# Detection of circulating cells in prostatic cancer by reverse transcriptase-polymerase chain reaction of prostate specific antigen and prostate specific membrane antigen

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**KEY WORDS**

prostate cancer ◗ reverse transcriptase polymerase chain reaction ◗ prostate specific membrane antigen ◗ prostate specific antigen

## INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer mortality in men [1]. Under-staging of clinically localized PCa is common, and in some cases reaches 50% due to the inadequacy of current staging modalities in terms of accurate determination of the local extent of this disease [2, 3, 4]. An important proportion of these patients are recurring within the first decade after prostatectomy [5]. These recurrences are attributed to presence, even pre-operatively, of locally invasive, circulating, or distant micrometastatic tumor cells, which are not detectable by conventional staging modalities [6, 7]. Thus, new defining reliable staging modalities are required for accurate staging of PCa to adapt appropriate treatment. It has been known that haematogenous dissemination of tumor cells occurs in cancer as well as in vivo models [8]. Reverse transcriptase polymerase chain reaction (RT-PCR) is a sensitive and useful method for detecting the presence of a specific cell type such as those cells that tumor-specific mRNA. RT-PCR PSA and PSMA have been shown to be both tumor and prostate specific [9]. The aim of this study is to compare clinical stages of the prostate cancer patients with PSA and PSMA results of cells in serum of these patients achieved by RT-PCR method and to search to what degree this can predict distant metastasis and lymph node invasion.

## MATERIALS AND METHODS

Written informed consents were obtained from all subjects. The study included 291 patients with biopsy-proven PCa who were treated and followed up between January 2002 and May 2008. Control patients were included, 60 healthy males, 20 healthy females, and 20 Benign Prostate Hyperplasia patients without prior history of malignancy nor any urologic disease. Bone scan and abdominopelvic computed tomography (CT) scans were obtained for patients with the serum PSA levels were higher than 10 ng/ml and 20 ng/ml, respectively. All patients who had localized prostate cancer underwent standardized radical retropubic prostatectomy. Pelvic lymphadenectomy was left out in those patients with low-risk characteristics (PSA <10 ng/ml, biopsy Gleason score ≤6 and clinical stage T1c/T2a). No patient was treated with either neoadjuvant hormonal or radiation therapy before radical prostatectomy. Because of the patient choice, radiotherapy was performed in 8 patients. Orchiectomy, hormonotherapy and transurethral resection of prostate (TUR-P) were performed in advanced prostate carcinomas.

From each patient and control, 10 cc of whole blood samples were collected in four tubes with EDTA and transported for RNA isolation to the laboratory within 2 h. We used 3 µg of total RNA for the synthesis of the first strand of cDNA with Superscript II reverse transcriptase (GIBCO-BRL, Life Technologies), according to the manufacturer's instructions. Briefly, RNA and random hexamers (GIBCO-BRL, Life Technologies) or downstream outer PSMA primer (10 pmol/µL) was first denatured for 5 min at 65°C, chilled on ice for 1 min, and then incubated for 1 h at 42°C in 20 µL of a reaction mixture containing 4 µL 5 x first strand buffer (0.25 mol/L Tris-HCl, pH 8.3, 0.375 mol/L KCl, 15 mmol/L $MgCl_2$), 250 mmol/L dNTP mix (Boehringer Mannheim, Germany), 10 mmol/L dithiothreitol and 200 U of superscript II reverse transcriptase.

For the amplification of PSA and PSMA, primer two base pairs were used. The outer upstream primer PSM1368: 5′CAG ATA TGT CAT TCT GGG AGG TC 3′ and the downstream primer PSM2015: 5′ AAC ACC ATC CCT CCT CGA ACC 3′, yielding a 647-bp PCR product, and the PSMA inner upstream primer PSM1689: 5′ CCT AAC AAA AGA GCT GAA AAG CCC 3′ and the downstream primer PSM1923: 5′ ACT GTG ATA CAG TGG ATA GCC GCT 3′, yielding a 234-bp product for the nested PCR. The outer upstream primer PS494: 5′TAC CCA CTG CAT CAG GAA CA 3′ and the downstream primer PS960: 5′ CCT TGA AGC ACA CCA TTA CA 3′, yielding a 486-bp PCR product, and the PS inner upstream primer PS559: 5′ ACA CAG GCC AGG TAT TTC AG 3′ and the downstream primer PS894: 5′ GTC CAG CGT CCA GCA CAC AG 3′, yielding a 355-bp product for the nested PCR. A total of 2.5 µL of the cDNA served as the starting material for the outer primer PCR reaction. The 50 µL of PCR mix consisted of 5 µL 10xPCR buffer (200 mmol/L Tris-HCl, pH 8.4, and 500 mmol/L KCl, pH 8.8), 1 µM of each primer, 250 mmol/L dNTPs, 10 mmol/L b-mercaptoethanol, 2 mmol/L MgCl2, and 1 U of Taq DNA polymerase (Promega). PCR was carried out the following program: 94°C for 1 min (3 min for the first cycle); 58°C for 1 min; and 72°C for 1 min (10 min for the last cycle) for 40 cycles. Then, 2.5 µL of the first PCR reaction products were used as the template for the nested PCR reaction. For internal control of RNA extraction, primer series with $\beta_2$microglobulin was utilized. These primer series were selected from exone 2 to exone 4 the obtained PCR product was in the length of 620 bp. The negative PCR patient with $\beta_2$microglobulin was accepted as not informative and taken into study. These primer series are; $\beta_2$ (Ekson2) 5′- AGCAGA GAA TGG AAA

**Table 1.** RT-PCR PSA and PSMA results in lymph nodes and distant metastases – sensitivity and specificity.

| | RT-PCR PSA | RT-PCR PSMA | RT-PCR PSA Lymph node metastasis | RT-PCR PSA Distant metastasis | RT-PCR PSMA Lymph node metastasis | RT-PCR PSMA Distant metastasis |
|---|---|---|---|---|---|---|
| Sensitivity | 52% | 59% | 67% | 78% | 76% | 88% |
| Specificity | 100% | 100% | 87% | 84% | 63% | 67% |
| Positive predicted value | 100% | 100% | 85% | 81% | 72% | 68% |
| Negative predicted value | 40% | 51% | 64% | 83% | 67% | 86% |

GTC AAA -3', $\beta_2$ (Ekson4) 5'- TGT TGA TGT TGG ATA AGA GAA -3'. In the last stage, PCR products were painted with etidium bromide and were examined under ultraviolet (UV) by the application of 2% agarose gel (Figure 1, Figure 2). The results obtained by the use of RT-PCR were stated as positive and negative.

## STATISTICAL ANALYSIS

The patients in different clinical stages of PCa and Gleason scores were compared with Mann-Whitney U test. RT PCR PSA and PSMA values in different clinical stages of PCa were compared with student *t* test. The correlation between the RT PCR PSA and PSMA values and lymph node positivity and distant metastasis were evaluated with Pearson correlation test. P values lower than 0.05 were considered as statistically significantly different.

## RESULTS

The clinical stages of 291 prostate cancer patients were varying between T1cN0M0 and T4N1M1. The mean age and the mean PSA level were 71.8±4.6 (range 63-81) years and 81.5±391.43 (range 2.21-2248 ng/mL, median 19.26) ng/mL, respectively. According to radiological and clinical diagnosis, we have found that 18 patients (6.1%), 87 patients (29.8%), 71 patients (24.3%) and 115 patients (39.5%) have been detected in stage 1 (T1N0M0), 2 (T2Nx-N0M0), 3 (T3Nx-N1Mx-M0) and stage 4 (T4N1M1), respectively. Bone metastases were detected in 126 (43.2%) patients with respect to bone scan evaluation. In accordance with the applied radiographic examination, pathologic lymph node was observed in 151 (51.8%) patients, while distant organ metastasis was found in 27 (9.2%) patients. Radical prostatectomy was applied to 18 patients in stage 1. Radical prostatectomy and radical radiotherapy were applied to 79 and 8 patients of 87 patients in stage 2, respectively. Hormonotheraphy + radiotherapy was applied to 35 patients of 71 which are in stage 3 and the remaining 36 patients underwent TUR-P+hormonotheraphy. 51 of 115 patients in stage 4 received hormonotheraphy, 39 of them underwent bilateral orchiectomy and remaining 25 patients underwent TUR-P+hormonotheraphy. When serum samples of patients were examined with RT-PCR, RT-PCR PSA was positive in a total of 126 patients (43.2%) and RT-PCR PSMA antigen was positive in 165 patients (56.7%). Both RT-PCR PSA and PSMA antigen were positive in 82 (28.1%) of the all patients. When RT-PCR PSA results were examined with respect to properties of the patients, no statistical difference has been found from the point of view of positive RT-PCR PSA when PSA value was taken as 0-10 ng/ml and higher than 10 ng/ml and f/t PSA ratio was taken less and higher than 15% (p = 0.17, p = 0.18). Positive RT-PCR PSA was statistically significant when Gleason score was less and higher than 7 and equal to 7 (p = 0.045). In a similar way, a significant difference was obtained as far as positive lymph node metastases (p = 0.0015) and distant metastases (p = 0.0036) were concerned. When RT-PCR PSMA results were examined with respect to the properties of patient, no statistical difference were found from the point of view of positivity of RT-PCR PSMA when PSA value was taken as 0-10 ng/ml and higher than 10 ng/ml and f/t PSA ratio was taken less and higher than 15% (p = 0.063, p = 0.06). For our patients, positivity of RT-PCR for PSMA was significant statistically when Gleason score was less and higher than 7 and equal to 7 (p = 0.022). A significant difference was found when lymph node positivity (p = 0.021) and RT-PCR PSMA positivity with regard to distant metastasis were examined (p = 0.0017). RT-PCR clinical stages were compared for RT-PCR PSA and PSMA positivity, statistically significant differences were observed, respectively (p = 0.0016, p = 0.0038). In this study, PSA and PSMA which were applied to the whole control patients with RT-PSA method were negative. The sensitivities and specificities for lymph node and distant metastasis in the presence of RT-PCR PSA and PSMA are given in Table 1.

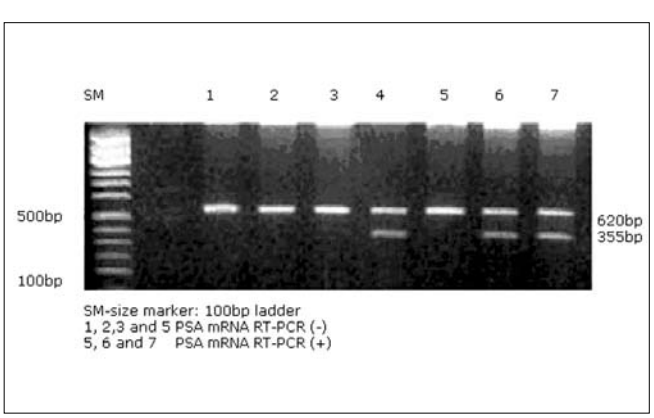


**Fig. 1.** PSA mRNA RT-PCR agarose-gel pictures results painted with etidium bromide.

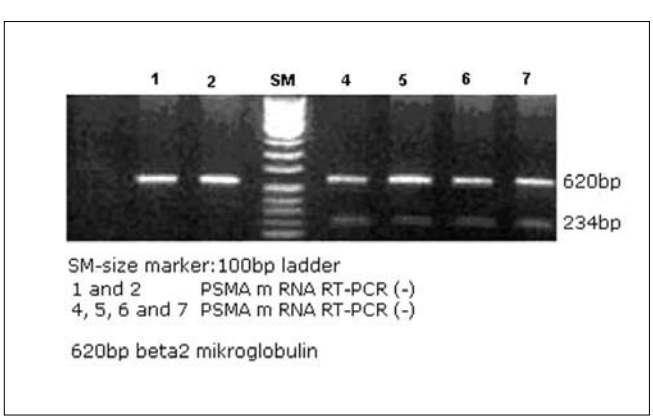


**Fig. 2.** PSMA mRNA RT-PCR agarose-gel pictures results painted with etidium bromide.

## DISCUSSION

Precise preoperative clinical staging of prostate cancer remains difficult and new prognostic factors are clearly needed [2]. These factors should define aggressive and metastatic potential of early prostate cancer and ideally predict the clinical outcome [10]. A major problem in anticipating the clinical course of prostate carcinoma was highlighted by the fact that the frequency of detecting lymph node metastasis by routine pathological analysis is not associated with the frequency of disease recurrence after radical prostatectomy [11]. The most important factor affecting the selection of the treatment in prostate cancer is the spreading towards or outside of the capsule. Partin et al showed that only the serum PSA value was increasing by 23% of the recurrence developed patients of the examined 955 patients with radical prostatectomy [12]. Because of incomplete tumor resection or clinically undetected micrometastasis, the recurrence may occurr after radical prostatectomy. It is impossible to apply local treatment in prostate cancers in which systemic disease is present in the form of clinically undetected micrometastases [12, 13]. Because of these conditions, researchers work on new methods to find out clinically undetected micrometastases. One of them is to detect prostate cells in circulation with RT-PCR method. The RT-PCR method was first used in the detection of Bcr/Abl mRNA produced by chronic myeloid leukemia (CML) cells in 1988 [14]. Actually, RT-PCR was used for the detection of micrometastatic disease, which the tissue source including specific mRNA defined and produced by the cell, and metastatic cells which one easily obtainable for sampling was required. Prostate cancer complies with all these criteria. Clinically, potential disadvantage of the use of mRNA is the hormonal regulation of PSA expression in patients on hormonal treatment [2, 15]. Recently, Ross et al showed that baseline RT-PCR PSA is a statistically significant predictor of time to progression in hormone-refractory prostate cancer [16].

PSMA is a membrane glycoprotein which shows increased expression in patients with metastatic prostate cancer [17]. On the other hand, it has been thought that the results of false positive PSMA mRNA may be brought about other tissues like liver, lung and normal peripheral blood [18]. It has been thought that PSMA expression is not affected by hormones and appears as an advantage to detect prostate cells circulating in patients on hormone treatment following radical prostatectomy [19].

Generally, the detection of prostate cancer cells circulating in peripheral blood and prostate specific mRNA series can be shown through RT-PCR PSA and PSMA method in 37-100% of the known metastatic disease patients [20, 21, 22, 23, 24, 25]. Control samples are negative in most of the studies. Cama et al used RT-PCR in molecular staging and discovered that the test was pertaining to capsular penetration, positive surgical margin and seminal vesicle invasion [26]. In another study, the authors used RT-PCR PSA to predict the pathologic stage with the help of preoperative PSA levels and the results of RT-PCR were found as 91% positive when the level of serum PSA was over 10 ng/ml and at stage pT3 [27]. Using the technique of RT-PCR, 100% positive results can be obtained with the examination of bone metastases in patients of known PSA and PSMA. RT-PCR was found positive in 81.5% of pT3 patients, it was found positive only in 37.5% of the patient with pT1-T2 stage disease [25]. Our study, however, when the serums of the patients with prostate cancer were examined with RT-PCR, showed that PSA was positive in 126 (43.2%) patients, while PSMA was positive in 165 (56.7%) patients. Both RT-PCR PSA and PSMA antigen were positive in 82 (28.1%) of all patients. These high values can only be expressed in case of lymph node invasion and distant metastases. Positivity of RT-PCR PSA and PSMA antigen was found to be statistically significant of lymph node invasion ($p = 0.0015$, $p = 0.021$) and distant metastases, respectively ($p = 0.0036$, $p = 0.021$). RT-PCR clinical stages were compared for RT-PCR PSA and PSMA positivity, statistically significant difference were observed ($p = 0.0016$, $p = 0.0038$).

Although our diagnosis shows that the determination of PSA with RT-PCR is more valuable than RT-PCR PSMA, this may be because of a small number of cases taken into the study which may have affected statistical analysis. Apart from that, since our clinic is a reference centre we believe that a considerable number of patients with metastatic prostate cancer were taken into our study. This method can be very valuable especially in the evaluation of patients with co-morbidity, who are at the upper limit of this age group and in advanced stage on examination, and who are treated according to real staging.

The results of detecting prostate cells circulating at a certain percentage in both localized and extracapsular patients can vary from centre to centre in a metaanalysis on molecular staging, which has been a subject of discussion on parameters affecting differences in RT-PCR results [28]. As can be understood from this, several problems such as sample collection and operation should be highlighted prior to the use of RT-PCR for staging of prostate cancer.

In conclusion, the multiplication of PSA and PSMA mRNA series with RT-PCR was quite successful in the detection of prostate cells circulating in peripheral blood. In our study, as a result of RT-PCR, PSA was examined with regard to the properties of the patient, positive values of both PSA and PSMA detected with RT-PCR were high in a significant respect. Besides, the RT-PCR results of patients with documented distant metastases, positive lymph node and Gleason score were showed to be significant in a statistical way. It was also found that serum PSA values had no statistical relation with the results of RT-PCR. Furthermore, the processes used in the method were highly sensitive. Technical difficulties could be overcome upon the standardization of protocols within centers and scrutinizing the process details.

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