

# Novel identification of the ETS-1 splice variants p42 and p27 in prostate cancer cell lines

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**Abstract.** ETS-1 is involved in cellular functions such as proliferation, migration, invasion, apoptosis and angiogenesis. The *ETS-1* gene encodes three distinct proteins, ETS-1 p51 encoded by a full-length mRNA, ETS-1 p42 and ETS-1 p27 encoded by an alternatively spliced mRNA lacking exon VII and exons III-VI, respectively. ETS-1 p51, commonly considered to be the active form, has been studied in prostate cancer (PCa). However, the ETS-1 p42 and p27 variants have not yet been identified in PCa. Therefore, we aimed in this study at investigating whether the splice variants p42 and p27 are expressed in the androgen-dependent VCaP and LNCaP and the androgen-independent PC3 and DU-145 PCa cell lines. Using RT-PCR, we found the expression of both splice variants p42 and p27 at the mRNA level in the VCaP, LNCaP, PC3 and DU-145 PCa cell lines. We then confirmed the expression of ETS-1 p51 and its splice variants p42 and p27 at the protein level using an anti-ETS-1 antibody directed against the DNA-binding domain (DBD) in lysates prepared from the latter-mentioned cell lines, as well as in PC3 cell nuclear extract. Moreover, differences in the expression ratios of the ETS-1 splice variants within each cell line were also found. In conclusion, we have demonstrated for the first time the novel identification of the ETS-1 splice variants p42 and p27 in PCa cell lines. It is very likely that the role of ETS-1 p51 in PCa is significantly influenced by the presence of its splice variants ETS-1 p42 and p27 as competition, abundance, affinity, interactions and cross-talk among them will eventually determine the genes that will be targeted and subsequently affect cellular functions. Follow-up studies will need to address in functional terms, the roles of these splice variants in PCa cell lines, as well as their expression in PCa tissues and its correlation with clinical outcome.

## Introduction

The ETS family of transcription factors which consists of 27 members in humans is characterized by an evolutionarily highly conserved DNA-binding domain, the ETS domain (1). ETS-1, the prototype of the ETS family is known to be involved in a broad variety of cellular functions (such as cell proliferation, migration, invasion, apoptosis and angiogenesis) (2). We have previously reported that ETS-1 promotes proliferation, migration or invasion of various neoplastic cell lines including PC3 prostate cancer cells, melanoma, HeLa, as well as glioma cells (3-6). Furthermore, ETS-1 has been reported to be overexpressed in latent and clinically manifest prostatic carcinomas and a strong expression of ETS-1 has been associated with poor tumor differentiation (7).

The *ETS-1* gene encodes three distinct proteins, ETS-1 p51 encoded by a full-length mRNA, ETS-1 p42 and ETS-1 p27 encoded by an alternatively spliced mRNA lacking exon VII and exons III-VI, respectively (8,9).

The ETS-1 p42 splice variant (without exon VII) lacks an essential part of the autoinhibitory module and the calcium-sensitive serines, which are phosphorylated upon calcium activation leading to the inhibition of DNA binding by reinforcing autoinhibition (2,10). Therefore, the lack of exon VII allows ETS-1 p42 to form stable complexes with DNA and contributes to the ability of the ETS-1 p42 protein to resist the negative action of calcium (2). ETS-1 p42 has been reported to bind more strongly to target DNA than ETS-1 p51 (11), commonly considered to be the active form, and to regulate different target genes in colon carcinoma cells (12). We have recently evaluated the functional effects of ETS-1 p42 in comparison to ETS-1 p51 expression in a well-characterized mouse fibroblast cell line, and found that ETS-1 p42 has different functions and target genes compared to ETS-1 p51 (e.g. TIMP-4, MMP-3, MMP-9 and MMP-13) (13).

On the other hand, the ETS-1 p27 splice variant (without exons III-VI) lacks the threonine-38 residue, the pointed (PNT) and the transactivation (TAD) domains affecting the transcriptional activity of ETS-1 and the transactivation of its target genes (2,9). Interestingly, both inhibitory domains that surround the DNA-binding domain are conserved in ETS-1 p27, indicating that ETS-1 p27 is autoinhibited for DNA binding like the full length ETS-1 p51 (9).

The ETS-1 p51 protein and its splice variant p27 have been investigated in functional terms in mammary carcinoma

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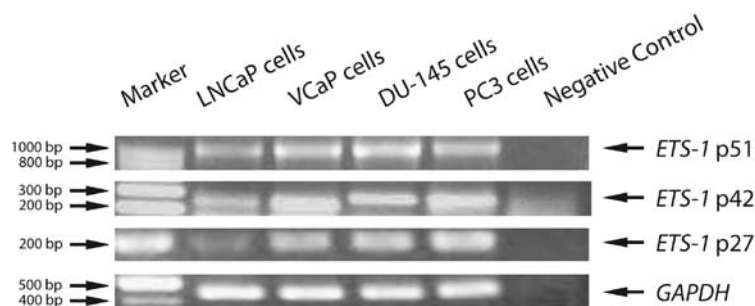


Figure 1. mRNA expression of *ETS-1* and its splice variants in prostate cancer cell lines. RT-PCR analysis showing the full length *ETS-1* p51 and its splice variants p42 and p27 in the androgen-dependent LNCaP and VCaP, and the androgen-independent DU-145 and PC3 prostate cancer cell lines. The gene *GAPDH* was used as a loading control, and amplification without a template was used as negative control.

cells (9). ETS-1 p27 has been shown to bind DNA in the same manner as ETS-1 p51 and to act both at a transcriptional and a subcellular level (9). More specifically, ETS-1 p27 has been shown to block ETS-1 p51-mediated transactivation of target genes in mammary carcinoma cells and to induce the translocation of ETS-1 p51 from the nucleus to the cytoplasm (9). Furthermore, the overexpression of ETS-1 p27s has been shown to repress the tumor properties of mammary carcinoma cells (9).

In PCa, the ETS family of transcription factors became a major focus of interest due to the frequent reporting of recurrent gene fusions occurring between the androgen-regulated prostate-specific serine protease *TMPRSS2* gene, and several members of the ETS family such as *ETV-1*, *ETV-4*, *ETV-5*, and most commonly *ERG* (14-16). In previous studies, we were able to show that the ETS family prototype, ETS-1 is important for the biological properties of PCa cells (6), as well as the regulation of other ETS family members which might thus constitute a complex transcriptional regulatory network (17) that possibly involves the ETS-1 splice variants.

Since both ETS-1 p42 and p27 have not yet been investigated in PCa cell lines, we aimed in this study at examining whether the ETS-1 splice variants p42 and p27 are expressed in the androgen-dependent VCaP and LNCaP and the androgen-independent PC3 and DU-145 PCa cell lines.

## Materials and methods

**Cell culture.** The prostate cancer cell lines LNCaP, VCaP, PC3 and DU-145 cells were grown in RPMI (Invitrogen, USA), DMEM (ATCC, USA), F12K (Invitrogen, USA) and MEM (Invitrogen, USA) media, respectively. The media were supplemented with 0.5% penicillin/streptomycin (Invitrogen) and 10% heat-inactivated fetal calf serum (Invitrogen). The MEM and RPMI media were additionally supplemented with 1% glucose (Invitrogen), 1% sodium pyruvate (Invitrogen) and 1% non-essential amino acids (NEIAA) (Invitrogen).

**Preparation of cell lysates.** Cell lysates were prepared from  $\sim 5 \times 10^6$  cells as follows. Briefly, the pelleted cells were resuspended in a lysis buffer containing 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40, and freshly added protease inhibitor (Complete Mini, Roche, Germany). The samples were then vortexed for 10 sec and incubated on ice for 15 min. Thereafter, the samples were centrifuged at  $14,000 \times g$  in a

pre-cooled centrifuge for 10 min at  $4^\circ\text{C}$ . The supernatants were then collected and labeled as cell lysates.

**Western blot analysis.** Following the determination of protein concentrations, the presence of ETS-1 p51, p42 and p27 in the cell lysates, and in PC3 cell nuclear extracts (Santa Cruz Biotechnology, Santa Cruz, USA) was determined by western blot analysis using rabbit polyclonal C-20 anti-ETS-1 antibody directed against the DNA-binding domain (DBD) (Santa Cruz Biotechnology). Furthermore, the housekeeping protein  $\beta$ -actin, an internal control was detected using rabbit polyclonal anti- $\beta$ -actin antibody (Sigma-Aldrich, Germany).

**RNA isolation and cDNA synthesis.** RNA was isolated from the prostate cancer cell lines using the RNeasy Mini kit (Qiagen, Germany) as described by the manufacturer. The amount of the isolated RNA was then measured with the ND1000 Nanodrop (Peglab, USA). Reverse transcription of total-RNA was performed with the SuperScript III First-Strand Synthesis SuperMix as described by the manufacturer (Invitrogen).

**PCR.** The polymerase chain reaction (PCR) was performed using the DNA-Engine Peltier Thermal Cycler (Bio-Rad Laboratories, Germany) to analyze the expression of *ETS-1* p51, p42, p27 and the house-keeping gene *GAPDH* (9), as well as ETS-1 p42 (13) were designed as previously described. All PCR products were analyzed on 2% agarose gels that were stained with ethidium bromide.

## Results

**mRNA expression of *ETS-1* and its splice variants in prostate cancer cell lines.** Using RT-PCR analysis, the mRNA expression of the full length ETS-1 p51 and its splice variants p42 and p27 was found in the androgen-dependent LNCaP and VCaP, and the androgen-independent DU-145 and PC3 PCa cell lines (Fig. 1).

**Protein expression of *ETS-1* and its splice variants in prostate cancer cell lines.** Western blot analysis using anti-ETS-1 antibody directed against the DNA-binding domain (DBD) (Santa Cruz Biotechnology) demonstrated that ETS-1 p51, p42 and p27 are expressed in PC3 cell nuclear extracts and in lysates prepared from the androgen-dependent VCaP and LNCaP and

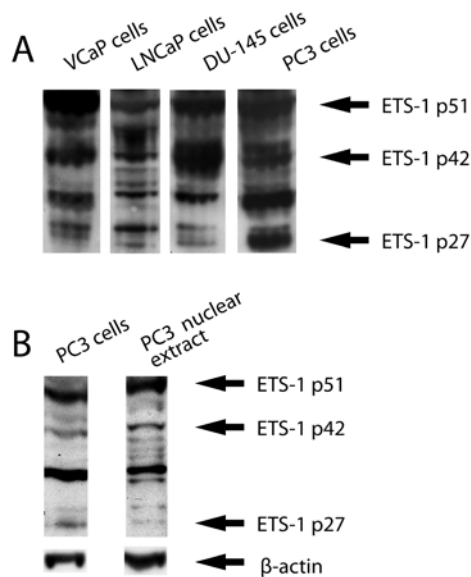


Figure 2. Protein expression of ETS-1 and its splice variants in prostate cancer cell lines. Western blot analysis using an anti-ETS-1 antibody directed against the DNA-binding domain (DBD) shows the protein expression of ETS-1 p51, p42 and p27 in VCaP, LNCaP, DU-145 and PC3 prostate cancer cell lines (A), and in PC3 cells/nuclear extract along with the housekeeping protein  $\beta$ -actin as a loading control (B).

the androgen-independent PC3 and DU-145 PCa cell lines (Fig. 2).

A strong band of ~35 kDa was also detected, and it is likely to correspond to another splice variant of ETS-1 (without exons IV and VII) (18), which has not yet been reported to result in the generation of a protein (Fig. 2). Additionally, the several weak bands that were also detected are very likely to be the phosphorylated forms of ETS-1 (Fig. 2).

## Discussion

ETS-1 overexpression has been associated with latent and clinically manifest prostatic carcinomas, as well as poor tumor differentiation (7). In previous studies, we found that ETS-1 is involved in a variety of cellular processes (e.g. proliferation, migration and invasion) of several neoplastic cell lines such as, melanoma, HeLa, glioma cells as well as PC3 prostate cancer cells (3-6). In PC3 cells, ETS-1 promotes their migration and regulates the expression of many genes involved in important steps of the so-called metastatic cascade (6,19).

Besides ETS-1 p51, commonly considered to be the active form, which is encoded by a full-length mRNA, *ETS-1* encodes two other distinct proteins, ETS-1 p42 and p27, which are encoded by an alternatively spliced mRNA lacking exon VII and exons III-VI, respectively (8,9). These two splice variants have been studied by us and others in functional terms in cell lines such mouse fibroblasts and mammary carcinoma cells as described above (9,11-13). However, both splice variants have not yet been investigated in prostate cancer cell lines.

Therefore, we investigated in this study whether the ETS-1 splice variants p42 and p27 are expressed in the androgen-dependent VCaP and LNCaP and the androgen-independent PC3 and DU-145 PCa cell lines.

Using RT-PCR, we observed the expression of both splice variants p42 and p27 at the mRNA level in the androgen-dependent VCaP and LNCaP and the androgen-independent PC3 and DU-145 PCa cell lines (Fig. 1).

We then confirmed the expression of ETS-1 p51 and its splice variants p42 and p27 at the protein level using an anti-ETS-1 antibody directed against the DNA-binding domain (DBD) in lysates prepared from the androgen-dependent VCaP and LNCaP and the androgen-independent PC3 and DU-145 PCa cell lines (Fig. 2). All 3 proteins were also expressed in PC3 cell nuclear extracts (Fig. 2).

Notably, a strong band of approximately 35 kDa was also detected in the lysates as well as in the nuclear extract, and it is likely to correspond to another splice variant of ETS-1 (without exons IV and VII) (18), which has not yet been reported to result in the generation of a protein (Fig. 2). The several weak bands that were detected close to the molecular weight area of ETS-1 p42 and p27 are very likely to be the phosphorylated forms of ETS-1, as ETS-1 is commonly regulated by phosphorylation (2) (Fig. 2).

Our results also show that there are differences in the expression ratios of the full length ETS-1 protein and of the ETS-1 splice variants within each cell line. These differences are most likely due to the different biological properties of these cell lines. The DU-145, PC3, LNCaP and VCaP cell lines are derived from different origins, brain metastasis, an advanced androgen-independent bone metastasis, a supraclavicular lymph node metastasis and a metastatic lesion to a lumbar vertebral body of a patient with hormone refractory prostate cancer, respectively.

It is worth noting that the differences in the expression ratios of the ETS-1 splice variants within each cell line is highly interesting in light of a previous study demonstrating that an increase in the ETS-1 p27/ETS-1 p51 ratio induces the delocalization of ETS-1 from the nucleus to the cytoplasm in mammary carcinoma cells (9). Therefore, the ratios that we found of the ETS-1 splice variants might also be significant in PCa, in the sense that they reflect their mechanistic complexity, competition among splice variants based on their abundance, affinity, co-factors and genes that may be regulated by different ETS-1 proteins in PCa.

Our preliminary findings about the expression level of a specific ETS-1 splice variant in a PCa cell line may provide us in further studies with insights into the molecular mechanisms of action of these splice variants in PCa, due to the already known unique properties of each splice variant (8,9), and their different target genes (9,12,13). For instance, in comparison to ETS-1 p51, ETS-1 p42 lacks an essential part of the autoinhibitory module and the calcium-sensitive serines, which are phosphorylated upon calcium activation leading to the inhibition of DNA binding by reinforcing autoinhibition (2,10), whereas the ETS-1 p27 lacks the threonine-38 residue, the pointed (PNT) and the transactivation (TAD) domains affecting the transcriptional activity of ETS-1 and the transactivation of its target genes (2,9).

Taken together, we have demonstrated for the first time the novel identification of the ETS-1 splice variants p42 and p27 in PCa cell lines. It is very likely that the role of ETS-1 p5, commonly considered being the active form, in PCa is significantly influenced by the presence of its splice variants

ETS-1 p42 and p27, as competition, abundance, affinity, interactions, and cross-talk among them will eventually determine the genes that will be targeted and subsequently affect cellular functions. Therefore, follow-up studies will address in functional terms, the roles of these ETS-1 splice variants in PCa cell lines, as well as their expression in prostate cancer tissues and their correlation with clinical outcome.

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