Identification of immunity-related genes in prostate cancer and potential role of the ETS family of transcription factors in their regulation

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Abstract. The role of the immune response in tumor progression, and disease outcome is still debated, and a lack of knowledge of the immune defenses in prostate cancer still exists. In addition, the ETS family of transcription factors which is involved in translocations frequently found in prostate cancer is reported to be essential for the regulation of immunity-related genes. In order to identify immunity-related genes in prostate cancer, we performed two microarrays using RNA extracted from laser microdissected glands of the normal prostate proper (or the peripheral zone) and moderately and poorly differentiated prostate carcinomas from patients who had undergone radical prostatectomy. Many differentially expressed genes were found, however, only immunity-related genes (B cell, innate, and T cell immunity) with an expression of more than 10-fold increase or decrease and a $P<0.01$ between the moderately differentiated tumors and the normal glands, and the poorly differentiated tumors and the normal glands were considered significant. Based on these two microarrays, we identified a set of 37 genes that were up- or down-regulated in tumors (moderately and poorly differentiated) compared to the normal glands. Analysis of these genes revealed, strikingly, that 31/37 of these genes have potential binding sites within their promoter regions for members of the ETS family of transcription factors, and some are reported to be targets of ETS members. These findings identified immunity-related genes in prostate cancer, and provided insights into their potential regulation, which may lead to a better early detection, immunotherapy, and therapeutic drug treatment of this disease. Unraveling the dynamics of the ETS-immunity-related genes will provide an invaluable insight into understanding prostate cancer immunology.

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Introduction

Prostate cancer is a clinically heterogeneous-multifocal disease with a clinical outcome difficult to predict, and one of the most prevalent malignancies in men (1-4). The role the host immune response plays in tumor progression is still in dispute, and studies have suggested that the immune system can promote or suppress tumor growth (5). It has been suggested that production of cytokines by the activation of innate immunity and inflammation can either stimulate or inhibit tumor growth and progression (6). The majority of pro-inflammatory cytokines produced by the host immune cells or tumor cells promote tumor development, whereas pro-apoptotic and anti-inflammatory cytokines usually interfere with tumor development (6). A weaker host-mediated antitumor activity than a tumor-mediated immunosuppressive activity, leads to immune escape and rapid growth of the tumor cells, whereas a stronger antitumor activity leads to the eradication of the tumor cells (6).

During the progress of cancer, many patients will develop innate and adaptive immunity responses (5). In prostate cancer, a combination of TH1-adaptive immunity and inflammation genes has been suggested to confer improved prognosis (5). A role of the immune system in determining disease outcome, and an association between the body's immune system and tumor behavior have also been suggested (5). The prostate is relatively predisposed to several degrees of inflammation, and inflammation is suggested to contribute to prostatic carcinogenesis (7,8). The reasons that lie behind such a predisposition are still unclear; however the ability of the body's immune response against prostate tissues is undeniable (7). A recent study using a mouse model, suggested that T regulatory lymphocytes down-regulated inflammatory cytokines, and that inflammatory cytokines are significantly elevated and linked with prostate cancer development, but without the presence of inflammatory disease in the prostate (9).

It has been reported that immunity against normal or cancerous prostate tissues can be induced by manipulating the immune system (7). Stimulating T-cell responses is the most immunotherapeutic approach in prostate cancer (7). Reduced infiltration of tumor-associated macrophages is associated with prostate cancer progression (10). Furthermore, absence

or weak tumor infiltrating lymphocytes are associated with high risk of tumor progression (11). Studies reported that an immune response in the form of autoantibodies against certain tumor antigens develops in many cancer patients (12-15), therefore subsequent studies attempted to use these autoantibody signatures to measure the immune response in prostate cancer and utilize it as a possible early detection method for the disease (15).

Prostate cancer can avoid the immune defenses by presenting a defective antigen, expressing immunosuppressive molecules, T cell receptor dysfunction, and active immune down-modulation (16). Most prostate cancer patients are immunocompetent, even though the tumor avoidance mechanisms of the immune defenses alter the host immunity (16). The failure of the immune defenses against the tumor can be interpreted as an immunological tolerance (16). Therefore, identifying and understanding the regulation of immunity-related genes in prostate cancer is of a great importance to be able to counter this disease.

The ETS family of transcription factors which is involved in translocations frequently found in prostate cancer (eg. TMPRSS2-ERG fusions 50-80% of occurrences, TMPRSS2-ETV-1 20% of occurrences) (17,18) is suggested to be essential for the regulation of the immune system, and the regulation of immunity-related genes (19). The ETS family of transcription factors has 27 members in humans and is characterized by an evolutionary highly conserved DNA-binding domain, the ETS domain, which consists of 80 amino acids with 4 tryptophan repeats (20,21). This common ETS domain interacts with nucleotide sequences that are centred around a GGAA/T motif (19). Binding preferences for distinct sequences flanking the GGAA/T motif facilitate specificity of the individual ETS-factor binding and may explain the unique biological functions of the different ETS factors (22,23). The ETS family is involved in various biological processes, such as development, differentiation, proliferation, apoptosis, migration, tissue remodelling, invasion and angiogenesis in a variety of cell types such as B cells, endothelial cells, fibroblasts as well as neoplastic cells (24-28).

The ETS family members ETS1, ETS2, ELF1, ELF3, ELF4, FLI1, GABP, SPI1 and SPIB are reported to regulate genes involved in immunity (19). While these ETS members usually activate transcription of pathogen and tumor defense-associated genes, some also repress gene transcription (19). Notably, ETS1, the family prototype and SPI1 seem to have the highest effect on immunity, primarily, by controlling immune cell development (19), whereas, other members such as ELF1 and FLI1 are implicated in autoimmunity, indicating that ETS factors can be negative regulators of immunity as well as positive ones (19).

Immunity-related genes regulated by ETS family members and their roles in immunity have been described in detail in a previous review (19). The ETS factors are reported to play roles in activation as well as repression of immunity-related genes (19). For instance, genes such as the ones encoding TCRα which facilitates T cell response to peptides presented on MHC, and CD3δ which is involved in T cell activation are activated by ETS1, whereas, genes such as the ones encoding TCRβ which is involved in T cell response, and IL2 which promotes lymphocyte proliferation, are repressed by ETS1 (19). Notably, the *c-fms* gene for instance which is involved in macrophage activation, can be activated by ETS1, as well as ETS2 and SPI1, while the gene encoding IL5, which promotes development and activity of eosinophil granulocytes, can be activated in cooperation with other factors by ETS1 and ETS2 (19). Other genes which can also be activated by multiple ETS members include, the *mb1* gene which is involved in facilitating B cell antigen receptor trafficking and signal transduction, and the *IL3* gene which is involved in promoting lymphoid and myeloid growth, by GABPα/ FLI1, and ELF1/ELF4, respectively (19). Other examples include the activation of the genes *CD49D* and *CD18* which are involved in facilitating leukocyte migration, by GABPα, and the genes encoding IL18/IL1β which promotes NK and Th1 cell activity and NK cell activation, respectively, by ELF3 (19).

In this study, we report the identification of immunity-related genes (B cell, innate, and T cell immunity) in prostate cancer, and their potential regulation by members of the ETS family of transcription factors. Identification of these immunity-related genes may have great implications into our understanding of prostate cancer immunology, and potentially lead to a better early detection, immunotherapy, and therapeutic drug treatment of this disease.

Materials and methods

Ethics statement. 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. Furthermore, written informed consents were obtained from all participants involved in the study, and were approved by the Faculty of Medicine's ethics review board of the University of Bonn/University Hospital.

Microarray raw data. The microarray raw data have been depostied at GEO under the accession number GSE28615 and we can confirm that all details are MIAME compliant.

Processing of human prostatectomy specimens. Prostate specimens were obtained from prostate carcinoma patients immediately after radical prostatectomy. The age of the patients ranged from 45-83 years (average of 67.1 years). Fresh tissue samples (0.5x0.5x0.3 cm) were taken out of the peripheral zones (prostate proper) of 5 moderately differentiated (Gleason-scores 6 and 7a) prostate carcinoma patients, 4 poorly differentiated (Gleason-scores 8 and 9) prostate carcinomas patients, as well as 10 normal peripheral zones of prostate cancer patients. The tissues were then shock-frozen in liquid nitrogen with ice-cold isopentane. Frozen sections of 6 μm were cut from the samples using a cryotome (LEICA, Germany) and mounted on membrane-coated slides (Membrane Slides, 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 as previously described (29-31). 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

Table I. Poorly differentiated tumors vs. normal glands of the peripheral zone: B cell immunity genes (up-regulated).

Short name	Long name	Fold change
HDAC9	Histone deacetylase 9	11.7
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	10.1
SH2D1A	SH2 domain protein 1A	10.1

Table II. Moderately differentiated tumors vs. normal glands of the peripheral zone: B cell immunity genes (up-regulated).

Short name	Long name	Fold change
SH2D1A	SH2 domain protein 1A	14.9
IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	11.7

Table III. Poorly differentiated tumors vs. normal glands of the peripheral zone: innate immunity genes (up-regulated).

Short name	Long name	Fold change
CRISP3	Cysteine-rich secretory protein 3	43.6
CXCL11	Chemokine (C-X-C motif) ligand 11	21.3
ADORA1	Adenosine A1 receptor	17.5
CXCL9	Chemokine (C-X-C motif) ligand 9	15
CXCL10	Chemokine (C-X-C motif) ligand 10	14
IDO1	Indoleamine 2,3-dioxygenase 1	13.4
IL2RA	Interleukin 2 receptor, α	12.1
HDAC9	Histone deacetylase 9	11.7
SH2D1A	SH2 domain protein 1A	10.1

-80˚C until use for laser-microdissection of normal glands and the stroma between them.

Quality control. The quality of the RNA was measured from every patient before laser microdissection using the laser capture microscope (LCM). The section was washed from the slide with 600 µl buffer RLT + 2 µM DTT (provided by the kit RNeasy mini kit, Qiagen, Germany) and vortexed for 30 sec. RNA-extraction was performed as described by the manufacturer. The recommended DNase digestion was made with the RNase-free DNase Set (Qiagen). The quality of the RNA was measured with the Agilent Bioanalyzer 2100 (Agilent Technologies, 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, Germany) and installed PALM MicroBeam (Zeiss). The LCM was performed under 10X objective. The glands from carcinomas and the normal peripheral zones were isolated from the stroma by laser microdissection, and collected in 200 µl Adhesive Cap tubes (Zeiss).

Microarray analysis of RNA isolated from laser microdisected moderately and poorly differentiated prostate carcinoma glands, as well as from normal glands of prostate cancer patients. RNA was isolated from laser microdisected moderately and poorly differentiated prostate carcinoma glands, as well as from normal glands from prostate carcinoma patients using the RNeasy micro kit (Qiagen) as described by the manufacturer. The recommend DNase digestion was included with the RNase-free DNase Set. The amount of the isolated RNA was measured with the NanoDrop photometer (Thermo Fisher Scientific, USA). Afterwards, an equal amount of RNA from the normal peripheral glands, moderately differentiated glands, and poorly differentiated glands were pooled to final concentrations of 300 ng of RNA, respectively. 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) according to the manufacturer's instructions. The microarray results were then validated by qRT-PCR of a subset of genes.

Transcriptional regulatory element database (TRED). The TRED database was used to determine whether members of the ETS family of transcription factors are known to target any of the 37 genes found (32). Hence, each gene was entered into the TRED database and a list of reported regulators was retrieved.

Transcription factor search (TFSEARCH). Search for ETS transcription factor binding sites within the promoter regions of the 37 genes was performed using the TFSEARCH (33,34). The promoter retrieval of each gene was performed using the TRED database (32), followed by insertion into the TFSEARCH database (33,34).

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 prostate cancer patients using Whole Human Genome Oligo microarrays. To identify immunity-related genes in prostate cancer, we analyzed the expression profile of genes involved in innate, B cell, and T cell immunity in the moderately and poorly differentiated prostate carcinoma glands compared to normal glands of the prostate proper (or the peripheral zone) from prostate cancer patients using two Whole Human Genome Oligo microarrays. Genes with an expression of >10-fold increase or decrease and a $P<0.01$ were considered significant and were selected for further analysis. A clustering analysis based on gene function placed each gene into an immunity-related category: B cell, innate, and/or T cell immunity.

Genes involved in B cell immunity. Genes found to be >10-fold up or down-regulated, with a $P<0.01$, and involved in B cell immunity are shown in Tables I, II, VII and VIII. The genes *HDAC9, CDKN2A, SH2D1A* and *IL5* were found to be up-regulated in either the moderately or poorly differentiated prostate carcinoma glands or in both compared to the normal glands (Tables I and II). On the contrary, *LTF, DMBT1, CD38,*

Table IV. Moderately differentiated tumors vs. normal glands of the peripheral zone: innate immunity genes (up-regulated).

Short name	Long name	Fold change
PGLYRP1	Peptidoglycan recognition protein 1	100
CRISP3	Cysteine-rich secretory protein 3	56.9
ALOX15	Arachidonate 15-lipoxygenase	21.5
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	15.2
SH2D1A	SH2 domain protein 1A	14.9
CXCL11	Chemokine (C-X-C motif) ligand 11	13.8
ADORA1	Adenosine A1 receptor	13.1
IDO1	Indoleamine 2,3-dioxygenase 1	12.3
IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	11.7

Table V. Poorly differentiated tumors vs. normal glands of the peripheral zone: T cell immunity genes (up-regulated).

Short name	Long name	Fold change
IDO1	Indoleamine 2,3-dioxygenase 1	13.4
IL2RA	Interleukin 2 receptor, α	12.1
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	10.1

Table VI. Moderately differentiated tumors vs. normal glands of the peripheral zone: T cell immunity genes (up-regulated).

Short name	Long name	Fold change
IDO1	Indoleamine 2,3-dioxygenase 1	12.3

Table VII. Poorly differentiated tumors vs. normal glands of the peripheral zone: B cell immunity genes (down-regulated).

Short name	Long name	Fold change
LTF	Lactotransferrin	-100
DMBT1	Deleted in malignant brain tumors 1	-27.2
CD38	CD38 molecule	-22.1
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	-10.9

BCL11A and *ITGA4* were down-regulated in either the moderately or poorly differentiated prostate carcinoma glands or in both compared to the normal glands (Tables VI and VIII).

Genes involved in innate immunity. Genes that were found to be >10-fold up and down-regulated, with a P<0.01 and involved in innate immunity are shown in Tables III, IV, IX and X. Among these genes, *CRISP3, CXCL11, ADORA1, CXCL9, CXCL10, IDO1, IL2RA, HDAC9, SH2D1A, PGLYRP1, ALOX15, FGR* and *IL5* were found to be up-regulated in either the moderately or poorly differentiated prostate carcinoma glands or in both compared to the normal glands (Tables III and IV). On the other hand, *LTF, CXCL2, ORM1, CCK, CXCL3, ORM2, DMBT1, SAA1, C5AR1, SERPINA3, SCGB1A1, CXCL6, SAA4, CXCL1, NOS2, NOX1, AOX1, ACE2* and *ABCC9* were found to be down-regulated in either the moderately or poorly differentiated prostate carcinoma glands or in both compared to the normal glands (Tables IX and X).

Table VIII. Moderately differentiated tumors vs. normal glands of the peripheral zone: B cell immunity genes (down-regulated).

Short name	Long name	Fold change
LTF	Lactotransferrin	-13.4
ITGA4	Integrin, α 4 (antigen CD49D, α 4 subunit of VLA-4 receptor)	-12.2

Table IX. Poorly differentiated tumors vs. normal glands of the peripheral zone: innate immunity genes (down-regulated).

Short name	Long name	Fold change
LTF	Lactotransferrin	-100
CXCL2	Chemokine (C-X-C motif) ligand 2	-87.9
ORM1	Orosomucoid 1	-72.5
CCK	Cholecystokinin	-60.5
CXCL3	Chemokine (C-X-C motif) ligand 3	-36.6
ORM2	Orosomucoid 2	-36.5
DMBT1	Deleted in malignant brain tumors 1	-27.2
SAA1	Serum amyloid A1; serum amyloid A2	-24.6
C5AR1	Complement component 5a receptor 1	-23.8
SERPINA3	Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 3	-21.9
SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin)	-19.6
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	-18.3
SAA4	Serum amyloid A4, constitutive	-17.4
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, α)	-16.5
NOS2	Nitric oxide synthase 2, inducible	-15.5
NOX1	NADPH oxidase 1	-10.7
AOX1	Aldehyde oxidase 1	-10.3

Genes involved T cell immunity. Genes that were >10-fold up or down-regulated, with a P<0.01 and involved in T cell immunity are shown in Tables V, VI, XI and XII. The genes *IDO1, IL2RA* and *CDKN2A* were found up-regulated in either the moderately or poorly differentiated prostate carcinoma glands or in both compared to the normal glands (Tables V and VI), whereas *SCGB1A1* and *BCL11A* were found to be down-regulated in the poorly differentiated, and *PRLR* down-regulated in the moderately differentiated carcinoma glands, compared to the normal glands, respectively (Tables XI and XII).

Table X. Moderately differentiated tumors vs. normal glands of the peripheral zone: innate immunity genes (down-regulated).

Short name	Long name	Fold change
C5AR1	Complement component 5a receptor 1	-41.8
ACE2	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	-33.6
AOX1	Aldehyde oxidase 1	-14.7
LTF	Lactotransferrin	-13.4
ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	-11.3
CCK	Cholecystokinin	-11.2

Table XI. Poorly differentiated tumors vs. normal glands of the peripheral zone: T cell immunity genes (down-regulated).

Short name	Long name	Fold change
SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin)	-19.6
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	-10.9

Table XII. Moderately differentiated tumors vs. normal glands of the peripheral zone: T cell immunity genes (down-regulated).

Short name	Long name	Fold change
PRLR	Prolactin receptor	-100

Genes with ETS transcription factors binding sites within their promoter regions. Search for ETS transcription factors binding sites within the promoter regions of the 37 genes using TFSEARCH revealed that 31/37 of these genes have potential binding sites for members of the ETS family of transcription factors (Table XIII).

Genes reported to be targets of ETS family members. Using the TRED database (32), we retrieved a list of 5/31 genes that were reported to be targets by members of the ETS family of transcription factors (19,32,35-38) (Table XIV).

Discussion

The role of the host immune response in tumor progression is still in dispute (5). The immune system has been suggested to promote or suppress tumor growth, and to play a role in determining disease outcome (5). It has also been suggested that there is an association between the body's immune system and tumor behavior (5). In prostate cancer, the majority of patients are immunocompetent, in spite of alterations of the host immunity by the tumor avoidance mechanisms of the immune defenses (16). The immune defense's failure to act against the tumor can be interpreted as an immunological tolerance (16).

Table XIII. Genes with potential ETS transcription factors binding sites within their promoter regions.

Gene name	Potential ETS family members
ABCC9	ETS1
ADORA1	ETS1
AOX1	ETS1
BCL11A	ETS1
CCK	ETS1
CD38	ETS1
CDKN2A	ETS1, ELK1
CRISP3	ETS1
CXCL1	ETS1
CXCL2	ETS1
CXCL3	ETS1, ELK1
CXCL6	ETS1, ELK1
CXCL9	ETS1
CXCL10	ETS1
CXCL11	ETS1
DMBT1	ETS1
FGR	ETS1, ELK1
HDAC9	ETS1
IDO1	ETS1, ELK1
IL2RA	ETS1, ELK1
IL5	ETS1, ELK1
ITGA4	ETS1
LTF	ETS1
NOS2	ETS1
NOX1	ETS1
ORM1	ETS1
PGLYRP1	ETS1
PRLR	ETS1
SAA1	ETS1
SAA4	ETS1
SH2D1A	ETS1

Table XIV. Genes reported to be targets of ETS family members.

Gene Name	ETS family members
IL5	ETS1, ETS2, SPI1
ITGA4	ETS1
LTF	ETS1, SPI1
NOS2	ELF3
SH2D1A	ETS1, ETS2

Therefore, identifying immunity-related genes in prostate cancer is of a great importance, as well as identifying their potential regulators. Activation and repression of immunity-related genes have been reported to involve members of the ETS family of transcription factors, as well as the regulation of the immune system (19).

In this study, we report the identification of 37 immunity-related genes in prostate cancer, and investigated their potential regulation by members of the ETS family of transcription

factors. We analyzed the expression profile of genes with more than a 10-fold increase or decrease in moderately and poorly differentiated prostate carcinoma glands compared to normal glands of the prostate proper (or the peripheral zone) using two microarrays, and a clustering analysis based on gene function placed each gene into an immunity-related category: B cell, innate and/or T cell immunity. We then searched for ETS transcription factor binding sites within the promoter regions of the 37 genes using TFSEARCH (33,34), and whether any of these genes have been reported to be a target of ETS family members using the TRED database (32).

In addition to the role that most of these genes play in the immune response, many have been implicated in a variety of cancers, including prostate cancer. However, it is not always clear from the findings published in the literature which precise functions these genes play in the network of cellular and molecular mechanisms of antitumor immunology according to current models. Furthermore, in the present study, we have not attempted to evaluate the expression of these genes in immune cells, which may provide further insights of their roles in cancer immunology.

B cell immunity. Our results show that *HDAC9, CDKN2A, SH2D1A*, and *IL5* are up-regulated in prostate carcinoma glands compared to the normal glands (Tables I and II). Examination of these genes revealed that most of them are implicated in a variety of cancers, including prostate cancer. Briefly, investigation of human *HDAC* family members in primary medulloblastoma samples revealed high *HDAC9* expression, which was significantly associated with poor survival, and a knockdown of *HDAC9* in medulloblastoma cells lead to a decrease in cell growth and viability (39). Other studies have also indicated that high expression HDAC9 is associated with poor prognosis in childhood acute lymphoblastic leukemia (40). A variant of the *CDKN2A* gene has been suggested to be associated with an increased risk of malignant melanoma development (41). *SH2D1A* is suggested to be involved in the regulation of B cell differentiation (42). Host-derived *IL5* has been implicated in promoting experimental malignant pleural effusions and suggested to be involved in its pathogenesis (43).

Analysis of the promoter regions of these genes for potential ETS factor binding sites revealed that *HDAC9, CDKN2A, SH2D1A* and *IL5* have binding sites for ETS1, ETS1/ELK1, ETS1 and ETS1/ELK1, respectively (Table XIII) (33,34). *SH2D1A* and *IL5* have been reported to be targets of the ETS factors ETS1/ETS2, and ETS1/ETS2/SPI1, respectively (32,35-38).

On the other hand, the genes *LTF, DMBT1, CD38, BCL11A* and *ITGA4* were found to be down-regulated in prostate carcinoma glands compared to normal glands (Tables VI and VIII). Strikingly, our results show that *LTF* is 100-fold down-regulated in prostate carcinoma glands compared to the normal glands, and in agreement with our findings, others have also reported that *LTF* was the most significantly down-regulated gene in prostate cancer cells (44). *DMBT1* is a commonly believed tumor-suppressor gene, that has been suggested to be involved in the carcinogenesis of the malignant brain tumors medulloblastoma and glibolastoma multiforme (45). Furthermore, the *DMBT1* gene is also reported to be highly unstable in cancer, and to be involved in the immune defense (46). It has been reported that decreased levels of CD38 are found in prostate adenocarcinoma cells (47). *BCL11A* is suggested to be involved in lymphoid malignancies (48). *ITGA4* has been suggested to be a suppressor of metastasis, as blocking it enhances cell migration in oral squamous cell carcinoma (49).

Analysis for potential ETS factors binding sites within the promoter regions of *LTF, DMBT1, CD38, BCL11A* and *ITGA4*, showed that all of these genes have a potential ETS1 binding site (Table XIII) (33,34). *LTF* and *ITGA4* have been reported to be targets of the ETS factors ETS1/SPI1, and ETS1, respectively (32,50-52).

Innate immunity. Our results show that *CRISP3, CXCL11, ADORA1, CXCL9, CXCL10, IDO1, IL2RA, HDAC9, SH2D1A, PGLYRP1, ALOX15, FGR* and *IL5* are up-regulated in prostate carcinoma glands compared to the normal glands (Tables III and IV). Most of these genes are implicated in a variety of cancers, including prostate cancer. Briefly, others have also reported that *CRISP3* is overexpressed in prostate cancer, and suggested that it may serve as a potential biomarker for the disease (53). Other studies also suggested that *CRISP3* is an independent predictor of recurrence following radical prostatectomy for localized prostate cancer (54). It has been reported that the chemokines *CXCL9, 10* and *11* were found to be significantly up-regulated in basal cell carcinoma tissue samples compared with non-lesional skin epithelium (55). Furthermore, high levels of CXCL9 and CXL10 are reported to be secreted in all and in most melanoma metastases, respectively, by tumor endothelial cells (56). *ADORA1* is found to be up-regulated in breast cancer cell lines, suggesting a role in tumor cell growth and survival (57). Furthermore, the ADORA1 protein expression is found to be higher in most human primary breast tumor tissues compared to normal tissues (57). It has been reported that inhibition of *IDO1* promotes growth of T and natural killer cells, leads to IFN-γ production increase, and reduction in conversion to regulatory T-like cells (58). It is further suggested that selective inhibition of *IDO1* effectively regulates mediators of antitumor immunity, making it a potential therapeutic target (58). *IL2RA* is expressed in various types of cancers, including prostate cancer. It has been reported that high expression of *IL2RA* in tumors correlates with poor prognosis (59). Peptidoglycan recognition proteins are implicated in innate immunity and anti-cancer defense and PGLYRP1 is reported to play a role in innate immunity (60-62). *ALOX15* is reported to have a higher expression in human prostate tumors compared to normal adjacent tissue, and a correlation between *ALOX15* and the Gleason-score was reported (63). Additionally, a protumorigenic role for *ALOX15* has been suggested in prostate tumor development (63-65). In an ovarian carcinoma study, FGR has been suggested to play a significant role in cancer growth (66). Lastly, *HDAC9, SH2D1A* and *IL5* were discussed in the previous section above.

Analysis for potential ETS factors binding sites within the promoter regions revealed only ETS1 binding sites in *CRISP3, CXCL11, ADORA1, CXCL9, CXCL10* and *PGLYRP1*, but both ETS1/ELK1 binding sites in *IDO1, IL2RA* and *FGR* (Table XIII) (33,34).

On the other hand, *LTF, CXCL2, ORM1, CCK, CXCL3, ORM2, DMBT1, SAA1, C5AR1, SERPINA3, SCGB1A1, CXCL6, SAA4, CXCL1, NOS2, NOX1, AOX1, ACE2* and

ABCC9 were found to be more than a 10-fold down-regulated in prostate carcinoma glands compared to the normal glands (Tables IX and X).

It was suggested that malignancy in prostate cancer could be associated with elevated synthesis of angiogenesis stimulating CXC chemokines (67). *ORM* is suggested to be involved in the protection of tumor cells against immunological attack. *ORM* was reported to be significantly increased in patients with various types of carcinomas, and was suggested to influence cancer progression via its immunosuppressive properties (68). Furthermore, ORM has been reported to be significantly increased in urine of urinary bladder cancer patients, especially, in patients with invasive tumor, and indications exist that tissue-resident inflammatory cells may play a role in the increase in *ORM* in addition to the cancer cells (69). Growth of human pancreatic adenocarcinoma has been suggested to depend on *CCK*, and it has been reported that a CCK receptor antagonist inhibits growth of human pancreatic cancer cells in tissue culture and in nude mice (70). It has been reported that during the acute phase response, SAA may be involved in enhancing the migration of monocytes and granulocytes into inflamed tissues (71). During tumor development, location and timing of SAA1 production could define an immune system-evasion strategy for the tumors (72). *SAA* (*SAA1* and *SAA2*) have been found to be highly expressed in lung cancer tissue (73). Furthermore, *SAA1* has been found to be overexpressed in ovarian carcinoma tissues, and cell lines, and ovarian carcinoma patients exhibit high SAA serum levels (74). *C5AR* is expressed on various cells and specifically on the surface of immune cells, and pro-inflammatory polypeptides binds to it (75). High expression level of *SERPINA3* has been reported in HLA-positive cervical carcinoma, while in breast cancer tissues, *SERPINA3* was found to be down-regulated (76,77). *SCGB1A1* loss of expression has been reported in prostate cancer and has been suggested to be a possible indicator of disease progression, and *NOS2* has been implicated and correlated with different prostatic diseases including prostate cancer (78,79). *NOX1* was found to have increased levels in most human prostate tumor samples, and there are indications that the increase correlates with increased tumorigenicity (80). Additionally, NOX1 protein overexpression is suggested to be an early event in the development of prostate cancer (81). *AOX1* is reported to be down-regulated in meningiomas, and hypermethylated in colorectal carcinomas (82,83). *ACE2* is reported to be down-regulated in renal tumors, and in pancreatic ductal adenocarcinoma (84,85). The ATP-binding cassette *ABBCC8* was found to be amplified in resistant cancer cell lines in a study aiming at investigating chemoresistance (86). Lastly, *LTF* and *DMBT1* were discussed in the previous section above.

Analysis for potential ETS factor binding sites within the promoter regions revealed only ETS1 binding sites in *CXCL1, CXCL2, ORM1, CCK, SAA4, NOS2, NOX1, AOX1* and *ABCC9*, whereas both ETS1/ELK1 binding sites were present in *CXCL3* and *CXCL6* (Table XIII) (33,34). Additionally, the *NOS2* promoter has been reported to be activated by the ETS factor ELF3 (19).

T cell immunity. The genes *IDO1, IL2RA* and *CDKN2A* that were found to be up-regulated in the carcinoma glands and the down-regulated genes *SCGB1A1* and *BCL11A* have been previously discussed.

PRLR, a member of the cytokine receptor superfamily, which we found to be down-regulated, and PRL are reported to be expressed in both normal and malignant human prostates (87). PRL is recognized as a survival factor involved in the support of tumor growth and chemoresistance in prostate as well as breast cancer (87). PRL is reported to induce apoptosis in androgen-sensitive prostate cancer cells, but not in androgen-insensitive cells, leading to the suggestion that androgen responsiveness could be needed for PRL to be effective (88). Finally, our analysis of the *PRLR* promoter region, revealed a potential ETS1 binding site within the promoter.

The role of the immune response in tumor progression, and disease outcome is still in dispute (5), and there is a lack of knowledge of the immune defenses in prostate cancer in particular. Thus our study identified a set of 37 immunity-related genes (B cell innate and T cell immunity) that were found to be up or down-regulated in tumors (moderately and poorly differentiated) compared to normal glands of prostate carcinoma patients. We found that in addition to the role that most of these genes may play in the immune response, most have been implicated in a variety of cancers, including prostate cancer. Some of the genes have been reported to be down-regulated in one type of cancer, while up-regulated in another, for example in prostate cancer. These observations may indicate that some of these genes possess unique functions that are specifically related to the type of cancer, as is the case here for prostate cancer, and knowledge of their regulation may shed more light on the role of these genes in the immune response in prostate cancer. In this study, we did not evaluate the expression of these genes in immune cells which may provide further insight into their roles in the immunology of prostate cancer.

We found that 31/37 of these genes have potential binding sites within their promoter regions for members of the ETS family of transcription factors, which have already been reported to be involved in activation or repression of immunity-related genes (Table XIII) (19). Interestingly, all of the 31 genes have potential binding site for ETS1. ETS1, the prototype of the ETS family, is reported to play a key role in immune cell development, and the lack of ETS1 leads to a reduction of T lymphocyte numbers, and to abnormal B lymphocyte differentiation (19). Additionally, ETS1 is reported to be required for the development of natural killer cells (NK) and NK T cells, which are involved in the body's innate immunity defense against malignant cells (19,89-93). Further analysis of these genes revealed that 5/31 genes are reported to be targets of ETS family members (Table XIV) (19,32,35-38,50-52).

An observation that is worth noting is that almost all the genes that overlap between the moderately and the poorly differentiated tumors follow a trend, in which up-regulation or down-regulation in the moderately differentiated tumors is followed by an even higher up or down-regulation in the poorly differentiated tumors, respectively. This observation indicates that these genes may be part of the prostate tumor progression cascade, as the tumor progresses from moderately to poorly differentiated. It may perhaps also provide some hints into the immune response associated with these genes during prostate tumor progression, as well as identifying roles in immunosuppression, protection of tumor cells, or countering the tumor

cells. The identification and the potential regulation of these immunity-related genes in prostate cancer by ETS factors may provide a better insight into understanding prostate cancer immunology.

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