Differential expression of TGFβ-stimulated clone 22 in normal prostate and prostate cancer

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The transforming growth factor-β (TGFβ) superfamily and its downstream effector genes are key regulators of epithelial homeostasis. Altered expression of these genes may be associated with malignant transformation of the prostate gland. The cDNA array analysis of differential expression of the TGFβ superfamily and functionally related genes between patient-matched noncancerous prostate (NP) and prostate cancer (PC) bulk tissue specimens highlighted two genes, namely TGFβ-stimulated clone-22 (TSC-22) and Id4. Verification of their mRNA expression by real-time PCR in patient-matched NP and PC bulk tissue, in laser-captured pure epithelial and cancer cells and in NP and PC cell lines confirmed TSC-22 downexpression, but not Id4 overexpression, in PC and in human PC cell lines. Immunohistochemical analysis showed that TSC-22 protein expression in NP is restricted to the basal cells and colocalizes with the basal cell marker cytokeratin 5. In contrast, all matched PC samples lack TSC-22 immunoreactivity. Likewise, PC cell lines do not show detectable TSC-22 protein expression as shown by immunoblotting. TSC-22 should be considered as a novel basal cell marker, potentially useful for studying lineage determination within the epithelial compartment of the prostate. Conversely, lack of TSC-22 seems to be a hallmark of malignant transformation of the prostate epithelium. Accordingly, TSC-22 immunohistochemistry may prove to be a diagnostic tool for discriminating benign lesions from malignant ones of the prostate. The suggested tumour suppressor function of TSC-22 warrants further investigation on its role in prostate carcinogenesis and on the TSC-22 pathway as a candidate therapeutic target in PC.

Key words: prostate cancer; TGFβ superfamily; TSC-22; basal cell marker

The transforming growth factor-β (TGFβ) and other members of the related TGFβ superfamily are key factors in maintaining epithelial cell homeostasis. Cellular actions of TGFβ include growth inhibition, stimulation of differentiation and induction of apoptosis.1 These actions are mediated by receptor-activated phosphorylation of the smad signalling pathway, which cross-talks with many other signalling pathways leading to the final activation of more than 300 downstream effector genes.2 In the prostate, TGFβ receptors (TGFβR) are ubiquitous but predominantly found in the basal cells,3,4 which harbour the stem cell compartment of the glandular epithelium.5 In cancer the role of TGFβ is complex, acting both as a tumour suppressor and as a tumour promoter. This ambiguity depends on accumulation of alterations that progressively unbalance TGFβ signalling towards increased oncogenic effects.2 Alterations in the TGFβ signalling pathway have been reported in prostate cancer (PC),6,7 and may contribute to the development and progression of PC. This is supported by the fact that overexpression of TGFβ and loss of TGFβRII expression are associated with poor clinical outcome.4

There has been no systematic survey focused on the expression of the TGFβ superfamily and downstream genes in PC. Therefore, we investigated differential gene expression in patient-matched noncancerous tissue of the prostate (NP) and PC by using cDNA arrays displaying an extended list of genes functionally associated with the TGFβ superfamily. We found TGFβ-stimulated clone-22 (TSC-22), a TGFβ target gene,9 to be differentially expressed in NP and PC tissue, and TSC-22 protein to be exclusively localised in the basal cells of the acinar epithelium of NP.

Material and methods

Tissue sampling and cell culture

Tissue sampling was approved by the local ethical committee and informed consent was obtained from all patients. Radical prostatectomy specimens (n = 21; mean age, 65 year) were collected at the Department of Urology, University of Bern, Switzerland. Within 5–10 min from surgical excision, samples of PC tissue or NP tissue were taken and either snap-frozen or immersed in RNA-later (Qiagen, Basel, Switzerland). Tissue adjacent to the respective samples was processed for paraffin embedding and served as histological control. Histological diagnosis and grading were performed by a pathologist (R.M.). The various specimens were comprehensive of Gleason patterns 2–5 (Gleason scores 5–9). The human PC cell line PC-3 (ATCC-LGC Promochem, Molsheim, France) was grown in DMEM supplemented with 10% FCS (BioWittaker, Verviers, Belgium). The normal rat ventral prostate cell line NbE,10 the human PC cell line LNCaP and the LNCaP-derived cell lines C4-2 and C4-2B4 (Urocor, Oklahoma City, OK, USA) were grown in T Medium.11 Cells at 70–80% confluence were used for RNA and for whole cell protein preparations.

cDNA array analysis and semi-quantitative real-time PCR analysis

Total RNA from tissue stored in RNA-later (Qiagen) and from cell lines was isolated using the RNeasy Midi Kit (Qiagen). For array analysis, quality was verified on a RNA LabChip (Agilent Technologies, Basel) with an Agilent Bioanalyzer 2100 (Agilent Technologies), and high quality total RNA was reverse-transcribed with addition of [32P] dCTP (Amersham Biosciences, UK). The labelled cDNA was hybridised on a human specific gene array including 96 genes directly or functionally related to the TGFβ superfamily (GEArray Q Series Human TGFβ BMP Signalling Pathway Gene Array, SuperArray, USA). Its functional gene grouping is available online (http://www.superarray.com/gene_array_product/HTML/HS-023). Washed filters were exposed for 24 hr to a PhosphorImager screen (Molecular Dynamics, San Francisco, CA).

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CA), scanned with a Storm 860 (Molecular Dynamics), quantified with the Image Quant (Molecular Dynamics) and analysed with the GEArray Analyzer software (Superarray). After background subtraction, intensity for each of the single dots was normalised to the hybridisation signal of GAPDH. The threshold for differentially expressed genes was arbitrarily set ≥2. Real-time PCR was performed with an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) using exon specific primers (Applied Biosystems) for TSC-22 (Hs_00234686_m1) and ID4 (Hs_00155465_m1). The resulting values were normalised to GAPDH (Hs_99999905_m1) or 18s (Hs_99999901_s1).

**Laser capture microdissection (LCM)**

Six micrometers cryosections were treated according to the Histo Gene LCM Frozen Section staining Kit (Arcturus, Bucher Biotech, Basel, Switzerland). The P.A.L.M. Micro-Beam system (P.A.L.M. Microlaser Technologies, Bernried, Germany) was used to excise ~1000 pure epithelial cells from patient-matched NP and PC tissues. Total RNA was extracted with the Pico Pure RNA Isolation Kit (Arcturus).

**Monoclonal antibody production**

Recombinant rat TSC-22 protein (rTSC-22) was produced as a GST fusion protein from the pGEX-T4 vector (kindly provided by Dr. L. Raftery, Cutaneous Biology Research Center, Massachusetts General Hospital, Charlestown, USA) as described previously. Recombinant human TSC-22 protein (hTSC-22) was generated with the FreeStyle 293 expression system (Invitrogen, Breda, Netherlands) according to the manufacturers protocol, using a pcDNA3.1 vector (Invitrogen) containing the human TSC-22 coding sequence. The sequence was amplified from cDNA of NP tissue using specific primers including a HindIII upstream (5'-AAGCTTataaataccactagtg-3') and a XhoI downstream (5'-CGTCGACTgtggtggtcctga-3') restriction site. Crude lysate of FreeStyle 293-F cells was harvested 62 hr after transfection. Female BALB/c mice kept according to the Swiss guidelines for the care and use of laboratory animals were immunised with the purified recombinant rTSC-22 protein. Monoclonal antibodies were prepared according to standard protocols. Specificity of the IgGs secreted by the various hybridomas was tested with recombinant rTSC-22 and with recombinant hTSC-22 proteins by ELISA and immunoblotting, respectively. Positive hybridoma cultures were subcloned twice by limited dilution. Clone IIIG12F6 (TSC-22-mAb-1) and clone VA2G8 (TSC-22-mAb-2) were further characterised as described later.

**Immunoblotting and immunoprecipitation**

Cells were lysed using RIPA buffer, supplemented with 1 mM phenyl-methyl-sulfonyl-fluoride (Sigma, Buchs, Switzerland) and total protein was loaded at a concentration of 30 μg/lane. rTSC-22 was loaded at a concentration of 50 ng/lane with or without β-mercaptoethanol (BME). Proteins were separated on SDS-polyacrylamide gels and blotted on Hybond-P membranes (Amersham Biosciences). TSC-22mAb-1 and 2 (5 μg/ml) and a monoclonal mouse anti-actin antibody (1:5,000) (Chemicon International, Juro Supply GmbH, Lucerne, Switzerland) were used as primary antibodies. Immunoreactivity was visualised with a peroxidase-labelled anti-mouse secondary antibody (Amersham Biosciences) and the ECL Advanced chemiluminescence substrate (Amersham). Mouse-IgG (Jackson ImmunoResearch, La Roche, Switzerland) and rabbit serum served as negative controls. Biotinylated anti-IgG antibodies followed by streptavidin/horseradish-peroxidase conjugate (Amersham Biosciences) were used as detection system. 3-Amino-9-ethyl-carbazole (AEC, Sigma, Buchs, Switzerland) served as chromogen. Specificity of the staining was further demonstrated by preabsorption of 2 μg/ml TSC-22mAb-1 with 10 μg/ml rTSC-22 for 10 min at room temperature. For colocalisation of cytokeratin 5 (CK5) and TSC-22, sections were first incubated with 0.3 μg/ml of the anti-CK5/anti-actin antibody. Anti-mouse-Cy3-diluted 1:200 (Amersham Biosciences) served to label in situ the anti-CK5/6 antibody (Chemicon International). After a blocking step with irrelevant IgG1, the sections were further incubated with 10 μg/ml TSC-22mAb-1 pre-labelled with the Zenon Alexa 546 fluorophore (Molecular Probes/Invitrogen).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism Version 4 (GraphPad Software Inc., USA), applying the paired t-test for the evaluation of patient-matched mRNA expression and the unpaired t-test for comparison of mRNA expression in bulk and laser-captured tissue. Expression levels of mRNA in cell lines were compared using one-way analysis of variance. A p-value equal or superior to 0.05 was considered as nonsignificant.
Results
cDNA array analysis and TSC-22 mRNA expression in patient-matched NP and PC tissue

Gene expression analysis showed that ~26% of the spotted cDNAs gave a detectable signal above background. The hybridisation pattern of gene expression among the 3 patients was more consistent in NP than in PC. Twenty-two genes (23% of the spotted cDNAs) were found to be expressed either in all 3 NP or in all 3 PC tissues. None of these genes was found to be uniquely expressed in NP or PC. TGFβ3, MADH2, c-fos, Id4, stat1, TGFB1I1, TIMP1 and TSC-22 attained a ≥2.5-fold differential expression in at least one patient. The mean expression ratios for the 14 genes displaying signal intensities above background in at least two matched cDNA samples for both NP and PC are shown in Figure 1. By setting the threshold factor at ≥2, three genes attained differential expression: Id4 (≥2.2), stat1 (≥2.3) and TSC-22 (≥4.3). Elevation of stat1 expression has already been reported in PC.15 Therefore, the expression of only TSC-22 and Id4 was further analysed by real-time PCR of cDNA derived from bulk tissue samples for a total of 9 patients, including the three patient tissue specimens that were previously investigated by cDNA array analysis. Six out of the 9 patients showed underexpression of TSC-22 in matched NP and PC tissues (Fig. 2a). However, this difference was not statistically significant. In contrast, significant underexpression of TSC-22 was found, analysing pure populations of epithelial cells isolated by LCM from sections of patient-matched NP and PC tissue. TSC-22 was underexpressed in the PC tissue of all 11 patient-matched samples analysed, (mean decrease 65%, ±21% SD; p = 0.0022; Fig. 2b). In NP, TSC-22 mRNA expression was 5.5 times lower in bulk tissue as compared to laser-captured epithelial cells (p = 0.0005; Fig. 2c). Instead, real-time PCR analysis could not confirm overexpression of Id4 (not shown).

Characterisation of anti-TSC-22 monoclonal antibodies

TSC-22mAb-1 and TSC-22mAb-2 recognise both rTSC-22 and hTSC-22 (Figs. 3a and 3b). Either antibodies recognises two bands of a molecular weight of approx. 15 and 30 kDa when electrophoresis of rTSC-22 is performed under nonreducing conditions (Fig. 3a). Under reducing conditions, both bands are still recognised, although the 30 kDa band to a much lower extent. LC-MS/MS revealed that TSC-22 was still present in this band (data not shown). In addition, TSC-22mAb-2 detected hTSC-22 immunoprecipitated with TSC-22mAb-1 from crude hTSC-22 protein preparations (Fig. 3b). Only TSC-22-mAb1 was found suitable for immunohistochemistry.

TSC-22 mRNA and protein expression in PC cell lines

The human PC cell lines LNCaP, C4-2, C4-2B4, PC-3 and the normal rat ventral prostate cell line NbE showed detectable TSC-22 mRNA levels (Fig. 4a). Only LNCaP and PC-3 TSC-22 mRNA expression levels significantly differ from each other. However, all cell lines show a 50 to 100-fold lower TSC-22 mRNA expression in comparison to the expression level of laser-captured NP cells (data not shown). NbE cells express TSC-22 protein, whereas in the other cell lines, TSC-22 is hardly detectable (Fig. 4b).
TSC-22 protein expression in the prostate

Clinical specimens of radical prostatectomy containing both NP and PC tissue (n = 21) were tested for immunoreactivity with the TSC-22-mAb-1. In NP acini, strong TSC-22 immunoreactivity is detected in the basal layer in all of the tissue samples, whereas no immunoreactivity is found in the secretory luminal cells and in the stromal compartment of the prostate (Fig. 5a). In basal cells, TSC-22 immunoreactivity is predominantly cytoplasmic, but scattered cells in the basal cell layer show nuclear staining as well (Fig. 5b). TSC-22 protein expression in the prostate of a healthy 25-year-old male showed an identical pattern of cellular distribution of the TSC-22 protein (Fig. 5c). The 21 samples analysed also contained representative regions of PC with a Gleason pattern ranging from 2 to 5. In these samples, TSC-22 immunoreactivity was completely and consistently absent in all malignant acini examined. Representative immunohistochemistry is shown in figures 5g and 5h.

Discussion

Here, we show for the first time differential expression of the TGFβ target gene TSC-22 in NP and PC. In normal prostate, TSC-22 protein expression is restricted to the basal cell layer of the acinar epithelium and, therefore, should be considered as a novel basal cell marker. Similarly to the loss of other basal cell markers, TSC-22 is not detectable in PC17.

The gene expression profile of the TGFβ/BMP superfamily, related signalling pathways and effector genes confirmed the differential expression between PC and NP for TGFβ36 and MADH2,18 c-fos,19 stat1,15 TGFB1I120 and TIMP121 already reported by others. Additional genes of the TGFβ superfamily, also reported as differentially expressed between PC and NP, such as the bone morphogenetic proteins,8,22,23 were represented on the cDNA array. However, the sensitivity of the array did not allow their consistent detection and, thus, precluded the analysis of these and, most likely, of other genes. Expression of Id4, a dominant negative acting helix loop helix (HLH) protein transcription factor,24 has been previously reported to be expressed in normal rat prostate epithelial cells.25 The over-expression of Id4 in PC observed on our cDNA arrays could not be substantiated by real-time PCR. In contrast, TSC-22, the most differentially and consistently expressed gene, as detected by cDNA array analysis, was confirmed to be down-regulated by real-time PCR. Since underexpression of TSC-22 in PC has not been reported previously, we focused our further investigation on TSC-22.

TSC-22 was originally identified as a TGFβ immediate early response gene in mouse osteoblasts.9 Several investigations have postulated an important role for TSC-22 in embryogenesis.26–28 Other studies have suggested a role for TSC-22 as tumour suppres-
also show nuclear localisation, which is in agreement with previous reports in cell lines and mouse tissue. This pattern of TSC-22 immunoreactivity in prostate acini was found to be independent of the age of the donors.

In contrast to NP, PC does not show any detectable TSC-22 protein expression, irrespective of the differentiation grade and Gleason score. Although larger clinical series are needed to confirm this finding, this observation suggests that loss of TSC-22 could be an early event in prostate carcinogenesis and, thus, a hallmark of malignant transformation in the acinar epithelium. The finding that all the PC cell lines examined lack TSC-22 protein, while detectable levels are found in the normal rat prostate cell line NbE, also supports this view. Indeed, it has been recently shown that down-regulation of TSC-22 expression is an acute molecular marker for hepatocarcinogenesis. Studies in human colon, gastric and salivary gland cancer cell lines indicate that TSC-22 may function as a growth suppressor possibly by acting on the cell cycle and apoptosis. As a potential tumour suppressor, TSC-22 may provide a further tool to investigate the potential involvement of basal cells in PC carcinogenesis.

Differently from the TSC-22 protein expression, TSC-22 mRNA is clearly detectable in all cell lines evaluated, although at much lower level than in laser-captured prostate cancer cells. This suggests that not only transcriptional but also posttranscriptional mechanisms regulate TSC-22 expression. Indeed, a recent study demonstrated that TGFβ can modulate TSC-22 expression by mRNA stabilisation.

Absence of the basal cell layer is an histopathological hallmark for PC acini. Thus, lack of TSC-22 expression may simply reflect the loss of the basal cell population in PC. On the other hand, recent evidence indicates that PC, especially when acquiring androgen independence, express basal cell-associated keratins and genes, and that PC cell lines almost invariably express a repertoire of genes more characteristic of basal than luminal cells. Accordingly, the current view on the origin of PC postulates that it arises from progenitor cells, termed ‘‘transit-amplifying/intermediate’’ cells, expressing markers of both basal and luminal cells. However, p63, considered to be an early basal cell marker since it is not found

**Figure 4** – TSC-22 mRNA and protein expression in cell lines. (a) Columns represent average mRNA levels, ± SD, of duplicate values for two different experiments. mRNA levels were normalised to 18s. * significantly different (p < 0.05). (b) TSC-22 protein expression in human PC cell lines. Immunoblot showing TSC-22mAb-2 reactivity in the cell lines according to A. Thirty micrograms of protein were loaded per lane and β-actin was used as a loading control. Although the same amount of protein was loaded, the expression of β-actin differs for LNCaP and its sublines C4-2 and C4-2B4 as compared to NbE and PC-3.
in intermediate cells, is very rarely expressed in PC. In analogy to p63, the complete and consistent lack of TSC-22 expression in PC suggests that TSC-22 may also be an early basal cell marker. Further investigation is warranted for elucidating whether TSC-22 is expressed in transit-amplifying cells or androgen-independent PC. Should this be the case, TSC-22 will be an additional marker for investigating cell lineage definition in the prostate epithelium.

Previous studies on TSC-22 expression during embryological development suggested a role not only in cell differentiation, but also in definition of tissue boundaries. The restricted expression of TSC-22 in basal cells, which exhibit the highest TGF-β receptor expression in the acinar epithelium, harbour the stem cell compartment of the epithelium and delineate the boundary to the stroma. may imply similar roles for TSC-22 in the prostate.
gland. Further studies are needed to evaluate whether TSC-22 is part of the TGFβ downstream effector genes controlling epithelial homeostasis of the prostate.

TSC-22 can mediate the growth inhibitory effect of TGFβ and of peroxisome proliferator-receptor-γ (PPARγ) agonists,30 Notably, TSC-22 is induced in PC cells by PPARγ agonists,30 which have shown to be growth-suppressive on PC cells.61 This suggests that TSC-22 may have a potential role as a pharmacological target gene also in prostate cancer.

In conclusion, we show that expression of TSC-22 in the prostate is restricted to the basal cell layer. Thus, TSC-22 should be considered as a novel marker for basal cells, which may help in delineating normal versus epithelial compartment of the prostate. Loss of TSC-22 expression was observed in prostate cancer cell lines and in all specimens of human prostate cancer analysed. Therefore, TSC-22 may be useful also as a marker for the differentiation between normal and malignant acinar epithelium of the prostate.

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References


