Genes differentially expressed in the peripheral zone compared to the transitional zone of the normal human prostate and their potential regulation by ETS factors

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Abstract. Prostate cancer (PCa) is a clinically heterogeneous and multifocal disease with a clinical outcome that is difficult to predict. Notably, predisposition to develop prostate cancer is different among the prostate zones, with the peripheral zone (PZ) or prostate proper as the most frequent site compared to the transitional zone (TZ). Translocations between the *TMPRSS2* gene and a number of members of the *ETS* family are frequently found in PCa. Here, we examined expression differences among the two zones by laser microdissection of normal tissues from PCa patients to separate glands from stroma, and investigated the gene expression differences in the glands of the PZ compared to those of the TZ using microarray analysis. We identified 9 genes involved in the so-called metastatic cascade, to be highly differentially expressed in the normal glands of the PZ compared to those of the TZ. The genes which were found to be up-regulated in the PZ compared to the TZ are commonly up-regulated in tumors. These findings may explain the lower susceptibility for PCa of the TZ compared to the PZ. According to bioinformatic analysis, 8/9 of these genes may be potentially regulated by ETS transcription factors.

Introduction

As a leading cause of cancer mortality, prostate cancer (PCa) is a clinically heterogeneous and often multifocal disease with a clinical outcome that is difficult to predict (1-4). Therefore, knowledge about the molecular basis of PCa may improve the prediction of prognosis, as genetic aberrations have been reported to drive the formation and aggressiveness of the disease (5).

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According to McNeal (6) and Joshua *et al* (7), the prostate consists of three anatomical glandular zones that are supported by a stroma: the peripheral (the so-called prostate proper), the transitional and the central zone (6,7). Notably, predisposition to develop PCa is different among these zones, with the majority of cases of cancer (75%) occurring in the peripheral zone (PZ), compared to only 20% in the transitional zone (TZ) (8,9), although the two zones show no histomorphological differences (6). Cancers originating from the PZ tend to be more aggressive and invasive compared to the cancers originating from the TZ, which are mostly non-aggressive (8,10). Although embryological bases have been suggested to perhaps contribute to the difference in susceptibility among the prostate zones (11), the molecular reasons that lie behind such differences in susceptibility for PCa in PZ compared to the TZ remain to be unraveled.

Current molecular models suggest that high-grade prostatic intra-epithelial neoplasia (HGPIN) is likely to represent a precursor of PCa (7). The relatively recent discovery in the last decade of the ETS family gene fusions in PCa has been a major step towards understanding the molecular basis of the disease (7). Translocations between the *TMPRSS2* gene, encoding an androgen-regulated prostate-specific serine protease, with a number of ETS family members, most commonly ERG, are frequently found in PCa (1,12-14). It has been reported that the *TMPRSS2-ERG* fusions account for 50-80% of PCa occurrences (7). As a result of these translocations, the expression of the rearranged ETS factors are increased in response to androgens (12). Furthermore, ETS factors are known to play significant roles in the various steps of the so-called metastatic cascade (proliferation, apoptosis, migration, invasion and angiogenesis) in different tissues (12,15-20).

A previous study reported that there are gene expression differences between the PZ and the TZ of the prostate (21). However, laser microdisection has not yet been used to separately analyze the stromal cells and the normal glands. In the present study, we examined gene expression differences among the two zones by laser microdissection of epithelial from stroma cells, and then investigated expression differences specifically in the glands of the PZ compared to those of the TZ using microarray analysis. We report the identification of 9 genes which are involved in the so-called metastatic cascade,

and which were found to be highly differentially expressed in the normal glands of the PZ compared to those of the TZ of the human prostate. Genes that were found to be up-regulated in the PZ compared to the TZ are commonly up-regulated in tumors, which may explain the differences of the prostate zones in susceptibility for PCa. We further report the potential regulation of these genes by ETS factors.

Materials and methods

This study has been approved by the Faculty of Medicine's Ethics Review Board of the University of Bonn/University Hospital, Germany, according to the principles expressed in the Declaration of Helsinki. Written informed consents were obtained from all participants involved in the study. All the raw microarray data used in this study have been deposited in a MIAME compliant database, GEO. The accession number for the manuscript is 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 (0.5x0.5x0.3 cm) were obtained from the PZs (prostate proper) and the TZs of 20 prostate cancer patients, 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 who confirmed the absence of prostate carcinoma in the two zones. Laser microdissection was performed as previously described (22-24). 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 of normal glands and the stroma between them.

Quality control. The quality of the RNA was measured for every patient prior to laser microdissection using the Lasercapture Microscope (LCM). The section was washed from the slide with 600 μl buffer RLT + 2 μM DTT (provided by the RNeasy Mini kit; Qiagen, Germany) and vortexed for 30 sec. Then RNA-extraction was performed as described by the manufacturer. The recommended DNase digestion was made with an RNase-Free DNase Set (Qiagen). The quality of the RNA was measured with the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Samples with a RIN factor >6 were used for LCM.

Laser-capture microscopy. 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 laser microdissected healthy glands of the PZ and TZ of the prostate. RNA was isolated from laser microdissected healthy glands of the PZs and TZs of the prostate from the patients using the RNeasy Micro kit (Qiagen) as described by the manufacturer. The recommended DNase digestion is included with the RNase-Free DNase Set. The amount of the isolated RNA was measured with the Nanodrop photometer (Thermo Fisher Scientific, USA). Thereafter, an equal amount of RNA from the glands of healthy PZ and TZ of 10 patients each was pooled, respectively, to a final concentration of 300 ng of RNA. These pools were sent to Miltenyi Biotec (Bergisch Gladbach, Germany) for the microarray analysis and bioinformatical interpretation. The RNA was labeled with Cy3 and hybridized on the Whole Human Genome Oligo Microarray 4x44K (Agilent, USA) according to the manufacturer's instructions. The microarray results were then validated by qRT-PCR of a subset of genes.

Transcription factor search. Search for ETS transcription factor binding sites within the promoter regions of the genes was performed using the Transcription Factor Search (TFSEARCH) (25,26). The promoter retrieval of each gene was performed using the TRED database (27), followed by insertion into the TFSEARCH database (25,26).

Results

Genes differentially expressed in normal glands of the PZ compared to the TZ of the prostate. Using Whole Human Genome Oligo Microarray, genes differentially expressed in normal glands of the TZ compared to the PZ of the prostate were identified (data not shown). Clustering analysis based on gene function placed each gene into various categories, and the genes that were placed into the steps of the so-called metastatic cascade (proliferation, migration and angiogenesis) and the extracellular matrix category (which is related to invasion) were examined. A total of 351 genes were identified in the previously described four categories (data not shown). However, only genes which were found to be highly differentially expressed (>10-fold increase or decrease and $p<0.01$) in the PZ compared to the TZ of the prostate were considered significant (Tables I-V). The genes that were found to fulfill these conditions include *MFAP5*, *ASPN*, *REG1A*, *KRAS*, *REG1B*, *F7*, *PKHD1*, *RUNX1* and *NOS1* (Tables I-V).

Genes with ETS transcription factor binding sites within promoter regions. A search for ETS transcription factor binding sites within the promoter regions of the 9 genes using TFSEARCH revealed that 8 of these genes have potential binding sites for ETS transcription factors (Table V).

Discussion

In the present study, gene expression differences between the normal glands of the PZ compared to those of the TZ of the human prostate were examined using microarray analysis. Due to the limited amounts of RNA that can be obtained from PCa tissues, RNA samples from individual patients had to be pooled to obtain enough RNA for microarray analysis. Genes

Table I. Genes involved in proliferation.

Gene	Name	Fold change
REG1A	Regenerating islet-derived 1 α; regenerating islet-derived 1 β	87.7
KRAS	v-Ki-ras2; Kirsten rat sarcoma viral oncogene homolog	39.0
REG1B	Regenerating islet-derived 1 β; regenerating islet-derived 1 α	19.4

The genes shown are up-regulated >10-fold ($p<0.01$) in the normal glands of the PZ compared to those of the TZ of the human prostate.

Table II. Genes involved in migration.

Gene	Name	Fold change
F7	Coagulation factor VII (serum prothrombin conversion accelerator)	17.3
PKHD1	Polycystic kidney and hepatic disease 1 (autosomal recessive)	14.2

The genes shown are up-regulated >10-fold ($p<0.01$) in the normal glands of the PZ compared to those of the TZ of the human prostate.

Table III. Genes involved in angiogenesis.

Gene	Name	Fold change
RUNX1	Runt-related transcription factor 1	100.0
NOS1	Nitric oxide synthase 1	32.8

The genes shown are up-regulated >10-fold ($p<0.01$) in the normal glands of the PZ compared to those of the TZ of the human prostate.

Table IV. Genes encoding components of the extracellular matrix.

Gene	Long name	Fold change
MFAP5	Microfibrillar associated protein 5	16.4
ASPN	Asporin	13.1

The genes shown are up-regulated >10-fold ($p<0.01$) in the normal glands of the PZ compared to those of the TZ of the human prostate.

Table V. ETS binding sites.

Gene	No. of potential ETS binding sites
ASPN	3
F7	1
KRAS	2
MFAP5	3
NOS1	5
PKHD1	1
REG1B	6
RUNX1	4

The genes shown are the ones with potential ETS transcription factor binding sites within their promoter regions.

that may be significantly affected among the patients rather than in an individual patient will remain significant in the pool.

A clustering analysis based on gene function placed each gene into various categories, and genes that were placed into the steps of the so-called metastatic cascade (proliferation, migration and angiogenesis) and the extracellular matrix category were examined. Out of 351 genes identified, only 9 were found to be highly differentially expressed (>10-fold increase or decrease and $p<0.01$) in the PZ compared to the TZ in these categories (Tables I-V).

Proliferation. The genes *REG1A*, *KRAS* and *REG1B* were found to be >10-fold up-regulated in the normal glands of the PZ compared to those of the TZ (Table I).

Briefly, the growth factor *REG1A* has been reported to be highly expressed within breast carcinomas and is suggested to be an independent predictor of poor prognosis (28). It has also been found to be up-regulated in colon carcinoma patients with unfavorable clinical outcomes (29). Furthermore, overexpression of REG IA protein in PCa cell lines has been shown to lead to accelerated cell proliferation and tumor growth *in vitro* and *in vivo* (30). Activation of oncogenic *KRAS* is frequently found in pancreatic ductal adenocarcinoma (PDAC), and has been recently suggested to contribute to PDAC initiation (31). Furthermore, *KRAS* activation is suggested to be a founder event in melanomagenesis (32). Lastly, *REG1B* along with the above-mentioned *REG1A* is reported to be up-regulated during colorectal carcinogenesis (33). Analysis for potential ETS factors binding sites within the promoter regions of these genes revealed that *KRAS* and *REG1B* have 2 and 6 potential ETS factor binding sites, respectively (Table V).

Migration. *F7* and *PKHD1* were found to be >10-fold up-regulated in the normal glands of the PZ compared to those of the TZ (Table II). Briefly, it has been reported that colorectal cancer is capable of ectopically synthesizing F7 (fVII), and a microenvironment with a high F7 protein could lead to promoting tumor metastasis (34). Furthermore, ectopic F7 expression is frequently reported in ovarian cancers as well (35). Finally, the protein polyductin, encoded by the *PKHD1* gene, was detected in cholangiocellular carcinoma (36). One potential ETS factor binding site was found within the promoter regions of *F7* as well as of *PKHD1* (Table V).

Angiogenesis. The genes *RUNX1* and *NOS1* were found to be >10-fold up-regulated in the normal glands of the PZ compared to those of the TZ (Table III). Briefly, *RUNX1*, which was found to be expressed 100-fold more in the PZ compared to the TZ, is reported to be expressed in PCa cell lines as well and

suggested to cooperate with the ETS transcription factor PDEF in regulating the expression of the prostate-specific antigen (PSA) (37). *NOS1* expression is reported to be associated with decreased survival rates in patients with chondrosarcoma (38). Lastly, 4 and 5 potential ETS factor binding sites within the promoter regions of *RUNX1* and *NOS1* were found, respectively (Table V).

Extracellular matrix. The genes *MFAP5* and *ASPN* were found to be >10-fold up-regulated in the normal glands of the PZ compared to those of the TZ (Table IV). *MFAP5*, also known as *MAGP2*, has been suggested to serve as a signature in advanced ovarian cancer and to promote tumor cell survival and angiogenesis (39). ASPN has been reported to be highly up-regulated in lobular carcinomas of the breast compared to normal ductal epithelium (40). Analysis for potential ETS factor binding sites within the promoter regions of these genes revealed three potential sites in *MFAP5* and *ASPN* (Table V).

Taken together, we identified 9 highly differentially expressed genes in the normal glands of the PZ compared to the TZ of the human prostate. The genes which were found to be up-regulated in the PZ compared to the TZ are up-regulated in various types of cancer, which may explain the differences in susceptibility for PCa of the TZ compared to the PZ. Eight out of 9 of these genes may be potentially regulated by ETS transcription factors, which is suggested by the presence of ETS factor binding sites in the promoters of these genes. Our assumption is further supported by our own findings showing that ETS factors are expressed more in the normal glands of the PZ compared to those of the TZ (unpublished data). Furthermore, ETS members have been reported to be involved in tumor angiogenesis, proliferation and invasion (41-44), and found to be overexpressed in latent and clinically manifest prostatic carcinomas (45). Future studies should examine the role of these selected genes and their potential regulation by ETS-1 in prostate cell lines derived directly from the normal glands of the PZ, the TZ and of PCa.

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