



Original contribution

Prostate-specific antigen mRNA and protein levels in laser microdissected cells of human prostate measured by real-time reverse transcriptase–quantitative polymerase chain reaction and immuno–quantitative polymerase chain reaction

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Summary Laser-assisted microdissection has mainly been used in cancer studies to excise pure cell populations from heterogeneous tissues. Cancer and normal cells selected by laser-assisted microdissection have frequently been used for mRNA expression studies usually by reverse transcriptase–quantitative polymerase chain reaction (qPCR). Recently, real time immuno-qPCR was developed as a new tool for highly sensitive measurements of proteins. Using reverse transcriptase–qPCR and immuno-qPCR, we measured the amounts of prostate-specific antigen mRNA and its corresponding protein in homogeneous and comparable cell populations, collected from normal and cancer prostates by laser-assisted microdissection. With these techniques, prostate-specific antigen mRNA and protein were quantified over a wide range of concentrations with a sensitivity sufficient to analyze single prostate cells (LNCaP). We did not find significant differences in prostate-specific antigen protein and mRNA between normal and cancer cells. The expression of prostate-specific antigen protein and mRNA was highly correlated in both normal and pathological cells. In microdissected peritubular stromal areas of prostate cancers, the concentration of prostate-specific antigen protein was about 100 times higher than in normal prostate, indicating an increased transit of secreted prostate-specific antigen. In the same samples, prostate-specific antigen mRNA was not detectable. Our data

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demonstrate, for the first time, the feasibility of simultaneous application of reverse transcriptase–qPCR and immuno-qPCR in studies of homogeneous cell populations, collected by laser-assisted microdissection. The approach is expected to become a very powerful tool for expression studies in human cancers at both mRNA and protein levels.

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1. Introduction

The study of nucleic acids as a mean to investigate the molecular basis of physiological and pathological conditions requires the development of assay methods characterized by high sensitivity, reliability, and practicability. As a quantitative tool, the polymerase chain reaction (PCR) meets these requirements. For many applications, however, studying nucleic acids is not sufficient to reveal the biological processes behind the physiopathologic events. Posttranslational mechanisms controlling gene expression, such as translational control [1] and protein half-life [2], strongly influence the relative concentrations of mRNA and its cognate protein. Several studies have been performed to find out if mRNA levels can be used to predict protein levels [3]. Data obtained for different proteins have not given consistent results [4–6]. The comparison is further hampered by the lower sensitivity of conventional methods used for protein quantification. Although nucleic acids can be amplified and accurately quantified with real-time PCR (qPCR), proteins cannot. The ultrasensitive qPCR method is excellent when analyzing limited amounts of sample, such as low-expressed genes and circulating heterogeneous nucleic acids in body fluids [7].

In recent years, laser-assisted microdissection (LAM) has been introduced to select pure cell populations from heterogeneous tissue samples. LAM of a tissue (ie, a typical glandular structure) and subsequent analysis of target genes by reverse transcriptase–qPCR (RT-qPCR) become a powerful tool in gene expression profiling studies and are likely to enhance their diagnostic and prognostic value [8]. This approach eliminates contributions to the gene expression data from fibromuscular tissue and tumor-infiltrating mononuclear cells [9]. An additional advantage is that LAM allows molecular analysis of cell populations in their native tissue environment and may be potentially applicable to biopsies obtained in preoperative diagnostic procedures. This new technique can thus overcome the problem of cellular heterogeneity that is a significant barrier to the molecular analysis of normal versus pathological tissues [10]. So far, few studies have reported on the protein content of microdissected tissue samples [11], mainly because of the limited sensitivity of protein immunoassays. This is expected to change because of the advent of immuno-qPCR technology, which is up to 1000-fold more sensitive than conventional immunoassays [12,13]. Immuno-qPCR is based on target detection with a specific antibody labeled with a DNA strand, where the detection of immunocom-

plexes is performed with qPCR. Conventional competitive or noncompetitive formats can be used.

Here we demonstrate for the first time an experimental approach to measure the levels of an mRNA and its cognate protein by RT-qPCR and immuno-qPCR in selected small homogeneous cell populations excised by LAM. The approach was used to measure prostate-specific antigen (PSA) expression in normal and cancer prostate tissues. Our results demonstrate the feasibility of this approach as a powerful tool to study, simultaneously and in the same cells, expression of both mRNA and protein in human cancers.

2. Materials and methods

2.1. Tissues and cells

The human prostate cancer cell line LNCaP (ATCC, CRL 1740) was cultured in RPMI 1640 containing 10% fetal bovine serum. Pathological prostate tissues for LAM were obtained from 3 patients operated for prostate cancer. All the 3 prostate cancers were graded as 3 + 5 according to the Gleason score. Seven normal prostates, without any evidence of cancer lesions, were obtained from patients who had undergone radical cystectomy for bladder cancer. Patients did not receive preoperative therapies. Tissues were flash-frozen in liquid nitrogen and stored at -80°C . The study was approved by the local ethic committee.

2.2. Laser microdissection

The optimal cutting temperature medium (OCT)-embedded tissue blocks were cut with a cryostat into 5- μm serial sections. Frozen sections were fixed with ethanol immediately after cutting, stained with hematoxylin-eosin followed by increasing ethanol concentration, and air dried. Only 1 section was thawed and dissected at a time to minimize degradation of proteins and RNA. The sections were mounted onto a polyethylene membrane slide according to the manufacturer's instructions.

For microdissection, we used the PALM Laser-Microbeam System (P.A.L.M. Microlaser Technologies AG, Bernried, Germany), which enables contact-free isolation of single cells or group of cells. The microdissected cells were catapulted into the lid of a 0.5-mL reaction tube using the laser pressure catapulting technique of the instrument.

An expert pathologist accurately selected from each patient pairs of contiguous tissue sections from which the same cellular areas were microdissected: 1 for mRNA and 1 for protein measurement. In the case of prostate cancers, at least 4 different areas of normal and tumor tissues were microdissected from each section. Using the same approach, we also collected paired contiguous areas of stromal tissue from normal and pathological prostates. For all tissue samples, the microdissected area was from 40000 to 80000 μm^2 .

Normalization of both RT-qPCR and immuno-qPCR results was achieved by referring to the theoretical number of cells that constitutes the microdissected tissue samples. This was done by dividing the microdissected area by the mean whole-cell area of 1 LNCaP cell (580 μm^2) derived from the morphometric measurements reported by Vona et al [14]. When dealing with different cell types (ie, when comparing results from prostate epithelium and the surrounding stroma), results were corrected for the microdissected tissue area.

2.3. RNA and protein extraction

To extract RNA from microdissected tissue samples, we used the RN easy micro Kit (Qiagen, Milan, Italy) following the manufacturer's instructions. Proteins were extracted by adding 20 μL CHAPS Buffer (Tris-HCl 10 mmol/L, pH 7.5, MgCl_2 1 mmol/L, EGTA 2 mmol/L, β -mercaptoethanol 5 mmol/L, DTT 1 mmol/L, glycerol 10%, CHAPS 0.5%, protease inhibitors 0.1 mmol/L) into the lid containing the microdissected samples. The tubes were then centrifuged, and the samples were analyzed by immuno-qPCR.

2.4. RNA quality evaluation

RNA quality was investigated by capillary electrophoresis on a Lab-on-chip device running in the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). One microliter from each isolated RNA sample was analyzed with RNA 6000 Pico Assay using RNA Pico LabChips (Agilent Technologies). The resultant electropherograms

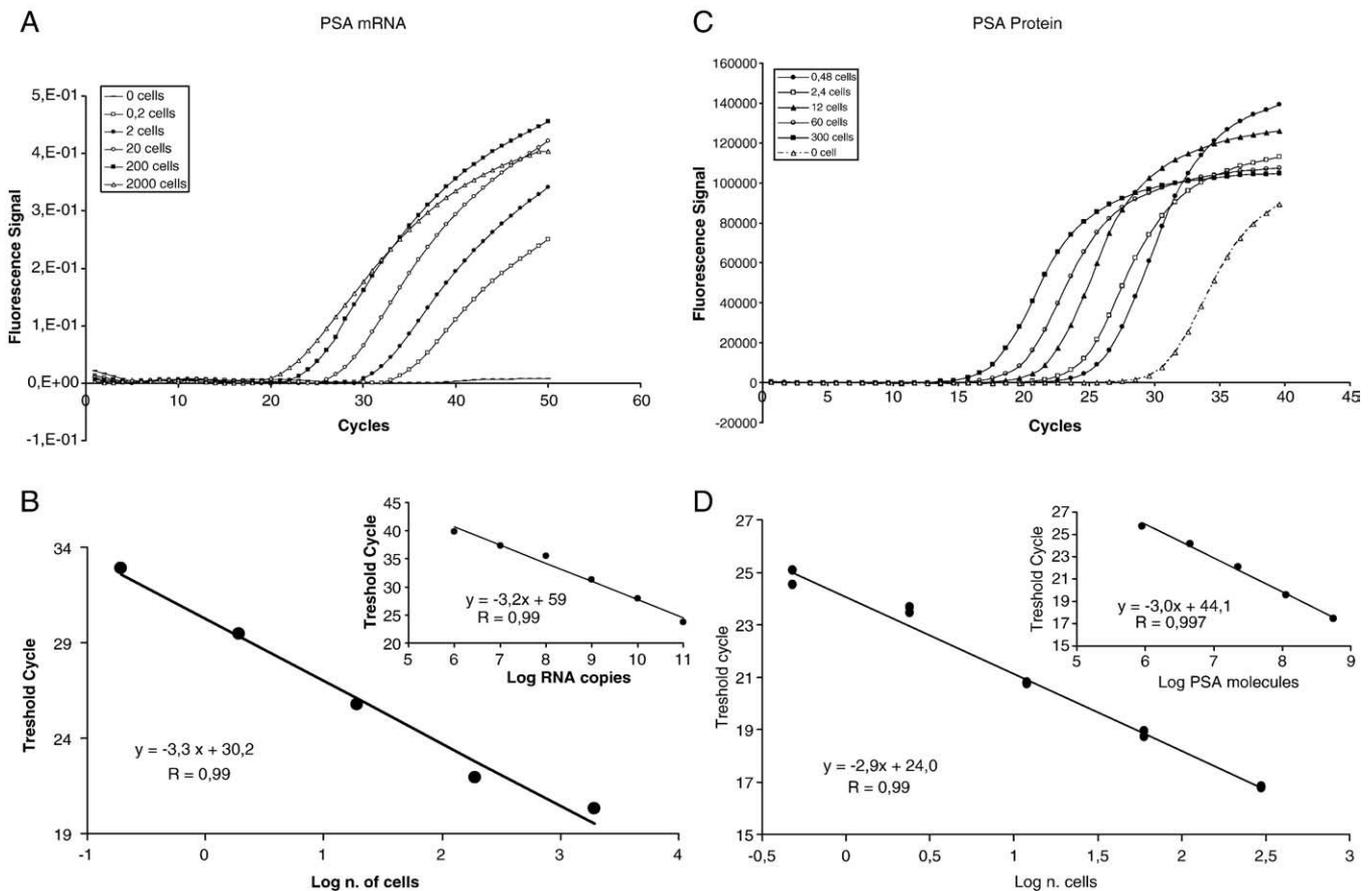


Fig. 1 PSA expression in LNCaP cells measured by RT-qPCR and immuno-qPCR. A, Amplification plots obtained of serial 10-fold dilutions of RNA extracted from LNCaP cell line (from 2000 to 0.2 cells/reaction tube). B, Linear regression analysis of the C_T value versus log of number of cells. The insert shows the external reference curve generated by serial dilutions of PSA cDNA. C, Amplification plots obtained by serial dilution of proteins extracted from LNCaP cell line suspension (from 300 to 0.5 cells/reaction tube). D, Linear regression analysis of the C_T value versus log of the number of cells. The insert shows the external reference curve generated by serial dilutions of PSA native protein.

were used to determine RNA integrity number (RIN), which is based on a proprietary Agilent Technologies algorithm [15] and is calculated by the 2100 Expert software (Agilent Technologies).

2.5. cDNA synthesis and RT-qPCR

First-strand cDNA synthesis was performed by using an RT-qPCR kit (Applied Biosystems, Foster City, CA) in a final volume of 20 μL following the manufacturer's instructions. PSA mRNA expression was measured by RT-qPCR with a Taqman assay using a premade kit from Applied Biosystems (kallikrein 3, Unigene ID Hs.171995; reference sequences: NM 001030047.1, NM 001030049.1, NM 001648.2). The amplicon generated by RT-qPCR Taqman assay is 65 bp in length. Moreover to check RNA integrity, we performed 18S RT-qPCR measurements using the predeveloped assay Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems). The PCR mixture contained 12.5 μL of Universal Master Mix (Applied Biosystems), 1.25 μL of the ready-made PSA probe and primers mix, and 5 μL of cDNA in a

final volume of 25 μL . The thermal cycle conditions were 1 hold at 50°C for 2 minutes, 1 hold at 95°C for 10 minutes, and 50 cycles of a 2-step amplification protocol (95°C for 15 seconds and 60°C for 1 minute). To calculate the expression of PSA mRNA, we referred to an external reference curve generated with synthetic cDNA obtained by cloning the target in the expression vector pcDNA3.1/CT-EGFP-TOPO (Invitrogen, Carlsbad, CA). The plasmid was linearized and used for the in vitro production of RNA by means of the "Ribo-Max" kit (Promega, Madison, WI) according to the manufacturer's instructions. PSA RNA reference standard concentration was determined by spectrophotometric measurements (Nanodrop, USA). A standard curve was obtained by serial dilutions yielding aliquots from 10^{11} to 10^6 mRNA copies following the same procedure as for testing the samples (2-step procedure).

Each sample was run in duplicate. Each experiment included a positive control (20 ng of reverse-transcribed RNA of LNCaP) and a negative control (with no template). The imprecision values of the assay (coefficient of variation [CV] of the C_T values of the TaqMan assay) evaluated on the

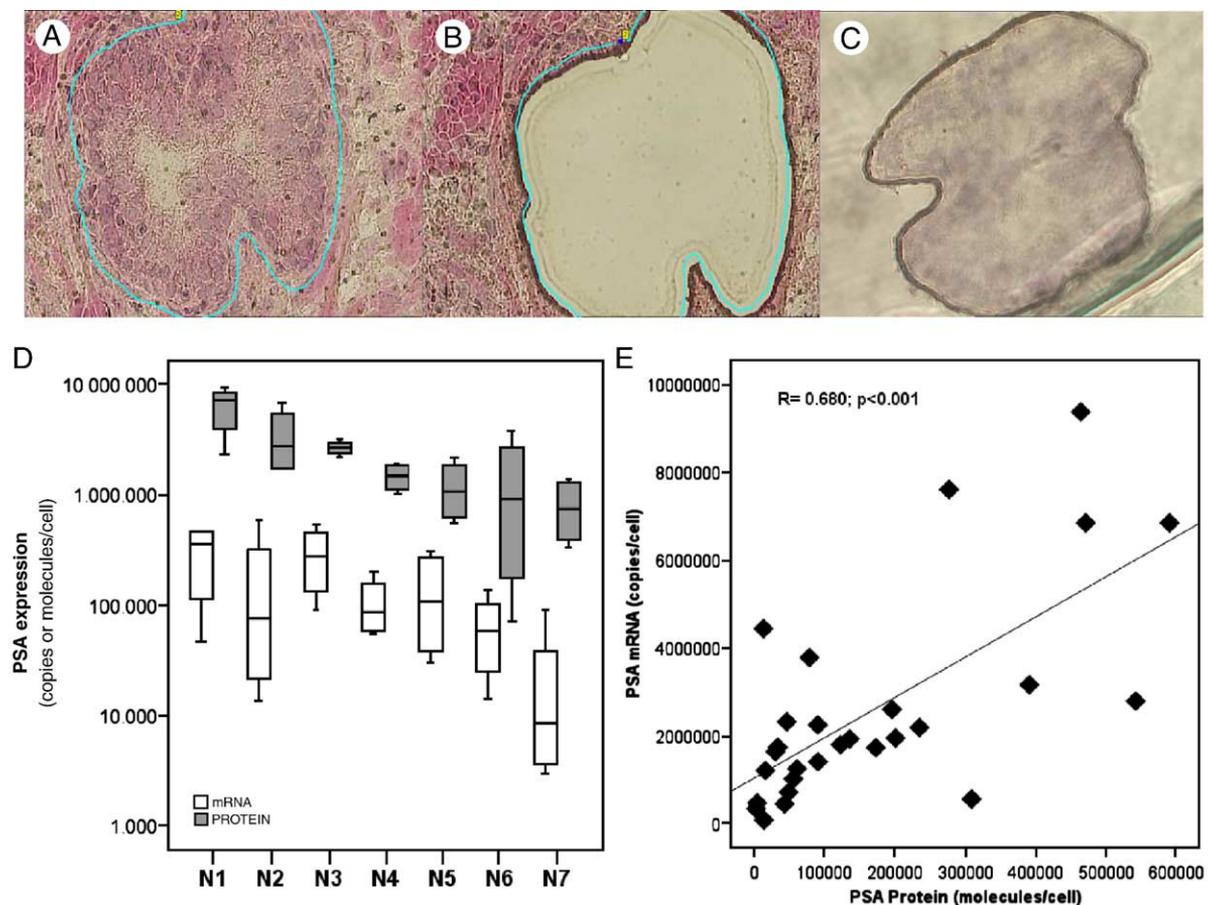


Fig. 2 PSA mRNA and protein expression in microdissected samples from normal prostates. Upper panel: Pictures illustrate the microdissection procedure. A, Selection of the area to be microdissected. B, The tissue after the catapulting of the sample. C, The microdissected sample collected into the lid of the reaction tube (magnification $\times 40$). Lower panel: Median mRNA and protein PSA expression levels of 4 dissected areas from the same section for 7 normal prostates (from N1 to N7) (D) and their linear relationship (E).

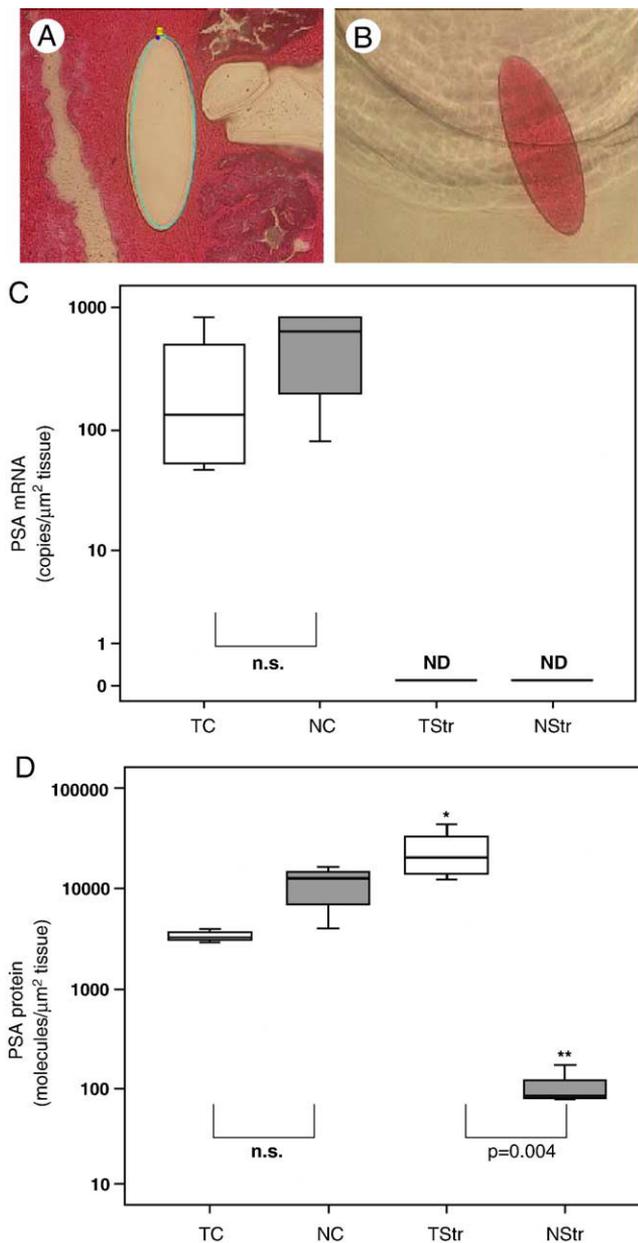


Fig. 3 PSA mRNA and protein expression in microdissected samples from normal and pathological prostates. Microdissection of a stromal area and its recovery are reported in panels (A) and (B) (magnification $\times 10$). Levels of PSA mRNA (C) and protein (D) measured in multiple sampling from TC (cancer cells), NC (epithelial cells from normal prostate), TStr (stromas from prostates with cancer), and NStr (stromas from normal prostates). Statistical differences were evaluated with the Mann-Whitney U test. Abbreviation: ND, mRNA was not detectable. $*P < .01$ versus TC; $**P < .001$ versus NC.

basis of the standard curve based on triplicates were always less than 2%.

2.6. Immuno-qPCR

Samples collected for protein measurement were analyzed for PSA with immuno-qPCR assay as previously described [13]. Capture antibody (anti-PSA10) and detection antibody (anti-PSA66) were provided by CanAg Diagnostics (Fujirebio Diagnostics, Göteborg, Sweden). Wells of the microtiter plate were coated overnight with first antibody (12.5 nmol/L in 0.2 mol/L NaH_2PO_4), then washed 3 times with 200 μL of wash buffer (0.154 mol/L NaCl, 5 mmol/L Tris, pH 7.75, 0.005% Tween 20, and 0.1% Germall II), and blocked with incubation buffer (phosphate-buffered saline [PBS] containing 1% bovine serum albumin [BSA] and 0.05% Tween 20) for 1 hour at 37°C. A PSA standard curve, spanning from 0.01 to 10 ng/mL, was generated by serial dilution of native PSA protein. Laser microdissected prostate samples were diluted 1:4 in incubation buffer. Standards/Samples (5.5 μL) in triplicates were incubated for 1 hour at room temperature with 22 μL of DNA-labeled anti-PSA66 [13]. Twenty-five microliters was dispensed per well in the coated microtiter plate and incubated for 1 hour at room temperature. The plate was finally washed 6 times with 200 μL of wash buffer and 10 times with 200 μL of Milli-Q. The amount of bound DNA was finally quantified by real-time PCR using the Bio-Rad iCycler iQ system with the primers and conditions described previously [13].

2.7. Statistical analysis

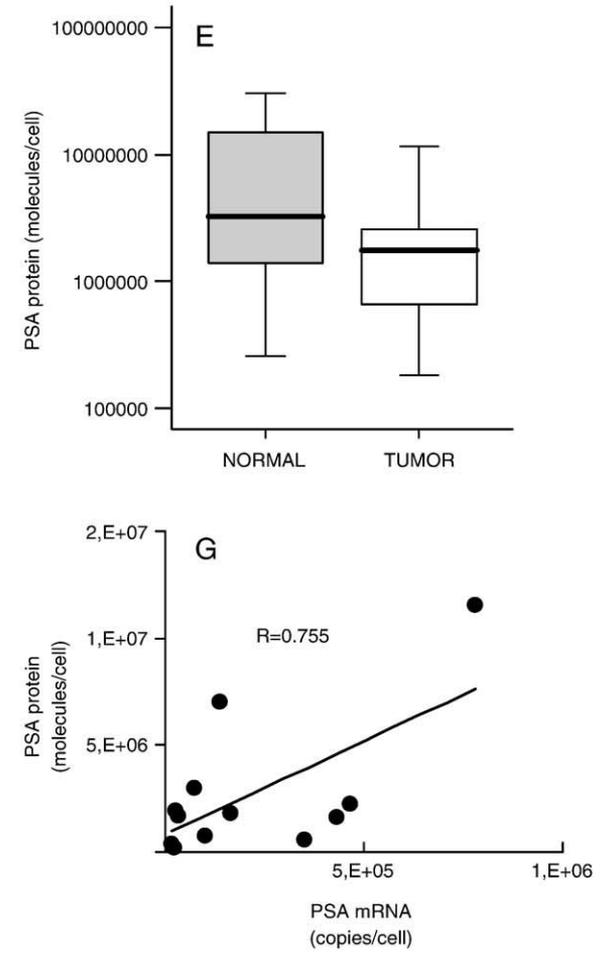
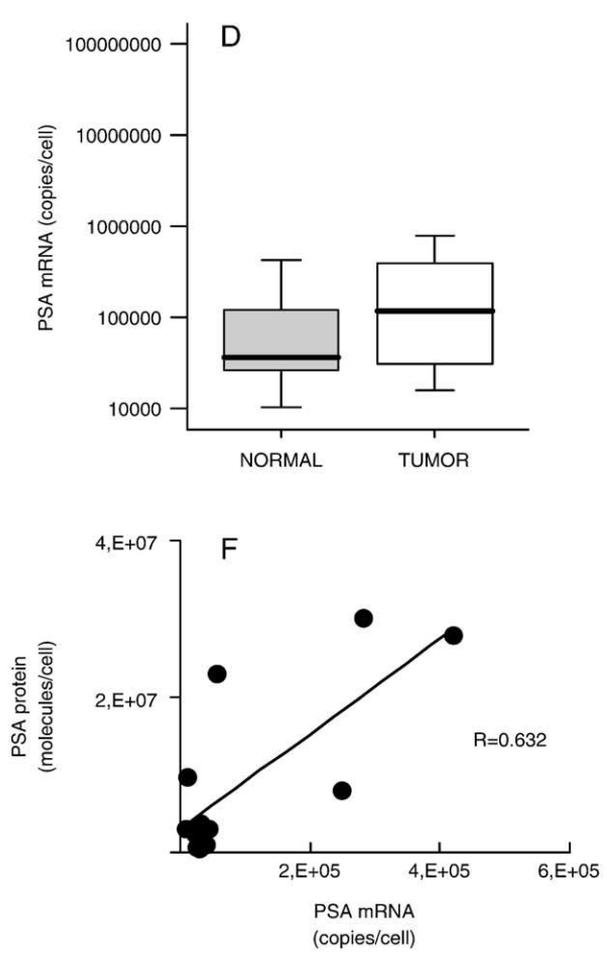
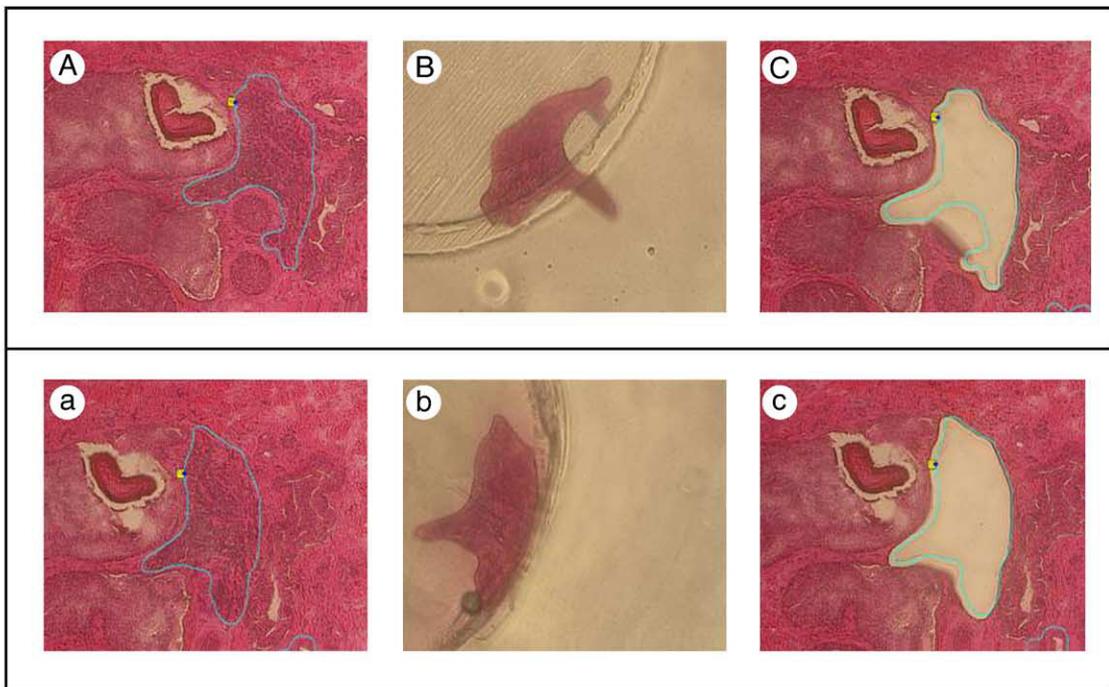
Nonparametric tests were used to compare RT-qPCR and immuno-qPCR results. Linear regression analysis and evaluation of the Pearson coefficient were applied to establish the correlation between PSA mRNA and protein expression. P values less than .05 were considered statistically significant. Data analysis was carried out with the SPSS statistical package, version 15.0 (SPSS Inc, Chicago, IL).

3. Results

3.1. RNA quality

The quality of the samples was evaluated on each RNA extracted from the tissues collected within this study. For each sample of each subject, RNA was extracted from a

Fig. 4 PSA mRNA and protein expression in microdissected samples from prostate cancers. An example of the different phases of tissue microdissection is reported illustrating corresponding areas on 2 serial sections from the same tissue (A and a); the microdissected tissue samples into the lid of the extraction tube (B and b); and the tissue area after the catapulting process (C and c) were used for separate analyses of PSA mRNA and protein content (magnification $\times 10$). Comparison of PSA mRNA (D) and PSA protein levels (E) in prostate cancer cells and paired normal epithelial cells (differences were not significant). Correlation between PSA mRNA and protein in normal (F) and cancer (G) microdissected areas in 3 cancer patients (n = 4 samples each).



slice of tissue collected with a sterile scraper from the microdissection slide after hematoxylin-eosin staining, just before the microdissection procedure. The quality of the samples was evaluated on the basis of the RIN number as calculated from the Agilent 2100 Bioanalyzer.

The median value of RIN numbers from the whole set of samples was 7.1 (range, 6.3-8.1) and thus considered fully suitable to real time RT-PCR analysis [16,17].

3.2. PSA mRNA and protein in LNCaP cell line

Real-time RT-qPCR for PSA mRNA was initially tested on serial dilutions of LNCaP cells. The counting of LNCaP cells was performed by a hemocytometer. The assay was linear from 2000 to 0.2 cells/reaction ($y = -3.27x + 30.26$; $R = 0.993$) and parallel to the corresponding external reference curve generated with PSA cDNA serial dilutions (Fig. 1). Based on 5 consecutive experiments, the median level of PSA mRNA was 1.3×10^7 copies per LNCaP cell (range, 6.0×10^6 to 3.0×10^7).

Immuno-qPCR for PSA protein measurement was linear in a range of 300 to 0.5 LNCaP cells/reaction ($y = -2.9x + 24.0$; $R = 0.99$), and the dilution curve was parallel to the reference curve obtained by serial dilution of purified native protein. The sensitivity of this method was 4.8×10^5 molecules, as previously reported [13]. This corresponds to about 1 cell/reaction tube (Fig. 1). Median level of PSA protein was 3.3×10^6 molecules/LNCaP cell ($n = 5$; range, 2.3×10^6 to 4.3×10^6).

3.3. PSA mRNA and protein in normal prostate

PSA mRNA and protein measurements were performed in 4 paired samples, microdissected from serial sections and from different glandular areas of 7 normal prostates for 28 sections (Fig. 2). Median level of PSA mRNA in these microdissected areas was 9.1×10^4 copies/cell (range, 2.9×10^3 to 5.9×10^5), whereas PSA protein level in the paired microdissected areas was 1.8×10^6 molecules/cell (range, 7.0×10^4 to 9.4×10^6). The levels of PSA mRNA and PSA protein in these samples were significantly correlated ($R = 0.68$) (see Fig. 2). The same sections of normal prostate were used for microdissection of 4 randomly selected areas of stroma. In these samples, we found no PSA mRNA, whereas PSA protein was present, although its concentration expressed as molecules/ μm^2 was lower than in the corresponding epithelium ($P = .004$) (Fig. 3).

3.4. PSA mRNA and protein in prostate cancers

Using the same procedure, PSA mRNA and protein levels were measured in microdissected samples obtained from 3 prostate cancers. Under the control of an expert pathologist, from each patient, we microdissected 4 areas of cancer tissue and 4 areas of apparently normal epithelium collected from the same sections.

The levels of PSA mRNA within tumor tissue (median, 1.0×10^5 copies of PSA mRNA/cell; range, 1.6×10^4 to 4.7×10^5) were not statistically different from those found in corresponding normal epithelia (median, 3.7×10^4 copies/cell; range, 1.0×10^4 to 4.2×10^5). Likewise, the median level of PSA protein in cancer cells (median, 1.7×10^6 molecules/cell; range, 1.8×10^5 to 7.0×10^6) was comparable to that found in normal epithelia (median, 3.3×10^6 ; range, 4.7×10^5 to 3.0×10^7) (Fig. 4). When all results from the different patients and the different microdissected areas were combined ($n = 12$), we found that the expression of PSA mRNA and protein was significantly correlated both in normal ($R = 0.632$, $P = .014$) and in cancer ($R = 0.755$, $P = .002$) cells (Fig. 4).

Using this procedure, we also microdissected stromal areas in neoplastic prostates. We did not detect any

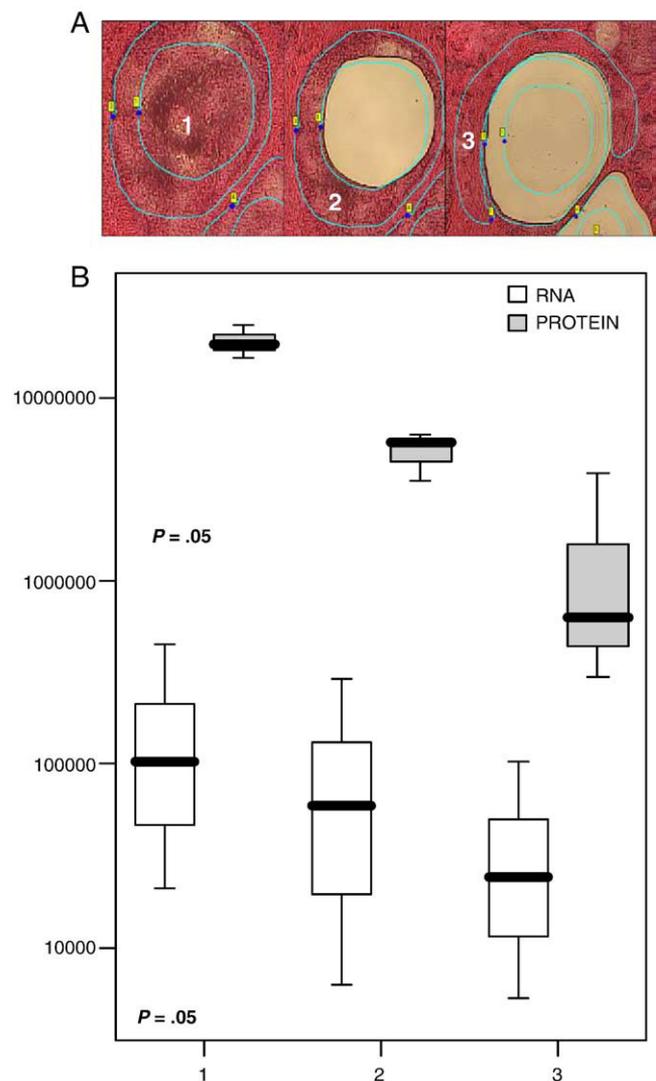


Fig. 5 PSA expression in concentric microdissected fragments of 1 representative prostate cancer lesion (A) (magnification $\times 10$). Significant decrease of mRNA and protein expression from the core to the outer areas of three neoplastic lesions is illustrated (B).

PSA mRNA in these samples either, but the PSA protein levels were more than hundred fold higher than in the normal prostate ($P = .004$) (Fig. 3). Surprisingly, in the cancer prostates, protein concentration appeared to be more elevated in the stromal compartments than in the cancer cells when compared with PSA molecules/ μm^2 ($P = .009$).

Finally, we studied in 3 pathological samples the gradient of PSA expression in typical prostate cancer lesion from which we microdissected concentric fragments, from the internal to the outer tumor area. As seen in Fig. 5, we observed the maximal PSA expression, both as mRNA and protein, in the core of the neoplastic lesion with progressive reduction toward the outer parts ($P = .05$).

4. Discussion

LAM has become a highly versatile technology to obtain pure samples of cells of interest for downstream molecular applications [18]. Same studies have extended LAM to measure changes in cellular protein content by 2-dimensional gel electrophoresis [19-21], which allows for qualitative profiling of protein content. Only a few studies have been reported on quantitative measurements of proteins in microdissected tissue samples by sensitive immunoassays [11], even when based on ultrasensitive assay procedures to detect low-abundance proteins in serum [22,23] or in cell lines [24]. Real-time PCR technology has been demonstrated to be suitable for measurements of gene expression with very high sensitivity both at the mRNA [25] and the protein levels [12,13], and in a recent study, telomerase activity was assessed [26].

The aim of this study was to demonstrate for the first time the feasibility of simultaneous measurements of PSA mRNA and its cognate protein in the same normal and cancer microdissected cells of human prostate. Both RT-qPCR and immuno-qPCR were proven to be sufficiently sensitive to detect PSA expression in 1 LNCaP cell as well as in a few microdissected normal or cancer cells. PSA protein levels measured in prostate microdissected cells were comparable to those reported previously by Simone et al [11], using a sensitive immunoassay.

Using LAM, in conjunction with real-time PCR methods, PSA can be localized and quantified in specific cells or areas of the prostate by serial sections. This way the PSA mRNA and protein levels were shown to be significantly correlated in normal and cancer prostate cells.

With our approach, it was also possible to measure PSA expression in different areas of the same histological section allowing comparison of mRNA and protein levels in normal epithelium and contiguous adenocarcinoma cells. Even if the number of samples examined in this study is very low, our results exclude dramatic differences of PSA expression between normal and cancer cells in the prostate.

Some interesting findings seem to derive from the analysis of microdissected areas of prostate stroma. As expected, we

were not able to detect any expression of PSA mRNA in stromal areas collected from normal and cancer prostates. Conversely, the concentration of PSA protein (expressed as molecules/ μm^2) is about 100-fold higher in stromal microdissected areas recovered from prostate cancer in comparison to normal prostate. This phenomenon seems to reveal an increased transit of the protein across the peritubular areas to reach capillaries. Our results reinforce the hypothesis previously presented by Webber et al [27] on the participation of PSA in tumor invasion. According to this hypothesis, PSA could play an active role in the prostate proteolytic cascade because disorganization of prostate epithelial cells should lead to a loss of polarized secretion, so that cells begin to secrete PSA and other proteases at their apical as well as basal ends [28]. This mechanism of abnormal secretion could contribute to the progressive proteolysis of basal membrane and extracellular matrix, promoting invasion and metastasis. According to this model [27], apparently supported by our data, the increased blood plasma PSA detectable in a large percentage of patients with prostate carcinoma is not necessarily the result of an increased expression by carcinoma cells, but it may be due to its diffusion through prostate stroma and leakage into the blood vessels.

Because of the limited number of subjects enrolled in our study, we cannot draw any new information on the physiopathologic role of PSA in prostate tissues. However, we think that our proposed model showed the feasibility of the simultaneous application of LAM in combination with the techniques for RT-qPCR and immuno-qPCR, for the sensitive, accurate, and specific quantification of cognate mRNA and protein in normal and pathological tissues.

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