The peripheral zone of the prostate is more prone to tumor development than the transitional zone: Is the ETS family the key?

DAVID ADLER[1], ANDREAS LINDSTROT[1], JÖRG ELLINGER[2], SEBASTIAN ROGENHOFER[2], REINHARD BUETTNER[1], SVEN PERNER[1] and NICOLAS WERNERT[1]

[1]Institute of Pathology, and [2]Department of Urology, University Hospital Bonn, 53127 Bonn, Germany

Abstract. Predisposition to develop prostate cancer (PCA) varies among the prostate zones, with the peripheral zone (PZ) more prone to tumor development than the transitional zone (TZ). In view of the fact that molecular differences between the zones may explain this difference, combined with the findings that translocations between TMPRSS2 and several ETS members are frequently observed in PCA, we hypothesized that the ETS family may be crucial to explaining this difference. Normal tissues from the PZ and the TZ of 20 PCA patients were laser microdissected to separate glands from stroma. Two oligo microarrays were performed in order to investigate the variation in ETS family gene expression between the glands and the stroma of the two zones. The ETS members, ELF-3, ELF-5, ERG, ETV-1, ETV-4, ETV-5, ETV-7 and FEV, were found to be differentially expressed. A striking observation was that ERG and ETV-1 were found to be up-regulated in the glands of the PZ compared to the TZ, particularly when considering that ERG and ETV-1 fusions account for 50-80% and 20% of PCA occurrences, respectively. These results indicate that the glands and stroma of the two zones display distinct molecular differences and zonal-specific expression of ETS members. Furthermore, ETS members up-regulated in PCA are already overexpressed in the normal PZ, suggesting that these members play a role in the development and progression of PCA.

Introduction

Prostate cancer (PCA) is a clinically heterogeneous and often multifocal disease with a clinical outcome that is difficult to predict (1,2). Therefore, advancements in the knowledge of the molecular basis of PCA should improve the prediction

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

of prognosis, as genetic aberrations drive the formation and aggressiveness of prostate carcinoma (3).

The prostate is supported by a stroma and can be divided into three anatomical zones: peripheral (the so-called prostate proper), transitional and central zones (4,5). The predisposition to develop PCA is different among these zones, as most manifest PCAs (75%) occur in the peripheral zone (PZ), at the dorsal and dorso-lateral side of the prostate, while only some (20%) occur in the transitional zone (TZ) (6,7).

The latter cancers are mostly so-called incidental carcinomas with a nonaggressive clinical course, whereas cancers from the PZ are more aggressive, tend to invade the periprostatic tissues and have higher biochemical recurrence rates (6,8). The molecular basis underlying the differences in the susceptibility to PCA between the different zones of the prostate remains unknown. However, it has been suggested that some of the differences in susceptibility may have an embryological basis (9). As PCA rarely develops in the central zone compared with the PZ, it has been suggested that the central zone is derived from the Wolffian duct, the same duct from which the seminal vesicle is derived, and in contrast to the remainder of the prostate, which is derived from the urogenital sinus (5,9,10).

In contrast to these varying predispositions of the TZ and the PZ to clinically manifest PCA, and to the fact that the two zones display no histomorphological differences (5), this suggests that there should be molecular differences explaining the various characteristics. A previous study reported that gene expression differences exist between the PZ and the TZ of the prostate and suggested that stromal-epithelial interactions may be responsible for the distinct zonal localization of PCA (11). It is already known that embryological prostatic development occurs through mesenchymal-epithelial interactions and is induced by androgens (12). The ability of the epithelial cells from the glands to respond to the stroma suggests that carcinoma development and progression may also be regulated by connective tissue and that the communication between the epithelium and stroma is multifactorial (12-14). Indeed, tumor-stroma communications consist of multiple dynamic interactions between neoplastic and stromal cells, and further evidence indicates that these communications also exist between metastatic PCA cell lines as well as their fibroblastic environment (15).

Understanding prostatic carcinogenesis remains a major challenge and the discovery of the ETS family of oncoproteins in PCA within the last decade has been the greatest breakthrough towards this goal (4). In human PCA, translocations between the TMPRSS2 gene, encoding an androgen-regulated, prostate-specific serine protease, with several genes of the ETS family including ETV-1, ETV-4, ETV-5 and, most commonly, ERG, are frequently reported (16-19). The TMPRSS2-ERG fusions appear to account for 50-80% of occurrences, while TMPRSS2-ETV-1 fusions account for up to 20% of occurrences (4,20). The known fusion genes are thought to occur in up to 60-70% of clinically manifest PCA diseases (4). The consequence of such translocations is the increased expression of the rearranged ETS factors as a response to androgens in PCA (16). It has been suggested that such ETS rearrangements are sufficient for initiation of prostate neoplasia, while, most recently, other studies have suggested that the rearrangements may foster progression rather than initiation events in prostate tumorigenesis (21,22).

In the present study, we hypothesized that the ETS family members may be the key to explaining the observation that the PZ of the prostate is more prone to tumor development than the TZ. Therefore, normal tissues from the PZ and the TZ of patients who had undergone radical prostatectomy for PCA were laser microdissected to separate the glands from the stroma. Two microarrays were then performed to investigate differences in ETS family gene expression between the glands and the stroma of the PZ and the TZ.

Our findings demonstrate that the glands and the stroma of the PZ and the TZ display distinct molecular differences, as well as zonal-specific expression of ETS members. Furthermore, the results also indicate that ETS members that are up-regulated in PCA are already overexpressed in the normal PZ, suggesting that these members may play a role in the development and progression of PCA. Taken together, these findings indicate that the ETS family members may be the key to explaining the observation that the PZ is more prone to tumor development than the TZ.

Materials and methods

The present study was approved by the Ethics Review Board of the Faculty of Medicine, University of Bonn/University Hospital, Germany, according to the principles expressed in the Declaration of Helsinki. Furthermore, written informed consent was obtained from all participants involved in the study.

All microarray data used in this study are MIAME compliant and the raw data have been deposited in a MIAME compliant database, GEO. The accession numbers for the manuscript are GSE29090.

Processing of human prostatectomy specimens. Radical prostatectomy specimens were obtained from PCA patients immediately following surgery. The patients were not exposed to any treatment prior to surgery. Fresh tissue samples (0.5 x 0.5 x 0.3 cm) were removed from the PZs (prostate proper) and the TZs of 20 PCA patients, and shock-frozen in liquid nitrogen with ice-cold isopentane. Frozen sections (6 μm) were excised 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 who confirmed the absence of prostate carcinoma in the two zones. Laser microdissection was performed as previously described (23-25). 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 their use for laser microdissection of normal glands and the stroma between them.

Quality control. The quality of the RNA was measured from each patient prior to laser microdissection, using a lasercapture microscope (LCM). The section was washed from the slide with 600 μl buffer RLT + 2 μM DTT (provided in the Kit RNeasy Mini Kit, Qiagen, Germany) and vortexed for 30 sec. Then RNA extraction was performed as described according to the manufacturer's instructions. The recommended DNase digestion was created with a RNase-Free DNase Set (Qiagen). The quality of the RNA was measured with the Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA). Samples with a RIN factor >6 were used for LCM.

LCM. The cresyl violet-stained sections were cut with an Axio Observer.Z1 microscope (Zeiss) and installed Palm MicroBeam (Zeiss). The LCM was performed under a x10 objective. The glands and the stroma of the PZs and the TZs were separated and collected in 200 μl AdhesiveCap tubes (Zeiss).

Microarray analysis of RNA isolated from lasermicrodissected healthy glands and stroma of the PZs and TZs of the prostate. RNA was isolated from laser microdissected healthy glands and stroma of the peripheral and TZs of the prostate from patients using the RNeasy Micro Kit (Qiagen) as described according to the manufacturer's instructions. The recommended DNase digestion was included with the RNase-Free DNase Set (Qiagen). The amount of the isolated RNA was measured with the Nanodrop photometer (Thermo Fisher Scientific, USA). Thereafter, an equal amount of RNA from healthy PZs and TZs of the glands of 10 patients was pooled, respectively, to a final concentration of 300 ng of RNA. Furthermore, an equal amount of RNA was also taken from the stroma of healthy PZs and TZs of the patients and pooled, respectively, to a final concentration of 300 ng of RNA. These pools were sent to MiltenyiBiotec (Bergisch Gladbach, Germany) for microarray analysis and bioinformatical interpretation. The RNA was labeled with Cy3 and hybridized on the whole human genome oligo microarray 4x44K (Agilent Technologies), according to the manufacturer's instructions. The microarray results were then validated by quantitative reverse transcriptase PCR (qRT-PCR) of a subset of genes.

Results

ETS family members are differentially expressed in normal glands of the TZ compared to the PZ of the prostate. Using

whole human genome oligo microarray, we identified a set of 5/27 ETS family members to be differentially expressed (p<0.01) in the healthy glands of the PZ compared to TZ (Table I). We found ELF-5, ERG and ETV-1 to be up-regulated in normal glands of the PZ compared to the glands of TZ, whereas ETV-4 and ETV-5 were found to be down-regulated in the normal glands of the PZ compared to the TZ.

Differentially expressed ETS family members in the stroma of the TZ compared to the PZ of the prostate. A set of 5 ETS family members was found to be differentially expressed (p<0.01) in the stroma cells of the PZ compared to those of the TZ using whole human genome oligo microarray hybridization (Table II). We found FEV, ELF-3 and ERG to be up-regulated in the stroma of the PZ compared to those of the TZ. On the other hand, ETV-5 and ETV-7 were down-regulated in the stroma of the PZ compared to that of the TZ.

Discussion

In this study, we examined the differences in gene expression of all ETS family members between the PZ and the TZ of the normal glands and stroma of the prostate. We report that 5/27 ETS family members were differentially expressed between the normal glands of the PZ compared to those of the TZ (Table I). Furthermore, 5/27 were found to be differentially expressed between the normal stroma of the PZ compared to that of the TZ (Table II). These results indicate that normal prostate glands and stroma of the PZ and the TZ display distinct molecular differences in regard to the expression of a unique set of ETS family members.

Previous studies have reported an overlap between genes that are overexpressed in prostate carcinoma and preferentially expressed in the normal PZ (11). In addition, tumor-associated genes were found to overlap with genes more highly expressed in the PZ compared to the TZ (11,26). This overlap was suggested to be either a result of gene-expression signatures of the normal PZ that remains dominant in PCA, displaying its origin, or that the unique expression profile of the PZ shows a zonal-specific microenvironment that supports tumor growth (11). Based on these studies, it was proposed that genes that are up-regulated in prostate carcinoma are already overexpressed in the PZ, suggesting that these genes may play a role in the origin and progression of PCA (11). It was further suggested that the rarity of tumor development in the TZ, might be due to the distinct expression profile in the TZ of specific genes and pathways that are involved in antitumor mechanisms (11).

Our results, which revealed that the ETS family members ERG and ETV-1, were up-regulated in the normal glands of the PZ compared to the TZ (Table I), combined with reports that ERG and ETV-1 fusions account for 50-80% and for up to 20% of PCA occurrences, respectively (4,20), support the proposed hypothesis of a predisposing cancer susceptibility signature in the PZ and a possible unique expression profile of the PZ microenvironment that supports tumor growth (11). This hypothesis can also be supported by our finding that ELF-5 is up-regulated in the glands of the PZ compared to the TZ (Table I), combined with previous studies reporting that increased expression of ELF-5 is associated with advanced

Table I. Differentially expressed ETS members in the normal glands of the PZ compared to the TZ.

ETS family member	PZ glands	TZ glands
ELF-5	+2.2	1
ERG	+12.7	1
ETV-1	+2.2	1
ETV-4	-3.7	1
ETV-5	-2.3	1

Values indicate the fold-change in gene expression with positive values indicating up-regulation and negative values indicate down-regulation. PZ, peripheral zone; TZ, transitional zone.

Table II. Differentially expressed ETS members in the normal stroma of the PZ compared to the TZ.

ETS family member	PZ stroma	TZ stroma
FEV	+2.4	1
ELF-3	+3.9	1
ERG	+6.5	1
ETV-5	-2.1	1
ETV-7	-2.1	1

Values indicate the fold-change in gene expression, with positive values indicating up-regulation and negative values indicating down-regulation. PZ, peripheral zone; TZ, transitional zone.

stages of PCA (27). In addition, ETV-4, which is found to be down-regulated in the glands of the PZ compared to those of the TZ (Table I), is reported to have decreased expression or genomic loss that is associated with advanced stages of PCA (27). Notably, ERG is found to be up-regulated in the stroma of the PZ, as it is in the glands, along with its closely related ETS member FEV (28) and ELF-3 (Table II). In support of our hypothesis, ELF-3 is reported to have elevated expression in breast cancer (27).

Immunohistochemistry, and/or *in situ* hybridization studies, have reported on the expression of ETS family members in PCA (27). For instance, ERG, which we found to be up-regulated in the PZ compared to the TZ, has been shown to be overexpressed in PCA tissues, whereas ETV-4, which we found to be down-regulated, was reported to show negative staining in PCA (27). We recently reported the expression pattern of all 27 ETS family members in androgen-sensitive VCaP and LNCaP, and androgen-insensitive PC3 and DU-145 PCA cell lines (29). We reported an expression specificity of various ETS family members in different cell lines (29). For instance, ERG, ETV-1 and ELF-5, which we found to be up-regulated in the PZ compared to the TZ, were found to be highly expressed in PC3 and DU-145 (for ERG) and in LNCaP (for ETV-1 and ELF-5) (29). Knockdown of ERG in PCA cell lines induced morphological changes and caused inhibition of cell growth in culture and mice (30). ETV-1 overexpression in benign prostate

cells as well as in the mouse prostate conferred neoplastic phenotypes (21). Expression of ETV-4 leads to inhibition of cell growth and tumor development in cancer cells, suggesting a role as a tumor-suppressor gene (31).

Taken together, our findings indicate that the ETS family members, which are up-regulated in PCA, are already overexpressed in the normal PZ, suggesting that these ETS members may play a key role in the origin and progression of PCA and may explain the observation that the PZ is more prone to tumor development than the TZ.

Further studies must be performed to examine the transcriptional regulatory networks performed by various ETS family members in the normal prostate zones and in PCA, as well as the function of these genes. Ideally, it would be helpful to acquire prostate cell lines derived directly from the normal PZ and the TZ of the prostate, and to further examine the role of the ETS family members, as well as their potential targets and networks. These ETS candidates may then prove useful as diagnostic and prognostic markers, or even novel therapeutic targets, for PCA in the future.

Acknowledgements

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