Analysis of laser-microdissected prostate cancer tissues reveals potential tumor markers

DAVID ADLER[*], ANDREAS LINDSTROT[*], REINHARD BUETTNER and NICOLAS WERNERT

Institute of Pathology, University Hospital Bonn, D-53127 Bonn, Germany

Abstract. Prostate cancer (PCA) is a clinically heterogeneous and often multifocal disease with a clinical outcome difficult to predict. A deeper knowledge of the molecular basis of the disease may lead to a better prediction of prognosis. Therefore, in this study we investigated the molecular basis of PCA by identifying potential tumor markers in laser-microdisected PCA tissues. Among a group of PCA patients, quantitative RT-PCR analysis was performed to compare the expression of 70 genes. These genes were selected from the results of two microarrays which investigated the gene expression profile differences between moderately or poorly differentiated prostate carcinoma glands and the corresponding normal glands. Among the genes examined, *CDKN2A, GATA3, CREBBP, ITGA2, NBL1* and *TGM4* were down-regulated in the prostate carcinoma glands compared to the corresponding normal glands, whereas *TFF3, TMPRSS2* and *ERG* were up-regulated. Our findings indicate that these genes may play roles as tumor suppressor genes or oncogenes in PCA, and may serve as potential tumor markers and novel therapeutic targets.

Introduction

Prostate cancer (PCA) is one of the most common cancers in the Western world (1). As a result of both steadily increasing age of western populations and the introduction of systematic prostate specific antigen (PSA) screening since 1987, PCA incidence is steadily increasing (2,3). A major problem with PCA is its clinical heterogeneity. PCAs include both indolent carcinomas carrying a favorable prognosis and requiring just watchful waiting, and aggressive tumors with distant metastases and a necessary systemic treatment (4-6). Based

Correspondence to: Professor Nicolas Wernert, Institute of Pathology, University Hospital Bonn, Sigmund-Freud-Str. 25, D-53127 Bonn, Germany
E-mail: nicolas.wernert@ukb.uni-bonn.de

[*]Contributed equally

upon clinical and biopsy-findings alone, it is not possible to exactly predict the outcome of a PCA in individual patients (3). Therefore, considerable research efforts have been undertaken to identify molecular markers carrying independent prognostic information or serving as potential therapeutic targets (5,6).

As PCA is considered to be a clinically heterogeneous and often multifocal disease with a clinical outcome that is difficult to predict (7,8), an understanding of the molecular events of the disease may improve prediction of prognosis, as genetic aberrations drive the formation and aggressiveness of prostate carcinoma (9). In an attempt to understand these molecular events, a notable number of studies have investigated the roles that epigenetics, oncogene/tumor suppressors, androgen signaling and microRNAs (miRNAs) may play in PCA (10-42).

Epigenetic studies deal with the modifications of gene expression without alterations of DNA sequences (43). There are two epigenetic strategies, the direct methylation of the so-called CpG islands (cystins followed by guanosines preferably in the promotor regions of genes) (44,45) and the modification of histones (43). The modifications of histones include acetylation, ADP-ribosylation, ubiquination, phosphorylation and methylation (43,45). The degree of methylation in histones can be mono- to trimethylation and depending on the position of the modification, the gene can be silenced or transcribed (44). It has been proposed that every cancer has its own histone map (28). DNA methylation and histone modifications have been linked to prostatic carcinogenesis, and are suggested to influence tumor formation (10). However, the reasons that lie behind these epigenetic changes remain to be investigated, even though external influences such as diet and oxidative stress are suggested to be involved (10,46).

Both tumor suppressors and oncogenes are known to contribute to the carcinogenic process and to tumor development (11). In PCA, both tumor suppressors and oncogenes have been found, however genes that are uniquely involved in prostate-specific carcinogenesis have not yet been identified (11). The RAS gene family of oncogenes is reported to be activated up to 50% in human cancers, whereas it seems to have only a minor role in the development of PCA (32,38). On the other hand, tumor suppressors such as the p53 protein encoding gene, which regulates cell cycle arrest and apoptosis, has a low mutation frequency in early PCA, whereas a heterozygous loss of function mutations are often associated with the late stage (33). Other tumor suppressor genes such the

Table I. The sequences of primers used in the qRT-PCR analysis.

Name	Left	Right
CDKN2A	TGCCTTTTCACTGTGTTGGA	GAGCTTTGGTTCTGCCATTT
CREBBP	GGATGATCACTCTTAGACCATGC	CCACCCTTTTGTCTGTTG
ERG	GACGACTTCCAGAGGCTCAC	ATAAAAGCTGCACCCCCTGT
GATA3	GTCCTGTGCGAACTGTCAGA	GGGGAAGTCCTCCAGTGAGT
ITGA2	GCAGCTGCAGAAATCAACAC	CCGAGCTTCCATAAAATTGC
NBL1	CTCTTCCTCCCCTCTCATCC	AAATTGTGCAGCTTGGCTCT
rPL13A (control)	TACGCTGTGAAGGCATCAAC	CACCATCCGCTTTTTCTTGT
TFF3	AGTGCCTTGGTGTTTCAAGC	GAGCATGGGACCTTTATTCG
TGM4	GCACCCCAATAAAAACTGGA	TTTTTGACCTGCTGGCTCTT
TMPRSS2	GGCCTATTTCAGCTGCTTTG	GGAGGCAGAACCATGGTAGA

Glioma pathogenesis-related protein1 (*GLIPR1*), and *PTEN* have been found to be down-regulated in PCA, to be involved in p53-dependent and independent apoptotic pathways and to be associated with a poor clinical outcome, respectively (30,37,39,41,42).

Normal and neoplastic growth of the prostate gland depends on the androgen receptor (AR) (14). The AR is crucial for proliferation and progression of PCA (12,13). However the androgen-independent growth mechanisms are not yet well understood (14). It has been reported that the *AR* may be amplified and overexpressed (15,16), hypersensitive to androgens or activated by non-androgenic ligands in androgen-independent PCA growth (17).

miRNAs are small noncoding RNAs which target the mRNAs for cleavage or translational repression (47). In PCA, various miRNAs were found to be underexpressed or over-expressed suggesting that miRNAs may be involved in PCA development as well (27). Furthermore, miRNAs can act as onco-miRNAs (e.g. miR-21, -125b, -221/222) (35,36,40), inhibiting the translation of tumor suppressor genes, or as tumor suppressor miRNAs (e.g. miR-101, -126*, -146a, -200 family, -330) (31,34,48-50), inhibiting oncogenes (29).

In addition to efforts investigating the role that epigenetics, oncogene/tumor suppressors, androgen signaling and miRNAs may play in PCA, recent studies have focused on investigating translocations in PCA between the *TMPRSS2* gene encoding an androgen-regulated prostate-specific serine protease and several genes of the ETS family of transcription factors, most commonly ERG (51-54). These gene fusions are reported in up to 60-70% of clinically manifested PCAs (10), and it is known that such translocations lead to an increased expression of the rearranged ETS factors in response to androgens (52). Furthermore, the ETS family members were initially viewed as oncogenic factors, but recently, they have been suggested to play roles as tumor- or metastasis-suppressors as well (55).

In this study, we have attempted to investigate the molecular basis of PCA by identifying potential tumor markers. We report here the identification of a set of genes in PCA tissues, which may function as tumor suppressors or oncogenes. These identified genes may serve as potential markers for early tumor detection, and therapeutic drug treatment.

Materials and methods

Processing of human prostatectomy specimens. Fresh tissue samples out of the peripheral zone (prostate proper, sample sizes around 0.5x0.5x0.3 cm) from 11 prostate carcinoma patients were taken immediately after radical prostatectomy and shock-frozen in liquid nitrogen with ice-cold isopentane. Frozen sections (6 μm) were cut from the samples using a cryotome (Leica, Germany) and mounted on membrane-coated slides (MembraneSlides, 1 mm PEN, Zeiss, Germany) for subsequent laser-microdissection. One section was mounted on conventional slides and stained with hematoxylin and eosin (H&E) for diagnostic evaluation by an experienced pathologist. Laser-microdissection was performed according to our previously published method (56-58). Frozen sections were dried for 2 min in the cryotome, washed for 2 min with 70% ethanol in DEPC-treated water and stained for 30 sec in 1% Cresyl Violet diluted in 50% ethanol-DEPC-treated water. Slides were then washed briefly in 70 and 100% ethanol, dried for 10 min and stored at -80˚C until use for laser-microdissection.

Quality control. A frozen section from each patient prior to laser-capture microdissection (LCM) and the dissected slide after LCM were used for quality control. For RNA extraction with the RNeasy mini kit (Qiagen, Germany) the section was washed from the slide with 600 μl 2 μM DTT in RLT buffer and vortexted for 30 sec. The recommend DNase digestion was carried out with an RNase-free DNase Set (Qiagen). RNA integrity was measured with the Agilent Bioanalyzer 2100 (Agilent Technologies, USA). Samples with a RIN factor >6 were used for laser-capture microdissection and microarray.

Laser-capture microdissection. Laser-capture microdissection of Cresyl Violet-stained sections was performed using the Axio Observer. Z1 microscope (Zeiss) with the integrated Palm MicroBeam software (Zeiss). LCM was performed under a x10 objective lens. Isolated normal or cancerous glands were collected in 200 μl adhesive cap tubes (Zeiss).

Microarray analysis of RNA isolated from laser microdisected moderately and poorly differentiated prostate carcinoma
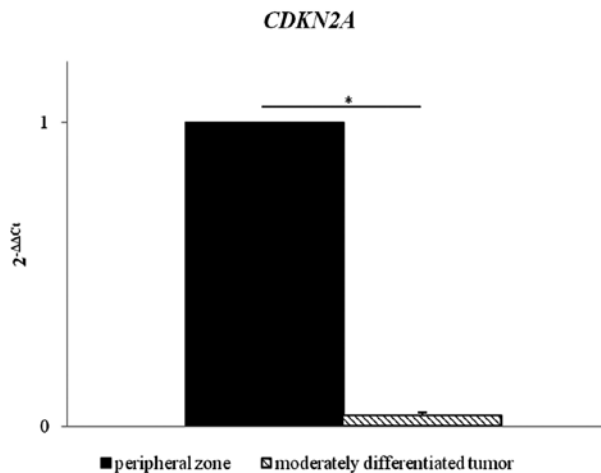
CDKN2A

Figure 1. Expression of *CDKN2A*. The relative expression of *CDKN2A* as measured by quantitative RT-PCR in the moderately differentiated prostate carcinoma glands compared to the normal peripheral zone glands. Significance was calculated with the Student's t-test; $^*P<0.05$.
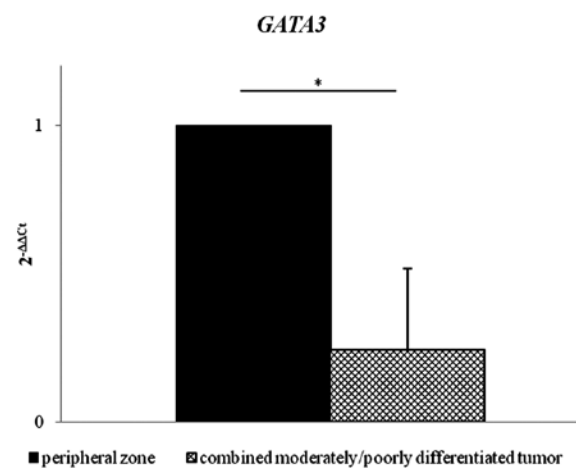
GATA3

Figure 2. Expression of *GATA3*. The relative expression of *GATA3* as measured by quantitative RT-PCR in combined moderately/poorly differentiated prostate carcinoma glands compared to the normal peripheral zone glands. Significance was calculated with the Student's t-test; $^*P<0.05$.

glands, as well as from normal glands of prostate cancer patients. RNA was isolated using the RNeasy micro kit (including the recommend DNase digestion with the RNase-free DNase set, Qiagen) from laser microdissected moderately and poorly differentiated prostate carcinoma glands, as well as from the normal prostate proper glands of prostatectomy specimens. The quantity of RNA was measured using the NanoDrop photometer (Thermo Fisher Scientific, USA). Thereafter, an equal amount of RNA from the moderately differentiated glands, poorly differentiated glands, and normal peripheral glands were pooled to final concentrations of 300 ng. The microarray analysis and bioinformatical interpretation were performed at Miltenyi Biotec (Germany) with the Whole Human Genome Oligo Microarray 4x44K (Agilent, USA). The microarray results were validated in a subset of genes by quantitative RT-PCR (qRT-PCR).

Quantitative RT-PCR using RNA isolated from laser micro-disected moderately and poorly differentiated prostate carcinoma glands, as well as from normal glands of prostate cancer patients. qRT-PCR was performed by comparing the expression of 70 genes that were selected from the microarrays in the moderately or poorly differentiated carcinoma glands to the corresponding normal glands of each of the 11 PCA patients. Briefly, following the reverse transcription of total-RNA with SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, USA), the quantitative real-time PCR was performed using SYBR-GreenER qPCR SuperMix (Invitrogen, USA) in a total volume of 10 µl. The PCR conditions in the Applied Biosystems 7900HT instrument were as follows: 2 min 50°C, 10 min 95°C, 40 cycles 15 sec 95°C and 1 min 60°C. At the end, a melting curve analysis with 15 sec 95°C, 15 sec 60°C and 15 sec 95°C was performed. The data were collected after the 60°C step at every cycle and after the melting point analysis. The primers of the genes that were used in the qRT-PCR analysis and were found to be significantly differentially expressed in the normal peripheral zone glands compared to the moderately or poorly differentiated carcinoma glands are listed in Table I.

Results

Gene expression analysis of moderately and poorly differentiated prostate carcinoma glands compared to normal glands of the prostate proper (or the peripheral zone) from PCA patients using Whole Human Genome Oligo microarrays. To identify potential tumor gene markers in PCA, we analyzed the gene expression profile in the moderately and poorly differentiated prostate carcinoma glands compared to normal glands of the peripheral zone from PCA patients using two Whole Human Genome Oligo microarrays. The two microarrays yielded 8,242 and 6,215 differentially expressed genes with a fold change >2 and a P-value <0.01 in the normal peripheral zone compared to the moderately and poorly differentiated prostate carcinoma glands, respectively (data not shown). A clustering analysis based on gene function placed each gene into one or more of following categories: oncogenes, tumor suppressors, apoptosis, AR pathway, development, angiogenesis, immunity genes, as well uncategorized genes (data not shown).

qRT-PCR analysis of selected genes in the moderately or poorly differentiated carcinoma glands compared to the corresponding normal peripheral zone glands of PCA patients. Due to the extremely limited amounts of RNA that can be extracted from PCA tissues of each of the 11 prostate carcinoma patients, we chose 70 highly differentially expressed genes from the various categories provided by the microarray, and examined their expression in each of the 11 patients by qRT-PCR. Among the 70 genes examined, 9 genes were found to be significantly differentially expressed in the normal peripheral zone glands compared to the moderately or poorly differentiated carcinoma glands (Figs. 1-8). Our data show that the genes *CDKN2A, GATA3, CREBBP, ITGA2, NBL1* and *TGM4* are down-regulated in the moderately or poorly differentiated carcinoma glands compared to the normal peripheral zone glands (Figs. 1-6). On the other hand, the genes *TMPRSS2, ERG* and *TFF3* are up-regulated in moderately or poorly differentiated carcinoma glands compared to the normal peripheral zone glands (Figs. 7 and 8).
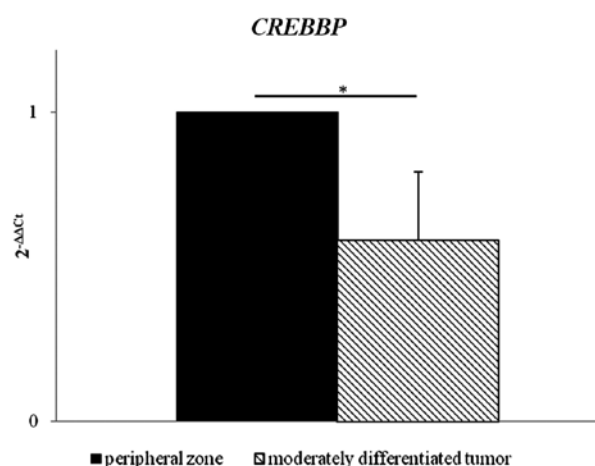
CREBBP

Figure 3. Expression of *CREBBP*. The relative expression of *CREBBP* as measured by quantitative RT-PCR in the moderately differentiated prostate carcinoma glands compared to the normal peripheral zone glands. Significance was calculated with the Student's t-test. P-values indicated as *P<0.05.

NBL1

Figure 5. Expression of *NBL1*. The relative expression of *NBL1* as measured by quantitative RT-PCR in the poorly and combined moderately/poorly differentiated prostate carcinoma glands compared to the normal peripheral zone glands. Significance was calculated with the Student's t-test. P-values indicated as **P<0.01.
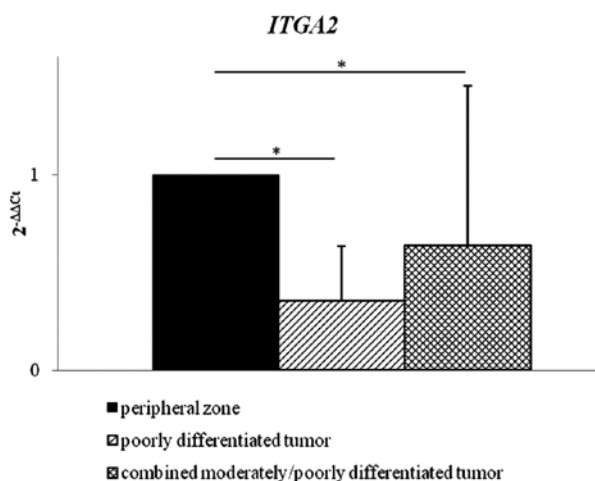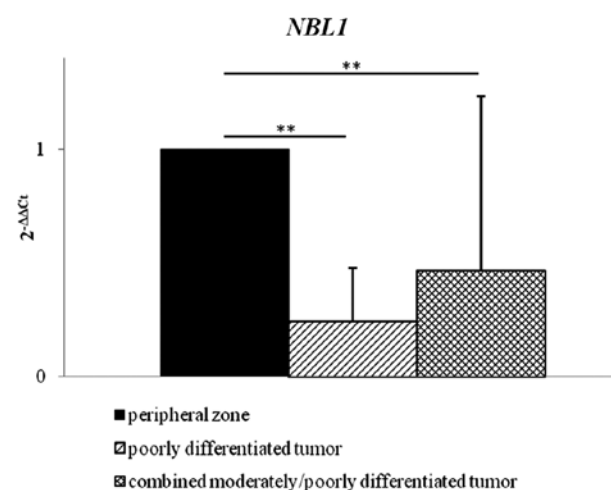
ITGA2

Figure 4. Expression of *ITGA2*. The relative expression of *ITGA2* as measured by quantitative RT-PCR in the poorly and combined moderately/poorly differentiated prostate carcinoma glands compared to the normal peripheral zone glands. Significance was calculated with the Student's t-test. P-values indicated as *P<0.05.
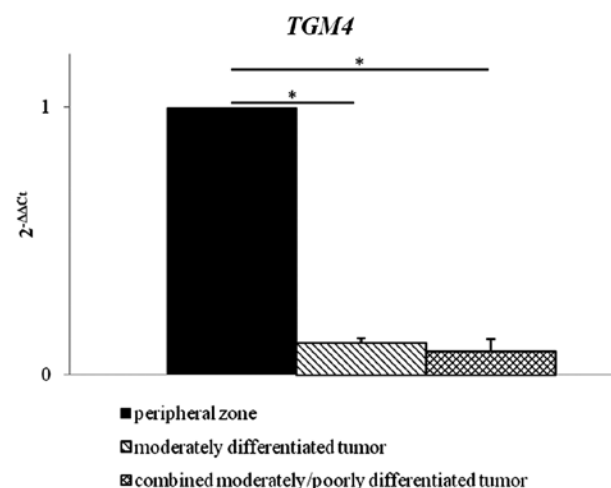
TGM4

Figure 6. Expression of *TGM4*. The relative expression of *TGM4* as measured by quantitative RT-PCR in the moderately and combined moderately/poorly differentiated prostate carcinoma glands compared to the normal peripheral zone glands. Significance was calculated with the Student's t-test; *P<0.05.

Discussion

Tumor development and progression involve changes in gene expression patterns in various pathways (59,60). Genes involved in such pathways may be classified into various categories, such as oncogenes, tumor suppressor genes, apoptosis-relevant genes or DNA-repair genes (60-63). PCA is a clinically heterogeneous and often multifocal disease with a clinical outcome difficult to predict (7,8). As genetic aberrations drive the formation and aggressiveness of prostate carcinoma, a knowledge of PCA at the molecular level may improve our understanding of the disease and lead to a better prediction of prognosis (9).

Therefore, in an attempt to identify potential tumor markers, in this study we investigated the gene expression profile in the prostate carcinoma glands compared to normal glands of the peripheral zone from PCA patients using microarrays and qRT-PCR analysis.

We report here the identification of 9 genes in PCA tissues including the well-known genes *TMPRSS2* and *ERG*, which may function as tumor suppressor genes, oncogenes, or apoptosis-related genes, and which may serve as potential tumor markers (Figs. 1-8).

The genes *CDKN2A*, *GATA3*, *CREBBP*, *ITGA2*, *NBL1* and *TGM4* were found to be down-regulated in prostate carcinoma glands compared to the normal peripheral zone glands, indicating that these genes may function as tumor suppressors in PCA (Figs. 1-6). On the other hand, the genes *TFF3*, *TMPRSS2* and *ERG* were found to be up-regulated in the prostate carcinoma glands compared to the normal peripheral zone glands, indicating that these genes may play the role of oncogenes in PCA (Figs. 7 and 8).

Briefly, *CDKN2A* which has been found to be down-regulated in the moderately differentiated carcinoma glands compared to the normal peripheral zone glands (Fig. 1), is
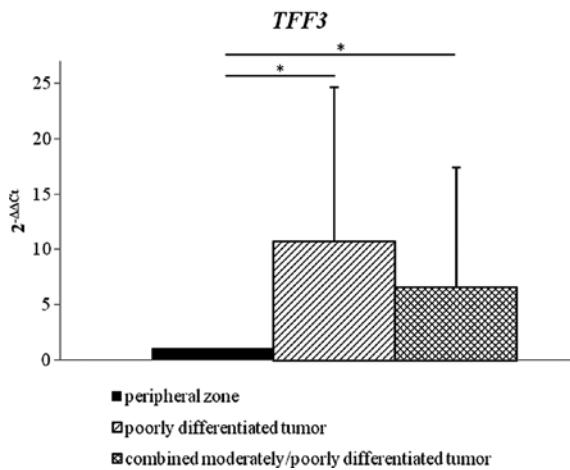
Figure 7. Expression of *TFF3*. The relative expression of *TFF3* as measured by quantitative RT-PCR in the poorly and combined moderately/poorly differentiated prostate carcinoma glands compared to the normal peripheral zone glands. Significance was calculated with the Student's t-test; *P<0.05.

reported to be a tumor suppressor gene (64,65). *CDKN2A* is known to be inactivated in most melanoma cell lines (66), and it is suggested that families carrying a *CDKN2A* mutation have an increased risk of multiple melanomas, pancreatic carcinoma, and breast cancer (65). Furthermore, inactivation of *CDKN2A* is implicated in primary central nervous system lymphomas (PCNSL) (67). Therefore, our finding that *CDKN2A* is down-regulated in PCA tissues suggests a role of *CDKN2A* as a potential tumor suppressor gene in PCA. The gene *GATA3* which is found to be down-regulated in the prostate carcinoma glands compared to the normal peripheral zone glands (Fig. 2) is reported to be involved in the androgen regulation of the PSA gene (68). *GATA3* is expressed at low levels in invasive breast carcinomas and suggested to be a potential breast cancer prognostic marker (69). The reported low expression of *GATA3* in breast carcinomas is similar to our findings in prostate carcinoma, indicating that *GATA3* may function as a tumor suppressor in both breast and PCA. The *CREBBP* gene has been found to be mutated in human cancers and encodes a protein which is reported to be a tumor

suppressor protein, that is essential in cell cycle control, differentiation and development (70). We found the *CREBBP* gene to be down-regulated in the moderately differentiated prostate carcinoma glands compared to the normal peripheral zone glands (Fig. 3). Our findings thus support the role of *CREBBP* as a tumor suppressor, and indicate that it may function as a tumor suppressor in PCA as well. *ITGA2* which is found to be down-regulated in the poorly differentiated prostate carcinoma glands compared to the normal peripheral zone glands (Fig. 4), has been reported to be down-regulated in breast cancer tissues as well (71). These findings indicate that *ITGA2* is very likely to play the role of a tumor suppressor in PCA. *NBL1* which is found to be down-regulated in the poorly differentiated prostate carcinoma glands compared to the normal peripheral zone glands (Fig. 5), has been suggested as a possible tumor suppressor gene of human neuroblastoma (72). Furthermore, electronic profiling of expressed sequence tags and qRT-PCR have reported that the expression of *NBL1* is reduced by over 80% in PCA (73). These previously reported studies support our findings and the notion that *NBL1* may play the role of a tumor suppressor in PCA. *TGM4* tissue-specifically expressed in the prostate (74), is found to be down-regulated in the moderately differentiated carcinoma glands compared to the normal peripheral zone glands (Fig. 6). Studies of normal prostate and human prostate carcinoma reported *TGM4* expression in the normal prostate, but not in PCA (75). Furthermore, *TGM4* expression was not detected in the human PCA cell lines, LNCaP and PC3 (74). These previous studies combined with our findings may indicate a possible role of *TGM4* as a tumor suppressor gene in PCA.

On the contrary, the genes *TFF3*, *TMPRSS2* and *ERG* were found to be up-regulated in the prostate carcinoma glands compared to the normal peripheral zone glands (Figs. 7 and 8), respectively. In agreement with our findings (Fig. 7), *TFF3* was reported to be overexpressed in PCA (76). Interestingly, *ERG* which is found to be up-regulated in the prostate carcinoma glands (Fig. 8A) is reported to be implicated in the regulation of *TFF3* in PCA progression (77). As *TMPRSS2* is already known to be overexpressed in PCA (78), and since translocations between *TMPRSS2* and several members of the ETS family, most commonly *ERG*, are frequently found in PCA (51-54),
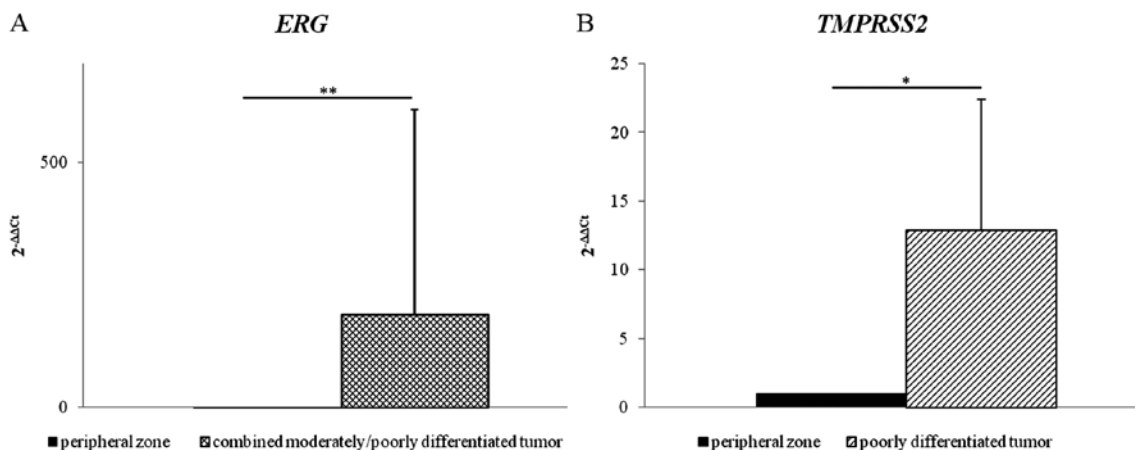

Figure 8. Expression of *ERG* and *TMPRSS2*. The relative expression of (A) *ERG* and (B) *TMPRSS2* as measured by quantitative RT-PCR in the combined moderately/poorly differentiated, and moderately differentiated prostate carcinoma glands, respectively, compared to the normal peripheral zone glands. Significance was calculated with the Student's t-test. P-values indicated as **P<0.01 and *P<0.05.

we investigated the expression of both *ERG* and *TMPRSS2* in our prostate carcinomas as well. Our findings that *TMPRSS2* and *ERG* are up-regulated in the prostate carcinoma glands compared to the normal peripheral zone glands (Fig. 8) further supports the previously reported studies, and may indicate a possible role of these genes as oncogenes in PCA.

Taken together, our analysis of laser-microdissected PCA tissues revealed a set of genes which may function as tumor suppressor genes or oncogenes in PCA. These genes may serve as potential tumor markers and novel therapeutic targets. Furthermore, investigating such candidate genes may add to connect state of knowledge of the molecular basis of PCA.

Acknowledgements

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