

Amplification of Urokinase Gene in Prostate Cancer¹

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Abstract

Prostate cancer is the most common male malignancy in the United States as well as in many European countries. It is curable as long as it is localized, but the invasion of prostate cancer and formation of metastasis turn it into a life-threatening disease. Urokinase-type plasminogen activator (uPA) is believed to play a key role in tissue degradation and cell migration under various normal and pathological conditions, including cancer invasion and metastasis. Increased expression of uPA has been reported in various malignancies including prostate cancer. However, the mechanisms of the overexpression have remained poorly understood. Here, we report increased copy number of *uPA* gene in 3 of 13 hormone-refractory prostate carcinomas, including 1 high-level amplification. Real-time quantitative reverse transcription-PCR showed that the increased expression of uPA coincided with the amplification of the gene in these tumors. Matrigel invasion assay showed that prostate cancer cell line PC-3, containing amplification of the *uPA* gene, was more sensitive to the urokinase inhibitor, amiloride, than DU145 or LNCaP cell lines, which do not have the amplification. The findings suggest that one of the mechanisms underlying the overexpression of the uPA is the amplification of the gene, which is associated with the increased invasive potential of the cells.

Introduction

Prostate cancer is the most common male malignancy in many Western industrialized countries. As a localized disease, it is curable by prostatectomy or radical radiation therapy. However, as the tumor invades through the prostate capsule and eventually metastasizes, it becomes a life-threatening disease (1). To invade and metastasize, cancer cells must effectively degrade ECM³ components. Plasminogen activation has been implicated as one of the mechanisms of the ECM degradation. Mammalian cells contain two types of plasminogen activators, the urokinase type (uPA) and the tissue type (tPA), of which uPA is primarily involved in ECM degradation (2, 3).

The uPA system consists of the serine proteases uPA and plasmin, their serpin inhibitors PAI-1, PAI-2, and α 2AP, as well as an uPA cell surface receptor, uPAR (4). uPA is synthesized and released by cells as an inactive, single-chain proenzyme, pro-uPA (5). It binds to an uPAR on cell surface (4, 6), which has an accelerating and positioning function for both the uPA and plasminogen activation (6, 7). Pro-uPA is cleaved to an active, two-chain protease form by plasmin (5). uPA has restricted substrate specificity, the main function of which is the cleaving of plasminogen to plasmin (7), which degrades several ECM

components and also activates many pro-matrix metalloproteases (7, 8). Plasmin is thus able to indirectly promote further matrix degradation. The plasminogen activation system is specifically controlled by their serpin inhibitors PAI-1, PAI-2, and α 2AP, of which PAI-1 has a more important role in cancer invasion and α 2AP is the main inhibitor of plasmin (2, 9). However, during cancer invasion and metastasis, this degradation system goes out of control, allowing cancer cells to cross the normal tissue boundaries. Indeed, uPA is believed to play a key role in the cancer invasion and metastasis (2, 10). Both the uPA secretion and presence of receptor bound uPA at the cell surface characterize prostate cancer cells that have invasive phenotype (11).

Increased expression of urokinase has been reported in various malignancies including prostate (11, 12), breast (13), colon (14), and lung (15) cancers. In many cases, its increased expression seems to be associated with an increased metastatic potential and poor survival (16–18). The mechanisms of increased expression of uPA have, however, remained unknown.

The urokinase gene has been mapped to chromosome 10q24–qter (19). By using CGH, we have shown previously that ~15% of the hormone-refractory recurrent prostate carcinomas contain gain or amplification at 10q (20). In addition, prostate cancer cell line PC-3 shows high level amplification at 10p12–q23 by CGH (Fig. 1A; Ref. 21). Here, we first remapped the *uPA* gene to 10q22 and showed that it is amplified in the PC-3 cell line. Subsequently, we analyzed the frequency of *uPA* gene amplification in hormone-refractory prostate carcinomas by FISH and the expression of the gene by Northern analysis and real-time RT-PCR (LightCycler methodology). Finally, we studied the association between *uPA* gene amplification and sensitivity of the cells to the uPA inhibition by invasion assay.

Materials and Methods

Cell Lines and Tumor Samples. Prostate cancer cell lines PC-3, DU145, and LNCaP were obtained from American Type Culture Collection (Manassas, VA) and cultured according to the manufacturer's protocols. Freshly frozen TURPs from 13 patients diagnosed with locally recurrent hormone-refractory prostate cancer were obtained from Tampere University Hospital. The TURPs were performed on the patients because of urethral obstruction during hormonal therapy. The endocrine therapy modalities were orchiectomy (4 cases), luteinizing hormone-releasing hormone analogue (4 case), estrogen (1 case), maximal androgen blockade (3 cases), or unknown (1 case). The time from the beginning of the therapy to TURP varied from 16 to 60 months. The presence of >60% of tumor cells in the tissue samples was histologically confirmed by H&E-stained frozen section slides.

Southern Hybridization. Twenty μ g of PC-3 and normal lymphocyte genomic DNAs were digested with *Hind*III and separated in 0.7% agarose gel electrophoresis. Equal loading of the samples was verified by ethidium bromide staining of the gel after electrophoresis. The gel was then blotted using routine techniques (22). The PCR-amplified insert of IMAGE-EST (GenBank accession number AI818478) was labeled with [³²P]dCTP using a random priming labeling method (rediprime II; Amersham Pharmacia Biotech, Uppsala, Sweden) and used as a probe for uPA. The overnight hybridization and washes were carried out as described previously (22). The blot was visualized and quantified with the Storm PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

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³ The abbreviations used are: ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; α 2AP, α 2-anti-plasmin; TURP, transurethral resection of the prostate; TBP, TATA-box binding protein; RT-PCR, reverse transcription-PCR; FISH, fluorescence *in situ* hybridization; PAI, plasminogen activator inhibitor; CGH, comparative genomic hybridization; BAC, bacterial artificial chromosome.

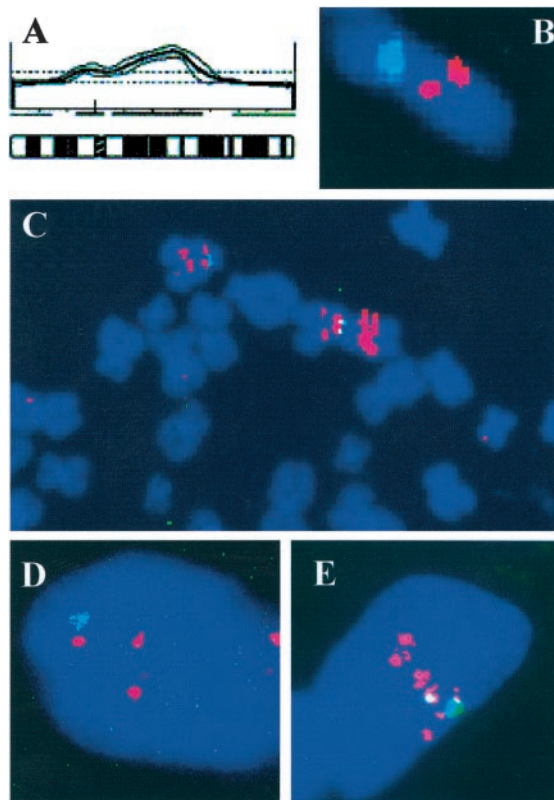


Fig. 1. A, mean (\pm SD) fluorescence ratio profile from p- to q-telomere for chromosome 10 in prostate cancer cell line PC-3 obtained by CGH. ---, the fluorescence ratio values 0.85 and 1.15. The profile indicates amplification at 10p12–q23 [according to Nupponen *et al.* (21)]. Two-color FISH analyses of normal (B) and PC-3 cell lines metaphase chromosome preparations (C), as well as interphase nuclei of two hormone-refractory prostate carcinomas (D and E) with BAC probe for the *uPA* gene (red signals) and chromosome 10 centromere-specific probe (green signals). B, the *uPA* maps to chromosome 10q22. C, multiple copies of *uPA* in the rearranged chromosomes are seen as a sign of high-level amplification in PC-3 cell line. D, three copies of *uPA* and one copy of the chromosome 10 centromere are seen in a hormone-refractory tumor. E, \sim 10 copies of *uPA* and 2 copies of the chromosome 10 centromere indicate 5-fold amplification of *uPA* gene.

Northern Hybridization. Total RNA was isolated from each of the cell lines using Trizol reagent (Life Technologies, Inc., Rockville, MD). Ten μ g of total RNA were separated in 1.2% denaturing formaldehyde-agarose gel electrophoresis and blotted (22). The [32 P]dCTP-labeled, PCR-amplified insert of IMAGE-EST (GenBank accession number A1818478) was used as a probe and hybridized overnight. After washing, the blot was visualized and quantified with the Storm PhosphorImager (Molecular Dynamics, Inc.). To verify equal loading of the gel, the blot was stripped and rehybridized using a 32 P-labeled probe for β -actin (Clontech, Palo Alto, CA) as described above.

FISH. A genomic clone for *uPA* was obtained by screening human BAC library with primers specific for the gene (5'-atc agc tgt aag aag agc tgg g-3' and 5'-atg ccc tgc cct ttt taa ct-3'). The authenticity of the clone (BAC-4613) was verified by partial sequencing. The BAC-*uPA* probe was labeled with digoxigenin-dUTP (Roche Diagnostics, Mannheim, Germany) by nick translation. SpectrumGreen-labeled, chromosome 10 centromere-specific probe (CEP10; Vysis, Inc., Downers Grove, IL) was used as a reference probe. Metaphase chromosome preparations from the prostate cancer cell lines and normal blood lymphocytes were prepared using routine techniques. Five- μ m

tissue sections from the freshly frozen tumor blocks were fixed on objective slides in a series of 50, 75, and 100% Carnoy's solution (3:1 methanol:acetic acid) 10 min each at room temperature. The dual-color FISH was performed essentially as described previously (23). Briefly, the slides were denatured in 70% formamide/2 \times SSC at 70.5°C for 2.5 min and dehydrated through an ethanol series. Hybridizations were carried out at 37°C for 48 h. After stringent washes, the slides were stained with anti-digoxigenin-rhodamine (Roche Diagnostics) and counterstained with an antifade solution (Vectashield; Vector Laboratories, Burlingame, CA) containing 4,6-diamidino 2-phenylindole. The FISH signals were scored from nonoverlapped epithelial cells using Olympus BX50 epifluorescence microscope (Tokyo, Japan). The criteria for high-level amplification was either the presence of the tight cluster of *uPA* signals or more than five copies of the gene.

Real-Time RT-PCR. The expression level of *uPA* in 12 tumor samples was studied using real-time RT-PCR. One to three 20- μ m frozen sections were cut from the tumor blocks using a cryotome. Total RNAs were isolated from the sections using Qiagen RNeasy MiniKit (Qiagen, Inc., Valencia, CA) and used for the first-strand cDNA synthesis with Superscript II reverse transcriptase and oligo d(T)_{12–18} primer according to manufacturer's protocol (Life Technologies, Inc.). For preparing the standard curve, 5 μ g of total RNA from normal mammary tissue (Clontech) were reverse transcribed as described above. After the first-strand cDNA synthesis, serial dilutions were made corresponding to cDNA transcribed from 500, 100, 20, 4, 0.8, and 0.16 ng of total RNA.

The primer and probe sequences used in the analyses of the expression of *uPA* and TBP genes are given in Table 1. The primers were designed to avoid amplification of any genomic DNA by choosing the forward and reverse primers for each of the genes from different exons. The PCR reactions were performed in the LightCycler apparatus (24) using the LC DNA Hybridization Probes kit (Roche Diagnostics). Thermocycling for each reaction was done in a final volume of 20 μ l containing 2 μ l of cDNA sample (or standard), 4 mM MgCl₂, 0.5 μ M of each primers, 0.2 μ M fluorescein, and 0.4 μ M LC Red640 labeled probes as well as 1 \times ready-to-use reaction mix including Taq DNA polymerase, reaction buffer, and deoxynucleotide triphosphate mix. After 30 s of initial denaturation at 95°, the cycling conditions of 55 cycles consisted of denaturation at 95° for 0 s, annealing at 57° (for *uPA*) or 58° (for TBP) for 13 s, and elongation at 72° for 9 (*uPA*) or 10 (TBP) s. The LightCycler apparatus measured the fluorescence of each sample in every cycle at the end of the annealing step. After proportional background adjustment, the fit point method was used to determine the cycle in which the log-linear signal is distinguished from the background, and that cycle number was used as a crossing-point value. Software produced the standard curve by measuring the crossing point of each standard and plotting them against the logarithmic value of concentrations. The concentrations of unknown samples were then calculated by setting their crossing points to the standard curve. The expression level of *uPA* was normalized by TBP. TBP was selected for the reference gene because there are no known retropseudogenes for it, and the expression of TBP is lower than in many commonly used abundantly expressed reference genes (25). After the PCR, all samples were also run in 1.2% agarose gel electrophoresis to ensure that the right size product was amplified in the reaction.

Invasion Assay. Invasion assay was done using Matrigel (BD Biosciences, Bedford, MA)-coated polyethylene terephthalate membrane with 8- μ m pore size (BD Biosciences). Matrigel was diluted to the concentration of 1 mg/ml in PBS. One hundred μ l of it were pipetted to a 24-well cell culture plate insert and allowed to solidified. The culture medium in the wells contained 5% FBS and fibronectin (5 μ g/ml) as chemoattractant. Cells (1 \times 10⁵; PC-3, DU145, or LNCaP) in culture medium containing 1% FBS were plated onto an insert. Half of the inserts were treated with 151 μ M amiloride (Sigma Chemical Co., St. Louis, MO) for 22 h. Subsequently, the cells inside the inserts were removed, and the invaded cells were fixed with methanol and stained with

Table 1 The primer and probe sequences used in the real-time RT-PCR

Gene	Primer sequences (5'-3')	Hybridization probe sequences (5'-3') ^a
<i>uPa</i>	TCACCACAAAATGCTGTGT AGGCCATTCTTCTTCTTGGT	TCCCCTGAGTCTCCCTGGCA-Fluorescein Red640-AATCTGTTTTCCACTGTGGGTCAGCAG
<i>TBP</i>	GAATATAATCCCAAGCGTTTG ACTTCACATCAGACTCCCC	TTTCCAGAAGTCAAATCAGTGGCC-Fluorescein Red640-TGGTTCGTGGCTCTCTTATCCTCATG

^a Purchased from Tib MolBiol (Berlin, Germany).

crystal violet. The number of invaded cells were counted under a microscope. The experiments were repeated four times per each cell line and treatment.

Results and Discussion

We have earlier shown by CGH that ~15% of the hormone-refractory tumors contain a gain at chromosome 10q (20). The *uPA* gene has been mapped previously to 10q24–qter (19), a region often deleted in prostate cancer. Because it has been found that old mapping data are sometimes inaccurate, we decided to remap the gene by FISH analysis. Indeed, we were able to show that the *uPA* gene actually maps to 10q22 (Fig. 1B). The “revised” localization of the gene is also supported by the recent human genome draft sequence assembly.

On the basis of the chromosomal localization of the gene, we speculated that the *uPA* gene might be amplified in prostate tumors. According to CGH analysis, the 10q22 region is amplified in the prostate cancer cell line PC-3 (Ref. 21; Fig. 1A). Thus, we first determined the copy number of the *uPA* gene in the cell line by FISH (Fig. 1C). It showed about 20–30 copies of the gene. In the analysis of the metaphase chromosomes, *uPA* signals were seen in several different chromosomes consistent with the spectral karyotyping data, which have earlier shown that chromosome 10 is involved in at least six different chromosomal rearrangements in the PC-3 cell line (26). Next, we confirmed the high-level amplification of *uPA* in PC-3 by Southern blotting, which showed ~2-fold increase in the copy number of urokinase gene as compared with the normal lymphoid DNA (data not shown). The Southern analysis was consistent with data published previously by Hollas *et al.* (27), suggesting a 2–3-fold amplification of *uPA* in PC-3 detected by Southern blotting. However, as compared with FISH analysis, the Southern blotting clearly underestimated the level of amplification. The DU145 and LNCaP cell lines showed three and four copies of both *uPA* and chromosome 10 centromere by FISH, respectively. The findings are consistent with the CGH data indicating no high-level amplification at 10q in these cell lines (21).

To evaluate the possible alterations of the *uPA* gene copy number in prostate tumors *in vivo*, we next studied the gene copy number in 13 locally recurrent hormone-refractory prostate carcinomas. We decided to analyze only hormone-refractory tumors because previous CGH studies have shown that the 10q gains are rarely found in androgen-dependent prostate tumors (28). FISH analyses of the tumors showed an increased copy number of the *uPA* gene in 3 of 13 cases. These included one high-level amplification (8%) and two moderately increased copy numbers (15%; Fig. 1, D and E). The FISH analysis of the tumor with high-level amplification showed tight clusters of signals making scoring the exact copy number difficult. However, we estimated that at least 10 copies of the gene and 2 copies of the centromere were present, indicating 5-fold amplification. In one of the cases with gain of *uPA*, the copy numbers of the *uPA* and centromere were 3 and 1, respectively. Whereas in the other case, both *uPA* and centromere probes showed four copies, suggesting tetraploidization. The observed frequency of increased *uPA* copy number in the locally recurrent hormone-refractory tumors is in good agreement with the published CGH studies. This is, at least to our knowledge, the first time that *in vivo* amplification of the *uPA* gene has been reported in solid tumors. Evidently, larger studies are needed to confirm the prevalence of the amplification of *uPA* and to study the whether the amplification is an early event or truly a late event, as current study and earlier CGH analyses have implicated.

Next, we studied whether the increased copy number of the *uPA* is associated with increased expression of the gene. Northern hybridization of three prostate cancer cell lines (Fig. 2) showed that the expression of urokinase is increased in the invasive PC-3, containing the high-level amplification of the gene. Also, the invasive DU145

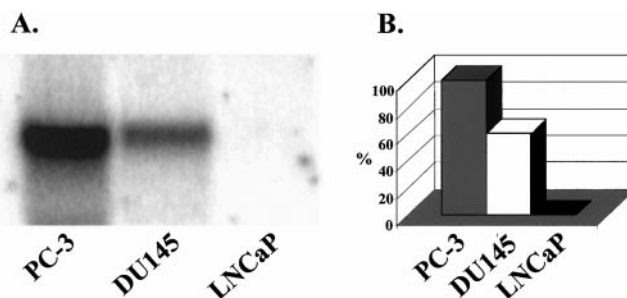


Fig. 2. A, Northern hybridization of *uPA* shows that invasive prostate cancer cell lines PC-3 and DU-145 express high levels of *uPA*, whereas the noninvasive LNCaP cell line does not. B, the relative expression level of *uPA* (*uPA*/β-actin) after quantification with the PhosphorImager. Results showed that the PC-3 cell line expressed two times more *uPA* than DU145.

cell line expressed a high level of the *uPA*. On the contrary, no expression was found in the noninvasive LNCaP cell line. According to quantification with the PhosphorImager, *uPA* was expressed two times more in PC-3 than in DU145. The findings are in consistent with a previous report of the levels of *uPA* mRNA in these cell lines (27).

The expression of *uPA* in the locally recurrent hormone-refractory prostate tumors was evaluated by quantitative real-time RT-PCR analysis. The results showed that the expression of urokinase was increased in two of three samples with the increased copy number of *uPA* as compared with *uPA* expression levels in tumors with normal gene copy number (Fig. 3). The case with the high-level amplification expressed two times more *uPA* than the other tumors on average. The association between the amplification and overexpression of the gene both in the cell lines and the tumors suggests that *uPA* is, indeed, a strong candidate target gene for the 10q amplification in prostate cancer. However, based only on the gene copy number and expression data, it is not possible to rule out completely the presence of other target genes for the 10q gain.

Van Veldhuisen *et al.* (12) have earlier shown by immunohistochemistry that ~70% of the prostate tumors with extracapsular extension express highly *uPA*, whereas only 27% of the tumors without capsular invasion do. The mechanisms and pathways by which tumor cells acquire increased *uPA* expression are most probably various. Our results suggest that amplification of the *uPA* gene is one of such mechanisms, at least in locally recurrent hormone-refractory prostate carcinomas. Other suggested mechanisms include thrombin, which has been shown to increase the expression of *uPA* in PC-3 cell line (29). In addition to the expression level, the activity of *uPA* is regulated by conversion of pro-*uPA* to an active two-chain form of *uPA*. This conversion may be enhanced by, *e.g.*, prostate-specific antigen, the serum levels of which increase in almost all patients with prostate cancer (30).

The high-level amplification of the *uPA* gene was found in an androgen-independent prostate cancer cell line and a tumor. The emergence of androgen-independent disease is considered to be a late event in the progression of prostate cancer. It almost always requires the presence of hormonal therapy (1). Whether the selection of the amplification is associated with the treatment, as amplification of androgen receptor gene as has been demonstrated to be (31), remains to be studied. However, based on the function of the *uPA*, it is more likely that the processes related to invasiveness rather than hormonal therapy are associated with the selection of the *uPA* amplification.

Because of the indications that *uPA* plays a central role in the invasion of cancer, it has been suggested that *uPA* could be therapeutically targeted. Several compounds that inhibit the activity of *uPA* in prostate cancer *in vitro* have been demonstrated. These include retinoic acid and maspin (32, 33). In addition, using prostate cancer

xenografts established by implanting DU145 and LNCaP cells into nude mice, Jankun *et al.* (34) showed that both PAI-1 and several small molecule uPA inhibitors do not just inhibit the invasion but also reduce tumor growth. On the other hand, there are examples indicating association between treatment sensitivity and amplification of the target gene of the treatment (35). To evaluate whether the amplification of uPA gene could be associated with treatment sensitivity, we studied the inhibition of uPA in three prostate cancer cell lines (PC-3, DU145, and LNCaP) using Matrigel invasion assay (Fig. 4). Both PC-3 and DU145 cells, which express uPA, showed invasion through Matrigel-coated filters, whereas LNCaP, which does not express uPA, had only very weak invasive capability. When a known uPA inhibitor, amiloride (34), was added to growth medium, the invasiveness of PC-3, containing the gene amplification, was reduced ~10-fold, whereas the number of invasive cells of DU145 was only marginally reduced. The reduction of invading cells in PC-3 was statistically significant. Thus, at least in these prostate cancer models, the uPA

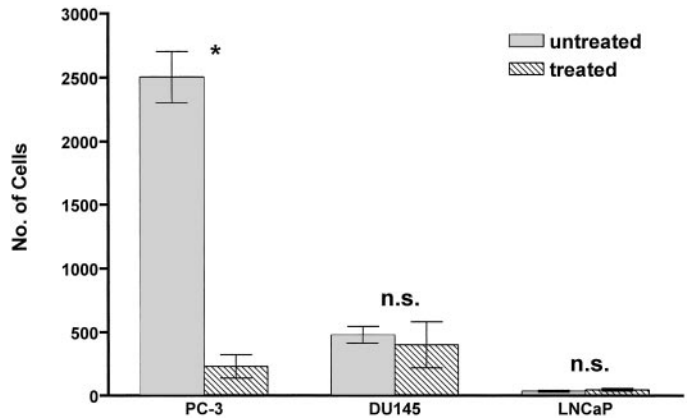


Fig. 4. Effects of amiloride on the invasive ability of PC-3, DU145, and LNCaP cells as examined by the Matrigel invasion assay. Cells were amiloride or mock-treated for 22 h. Subsequently, the cells invaded through the gel were counted. Data represent means of four experiments; bars, SE. *, $P < 0.001$; n.s., statistically nonsignificant difference (Student's *t* test).

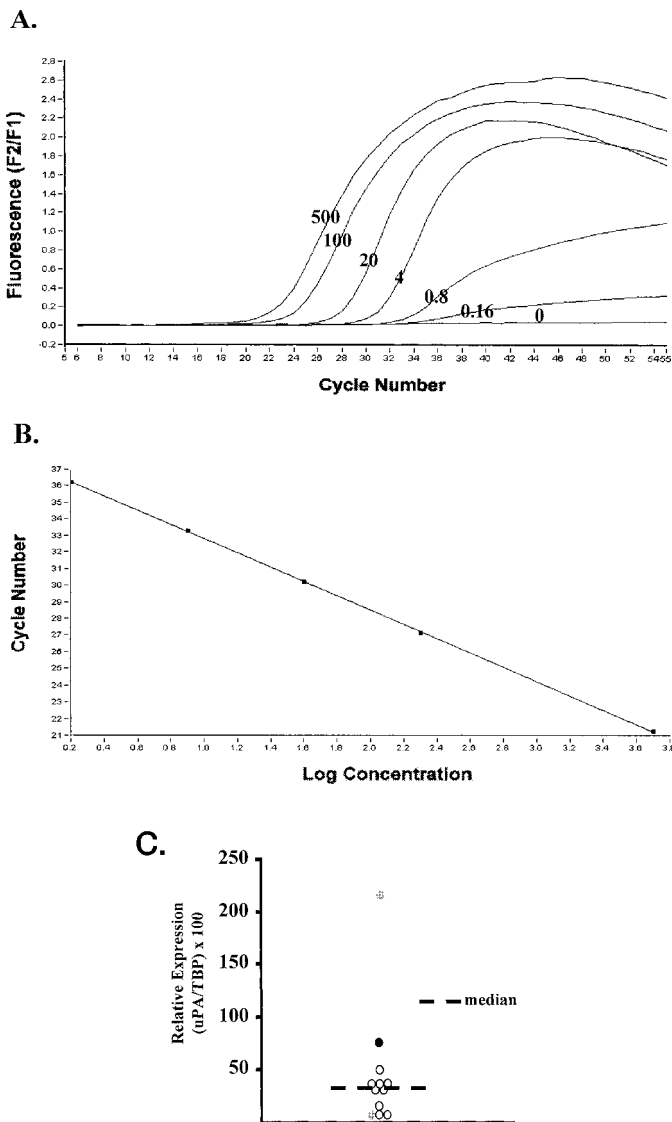


Fig. 3. Expression level of uPA measured by real-time RT-PCR. A, PCR curves of the serially diluted standard containing cDNA transcribed from 500, 100, 20, 4, 0.8, and 0.16 ng of total RNA (B). Standard curve blotting fractional cycle number at the fluorescent threshold for each standard curve presented in A. C, the relative expression of uPA genes in prostate tumor samples. ●, a tumor containing high-level androgen receptor gene amplification; gray circle, tumors with gain of the uPA gene; ○, tumors without uPA gene copy number alterations. Horizontal scattered line, the median value of expression.

gene amplification was associated with cells sensitive to the uPA inhibitors. The finding further supports the implication that uPA is one of the target genes for the 10q gain and that it may well be a potential treatment target.

In conclusion, we report here, for the first time, a high-level amplification of the uPA gene in hormone-refractory prostate carcinoma *in vivo*. The increased copy number was associated with increased expression of the gene and with the sensitivity of the cells to uPA inhibition. The findings support the suggestion that uPA is involved in the acquisition of invasive phenotype in prostate cancer cells.

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