Molecular Detection of Prostate Specific Antigen in Patients with Prostate Cancer or Benign Prostate Hyperplasia the First Investigation from Iran

*L Andonian [1], MR Khorramizadeh [2], DD Farhud [3], M Hashemzadeh Chaleshtori [4], K Holakouie Naieni [1], A Razi [5], J Sanadizadeh [6], G Pourmand [7], M Nouraie [8], S Rezaie [9], F Saadat [2], L Yepiskoposyan [10], M Norouzi [2], H Soleimanpour [11], A Berahme [2], N Aalizadeh [12]

[1] *Dept. of Epidemiology and Biostatistics, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Iran*
[2] *Div. of Biotechnology, Dept. of Pathobiology, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Iran*
[3] *Genetic Clinic, vallie Asr Sq., 16 Keshavarz Blvd., Tehran 14,Iran*
[4] *Cellular & Molecular Research Center, Shahrekord University of Medical Sciences, Iran*
[5] *Div. of Urology, Shariati Hospital, Tehran University of Medical Sciences, Iran*
[6] *Div. of Urology, Mehrad Hospital, Tehran, Iran*
[7] *Urology Research Center, Sina Hospital, Tehran University of Medical Sciences, Iran*
[8] *Digestive Disease Research Center, Tehran University of Medical Sciences, Iran*
[9] *Div. of Molecular Biology, Dept. of Medical Mycology & Parasitology, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Iran*
[10] *Institute of Man, Yerevan, Armenia*
[11] *Mitochondrial Research group, Department of Neurosciences, Medical school, University of Newcastle upon Tyne*
[12] *Dept. of Immunology, School of Medicine, Bandarabbas University of Medical Sciences, Iran*

Abstract

Prostate cancer is the second common form of cancer in men. Detection of circulating Prostate Specific Antigen (PSA) transcripts has effectively been used for early diagnosis of prostate cancer cells. This investigation employed a reverse transcriptase polymerase chain reaction (RT-PCR) technique to distinguish the patients with either localized or metastatic prostate cancer (CaP) vs. Benign Prostate Hyperplasia (BPH) and control subjects, as compared with clinical and pathological records. With reservation of ethical issues, blood samples were collected from 60 cases. Based on pathological and clinical findings, 25 patients (20 with localized cancer, 5 with metastatic), 22 with BPH, and 13 healthy (including 3 females) subjects as negative controls, were selected from Shariati, Mehrad, Sina,, Khatam and Atie Hospitals in Tehran, Iran. RT-PCR for a 260 bp PSA transcript was then performed. Clinical and pathological records were used for the assessment and comparison of PSA RT-PCR results. None of the control subjects and BPH (with 7 exceptions) were found positive by RT-PCR (Relative specificity= 72.7%). In patients with prostate cancer, 21 out of 25 were found PSA positive (Relative sensitivity= 83.4%) and the remaining 3 have been shown to be PSA negative (Positive predictive value= 83.4%). All of 5 metastatic patients (100%) revealed PSA positive results. Our data reflects the clinical relevance and significance of RT-PCR results as assessed with clinical and pathological examinations. PSA RT-PCR might be used as a powerful means for diagnosis, even when either pathological or clinical findings are negative, and could be employed for further molecular epidemiology surveys.

Keywords: *PSA, RT-PCR, BPH, Prostate cancer, Iran*

*Corresponding author: Tel: + 98 21 88989123, Fax: + 98 21 66462267, E-mail: andonian@sina.tums.ac.ir

Introduction

Prostate cancer is the second leading cause of cancer in men in the United States (1-3). It is the most common form of cancer in men (followed closely by lung cancer) in the world, and more than 300,000 new cases are detected every year (4, 5). Evaluation of the serum prostate specific antigen (PSA) levels has been considered as the early detection test of prostate cancer (6). Although PSA is effective at identifying prostate cancer (CaP) in men, it is also elevated in men with benign prostate hyperplasia (BPH) and other prostate disorders (5). Therefore to distinguish CaP from other non-malignant diseases, specific molecular markers are needed (5). For this reason, detection of circulation PSA transcripts has effectively been used for early diagnosis of malignant prostate cells (7-10). PSA is a 34-kDa serine protease, produced by the prostate epithelial cells belonging to the third member of human glandular kalikrein family hk1, hk2 and hk3 (PSA). Hk2 is strongly expressed in the prostate (11). They are all encoded by genes located on chromosome $19q_{13.3-13.4}$ (12). PSA is secreted into seminal fluid at concentrations of 0.5-3 mg/ml (i.e., about 1 million times those of plasma) (13). PSA dissolves the gel forming after ejaculation by digesting seminogelin -1 and -2 and fibronectin, causing release of the sperm, which is essential for sperm function (14).

In the normal prostate, natural barriers (including epithelial and basal cell layer and the basement membrane) minimize PSA leakage. In prostate carcinoma the basement membrane and the basal cell layer are disrupted (15). For this reason prostate epithelial cells loose organization and may infiltrate the surrounding stroma. These changes contribute to the increased passage of PSA into the blood of prostate cancer patients (16). Many investigators have focused on the use of a very powerful and sensitive molecular tool RT-PCR (reverse transcriptase polymerase chain reaction) to observe PSA transcript (17-20). This investigation employed an RT-PCR technique to distinguish the patients with either localized or metastatic prostate cancer CaP vs. BPH and control subject as compared with clinical and pathological records.

Materials and Methods

Patients and Samples   All patients in this study, except control subjects, were selected according to clinical and pathological records either for adenocarcinoma of the prostate or BPH at the urology section, Shariati, Mehrad and Sina Hospitals, Tehran University of Medical Sciences, Iran.

From 15 May 2002 to 5 January 2004, 60 patients, mean age 68.4 years (ranged 50-87), were included in this study. All patients except control subjects had been subjected to biopsy as a result of their abnormal digital rectal exam (DRE) and elevated serum PSA levels.

Six ml peripheral blood sample, treated with sodium-citrate, from all patients were collected either immediately before radical prostatectomy at surgery room or before biopsy. A questionnaire was filled in for all patients according to International Prostate Symptom Score (IPSS) standard (21). Whole blood samples were divided in two aliquots for further RNA extractions: one used freshly and, the other kept deep freezed for further repeats.

Statistical analyses: All collected data on RT-PCR results were compared separately with either clinical findings or pathological records. Relative sensitivity, relative specificity, and relative positive predictive value (PPV) were calculated there on.

Cell Lines   Two human prostate cancer cell lines, PC3 (22) and LNCaP (23), obtained from Pasteur Institute of Iran, were included in this study. These cells were used as positive controls.

RNA Extraction   The blood samples were received in 6 ml aliquots and kept in four different ways for further applications: 1-freezed whole blood, 2-freezed whole blood in equal volumes of guanidine isothiocyanate (GITC), 3-

freezed buffy coat in equal volume of GITC and 4-fresh buffy coat in equal volume of GITC, respectively. The fresh samples were immediately centrifuged at 10000 rpm for 15 min. The buffy coat transferred to a new tube, washed one time by an equal volume of phosphate buffer saline (PBS). Total RNA extraction from the above samples, either fresh or freezed, was performed according to Chomzinsky method with slight modifications (24). In brief, cells were lyzed in GITC solution (Bohringer Mannheim-Germany),then extracted with phenol, chloroform, isopropanol, and precipitated by absolute ethanol. The quality of the isolated RNAs was verified by control RT-PCR reactions using a house keeping gene, gamma actin. Extracted RNA was also evaluated by running an aliquot on a 1% agarose gel.

Reverse transcription reaction (cDNA synthesis) For cDNA synthesis a First Strand cDNA Synthesis kit was used (Roche Inc., GMBH, Germany). According to kit instructions, an aliquot of Reaction Mix was prepared containing 4μl 5x buffer, 2μl dithiothreitol (DTT), 1μl deoxyribonucleotide mix (dNTP10 mM), 1μl oligo deoxy thymidine (dt), 1μl random hexamer, and 0.15μl RNase inhibitor added by 1.5μl moloneymurine leukemia virus reverse transcriptase enzyme (MMLV) and nearly one microgram total RNA. The volume was reached to 25μl by H_2O. Using a programmable thermocycler, synthesis of cDNA was carried out for 55 min. at 38º C, followed by incubation at 95º C for 3min and equilibrated at 20º C for 1 min.

PCR (polymerase chain reaction) The primers for PSA gene were chosen as described earlier (21). The sequence of PSA sense primer with 18 mer length was as follows: 5'-GCA CCC GGA GAG CTG TGT-3', and the sequence of PSA antisense primer with 21 mer length was as follows: 5'-GAT CAC GCT TTT GTT CCT GAT-3', (MWG Oligo Synthesis Company). In the PCR reaction, 5μl of prepared cDNA was amplified in a 25μl aliquot containing 2.5μl (10X buffer+ $MgCl_2$), 2μl

$MgCl_2$ (50mM), 1μl dNTP (10 mM), 2μl (20 pM) of each sense and antisense primers and 0.5μl. Thermus aquaticus (taq) enzyme (500U super taq DNA polymerase), and the rest ddH_2O. After holding 3 min at 95º C (initial denaturation), 40 cycles were repeated at 94º C/20 sec (denaturation), 54º C/20 (annealing) and 72º C/ 30 (extension), respectively, followed by 10 min at 72º C and terminated for 1 min at 20º C.

Results

We used four different ways in order to save RNA during extraction (Fig.1). The visualized 28S and 18S ribosomal RNA bands were indicative of RNA integrity. As Fig. 1 shows in line 4, a better quality of RNA was achieved when fresh buffy coat was used as the starting sample. Thus the 4th way of sample collection was applied for the further steps during the study. The PCR products, containing 260 bp PSA primers (25) and 1104 bp gamma actin primer were evaluated by 1% agarose gel electrophoresis and visualized after staining by ethidium bromide (Fig.2). It was also evaluated by PAGE (Polyacrylamide- gel electrophoresis) and visualized after silver staining (Fig.3). As it is shown in these two figures, normally more bands are seen in the PAGE gel after silver staining, owing to the higher sensitivity of this method. However, a clear PSA band with identical electrophoresis characteristics is seen in the two gels.

RT-PCR for PSA transcript was performed on samples from 25 patients with CaP, 22 patients with BPH, 10 healthy male and 3 healthy female subjects. Of the 25 prostate cancer patients, 20 had clinically localized and the rest had metastatic prostate cancer.

RT-PCR showed no positive results for PSA in samples derived from normal individuals. The PSA transcript however, was detected in 31.8% BPH, 100% metastases and 80% localized prostate cancer patients, respectively. (Table1) When pathological data were considered as gold standard, the compared RT-PCR positivity was 27.3% for BPH, 100% for metastases, and

78.6% for localized patients, respectively (Table 2).

In summary none of the control subjects and BPH (with 7 exceptions) were found positive by RT-PCR (Relative specificity= 72.7%). In patients with prostate cancer, 21 out of 25 were found PSA positive (Relative sensitivity= 83.4%) and the remaining 3 have shown PSA negative (Positive predictive value= 83.4%).
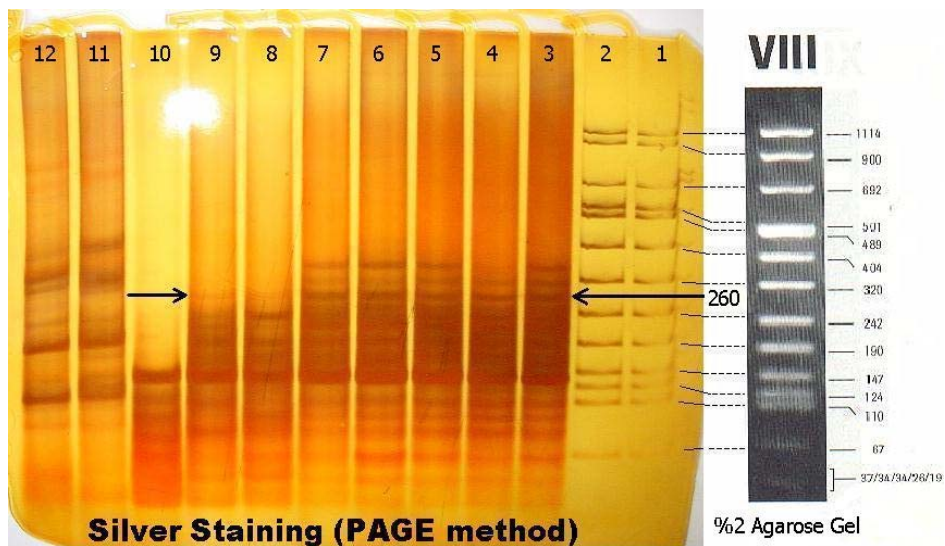
All of 5 metastatic patients (100%) revealed PSA positive results.
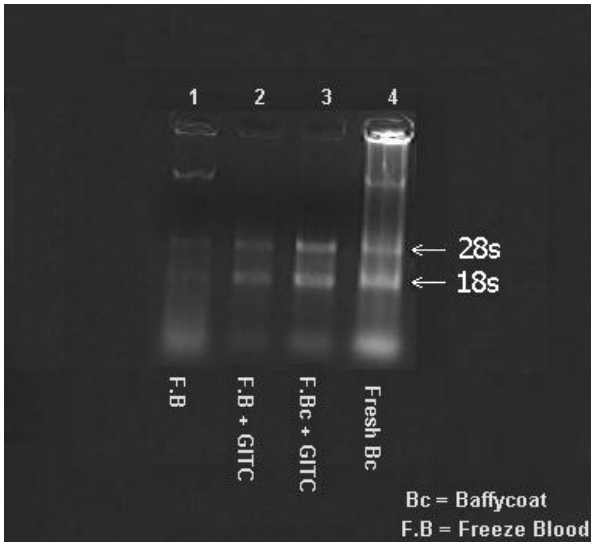
Fig. 1: Extraction of RNA by gel electrophoresis by fresh & freezed blood
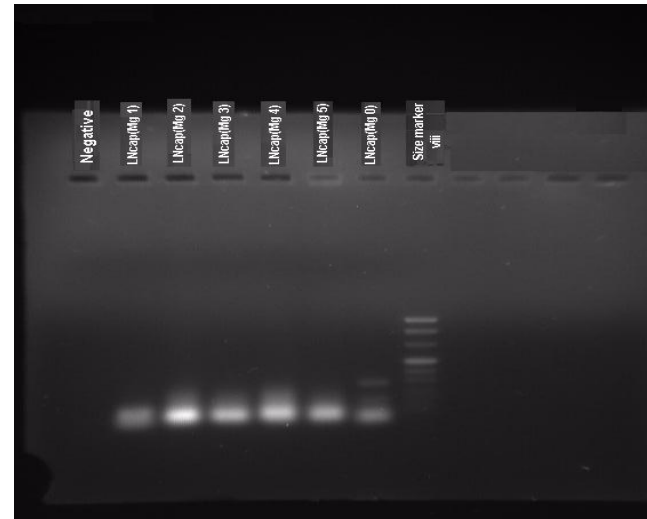
Fig. 2: Optimization of LNcap cDNA in the human prostate cancer cell line

Fig. 3: RT-PCR for PSA in patients as analyzed with silver staining method. line 1-2, 1114-bp molecular weight marker VIII (Life Technologies, Inc.). Line 3-7 localized cancer, line 8, 9 BPH; line 10-12 control subjects were analyzed. The PCR product is 260 bp for PSA.

Table 1: Correlation between PSA RT-PCR & clinical results

PSA RT-PCR	Total	Positive		Negative	
Classification of Patients	**No**	**No**	**%**	**No**	**%**
rostate cancer (Localized)	20	16	80	4	20
Prostate cancer (Metastatic)	5	5	100	0	0
Benign prostate hyperplasia (BPH)	22	7	31.81	15	68.18
Control negative subjects (men)	10	0	0	10	100
Control negative subjects (women)	3	0	0	3	100
Total	60	28	46.66	32	53.33

Table 2: Correlation between PSA RT-PCR & pathological results

PSA RT-PCR	Total	Positive		Negative	
Classification of Patients	**No**	**No**	**%**	**No**	**%**
Prostate cancer (Localized)	14	11	78.57	3	21.42
Prostate cancer (Metastatic)	4	4	100	0	0
Benign prostate hyperplasia (BPH)	11	3	27.27	8	72.72
Control negative subjects (men)	10	0	0	10	100
Control negative subjects (women)	3	0	0	3	100
Total	42	18	42.85	24	57.14

Discussion

In the present study, we performed peripheral blood- based RT-PCR assay for PSA in Iranian prostate cancer versus BPH patients compared to healthy individuals as control. For each included sample, the obtained RT-PCR result was then compared to the clinical examination record to evaluate their association with the pathological data. Collectively, our results showed that PSA RT-PCR was a more sensitive and specific method in screening prostate cancer patients that was in agreement with other studies (1, 2, 7). However, in 7 of 22 BPH cases, PCR was revealed positive. This result, which hinders PCR specificity, might be to some extent stemmed from the fact that pathological examinations were made on only 3 of the 7 PCR positive BPH patients. Clinical observations are subjective and usually prone to error. Based on pathological data gathered from biopsies, our data demonstrate that the presented RT-PCR method allows detection of circulating prostatic cells with PSA expression characteristics. Debate still exists on the validity and clinical significance of detecting circulating prostate cancer cells using RT-PCR technology (26, 27). However, in line with some reported studies, our data shows more significant correlation, than clinical criteria, between RT-PCR and Glisson Pathological Grading of

prostate cancer (2, 7, 9). In summary, none of the control subjects were found positive according to RT-PCR relative specificity (72.7%). In clinically identified BPH patients, 7 of 22, revealed positive RT-PCR results. This positivity however, was lowered down to 3 of 11 cases when pathological data included as gold standard. In patients with prostate cancer, either metastatic or localized, 21 of 25 were found PSA positive (Clinically relative sensitivity, 83.4%) and the remaining 4 have showed PSA negative (Positive predictive value (83.4%). All of 5 metastatic patients (100%) revealed PSA positive RT-PCR results. Collectively, these results advocate the promising applications of RT-PCR as a powerful gold-standard test for screening and monitoring patients suffering from prostate cancer. Our data reflects the clinical relevance and significance of RT-PCR results as assessed with clinical and pathological examinations. PSA RT-PCR might be used as a powerful means for diagnosis, even when either pathological or clinical findings are negative, and could be employed for further molecular epidemiology surveys.

Acknowledgments

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