find any significant association between the pre- or intraoperative positivity of circulating PSA mRNA and the pathological findings such as pT stage, seminal vesicle or lymph node involvement and organ-confined status. Some investigators have mentioned no attributable role of circulating PSA mRNA in predicting the pathological stage of localized prostate cancer [6, 28, 30]. Sokoloff et al. [28] noted that circulating PSA mRNA was detected in 42 (62%) of 68 patients with pT2 and pT3 stage diseases, but they found no significant relationship between the preoperative positivity of circulating PSA mRNA and the pathological parameters. On the contrary, investigators at Columbia University noted that the staging accuracy of PSA mRNA was greater than that of other modalities such as a digital rectal examination, transrectal ultrasonography or CT scan [14]. They also demonstrated that the circulating PSA mRNA signal was an independent factor in predicting extracapsular extension of the disease superior to any other established prognostic factors such as serum PSA, Gleason score or clinical stage [7]. Additionally, they

showed that the circulating PSA mRNA was a significant predictor of a PSA recurrence following RP [23]. Although the results from Columbia University are not consistent with ours, there is a possible reason for the discrepancy. The sensitivity of the assay used by the Columbia group was reported to detect one LNCaP cell in 10^5 mononuclear cells, which was lower than ours by 10^2. In fact, the positive rate (27%) of pretreatment circulating PSA mRNA in their recent report [7] on clinically localized prostate cancers is substantially lower than ours (48%). This may indicate that the assay employed in our study was too sensitive to differentiate a significant level of circulating cancer cells from a nonsignificant one. Our assay did not show any positive signal in the samples from any of the 15 controls, indicating a reasonable specificity. Some investigators, however, have claimed a lower specificity of the RT-PCR assay for circulating PSA mRNA as the sensitivity increases [10, 27]. An appropriate sensitivity of the RT-PCR assay will need to be established before the clinical implication of the results obtained can be assessed.

We found a high positive rate (78%) of intraoperative circulating PSA mRNA during RP, which is consistent with found by Eschwege et al. [8]. They first detected a PSMA mRNA-specific signal in 12 of 14 patients (86%) immediately after RP, including nine patients (81%, 9/11) showing positive PSMA mRNA conversion after RP. More recently, Oefelein et al. [20] reported a 45% (10/22) intraoperative positive rate of circulating PSA mRNA with 25% (4/16) negative-to-positive conversion during RP. These findings and ours suggest that a substantial number of patients receiving RP are at risk of hematogenous dissemination during surgical manipulation of the prostate.

We found a significantly lower rate of positive preoperative circulating PSA mRNA in the NHT group compared with the RP-alone group, despite there being no significant difference in the clinicopathological parameters between the two groups. Although we had no data on the pretreatment circulating PSA mRNA in the patients with NHT, hormonal therapies could be responsible for either a decrease in PSA mRNA transcripts in individual cancer cells or a decrease in the number of circulating cancer cells, or both [33]. A potential benefit of NHT is the downstaging of primary tumors [2, 15], and another might be the prevention of hematogenous dissemination during surgery. It is, however, intriguing that the positive rates of intraoperative circulating PSA mRNA in our study were similar between the groups with and without NHT. In addition, we failed to find any suppressive effect of NHT on the negative-to-positive conversion of PSA mRNA during RP. Although these observations may indicate that NHT is ineffective in preventing metastasis due to intraoperative hematogenous dissemination, there are two alternative possibilities. First, the regimen of NHT with antiandrogen monotherapy in our study might not be sufficient to suppress intraoperative hematogenous dissemination. Indeed, the average reduction rate in serum PSA by NHT was 46% (Table 1), which did not reach the levels (80–98%) achieved by other studies using an LH-RH agonist or estramustine phosphate [29, 31]. Thus, NHT with a total androgen blockade may have decreased the positive rate of circulating PSA mRNA during RP. Secondly, it is possible that the androgen-suppressed milieu created by NHT will exert an inhibitory effect on implantation of cancer cells in the distant organs even if the number of circulating cancer cells increases during RP. This possibility could be investigated by comparing the intraoperative PSA mRNA status with the outcomes of patients.

In summary, our results suggest that a substantial number of patients receiving RP are at risk of hematogenous dissemination caused by surgical manipulation of the prostate. In addition, we failed to find any suppressive effect of NHT with antiandrogens on the circulating PSA mRNA during RP. However, the clinical significance of circulating cancer cells remains to be determined, because the presence of cancer cells in the circulation is not a definitive step in the complex cascade of metastasis [16]. A study of long-term results following RP and their association with the circulating cancer cells detected by the RT-PCR is mandatory. Moreover, a randomized control study is required in order to establish the usefulness of molecular staging and the role of NHT.

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