Prognostic Implications of Important Genetic Alterations in Prostate Cancer

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Prognostic Implications of Important Genetic Alterations in Prostate Cancer

Prognostische Implicaties van Belangrijke Genetische Veranderingen in Prostaatkanker

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"Ik vind liever één duiding van een oorzaak dan dat het koninkrijk der Perzen mij toevalt."
Democritus, Grieks filosoof, 5e eeuw v. Chr.

Contents

List of Abbreviations

AKT1	v-akt murine thymoma viral oncogene homologue 1
AR	androgen receptor
BRFS	biochemical recurrence-free survival
CRPC	castration-resistant prostate cancer
CT	computerized tomography
DRE	digital rectal examination
DHT	dihydrotestosterone
ERG	v-ets erythroblastosis virus E26 oncogene homolog (avian)
ETV1	ETS variant 1 gene
ETV4	ETS variant 4 gene
ETV5	ETS variant 5 gene
FISH	fluorescence in situ hybridization
FOXP1	forkhead box P1
HDAC1	Histone deacetylase 1
HMBS	Hydroxymethylbilane synthase
IHC	immunohistochemistry
Kb	kilobases
LHRH	luneinizing hormone-releasing hormone
Mb	megabases
MRI	magnetic resonance imaging
PBGD	Porphobilinogen deaminase
PCR	polymerase chain reaction
PHD	pleckstrin homology domain
PI3K	phosphatidylinositol 3-kinase
PIN	prostatic intraepithelial neoplasia
PLND	pelvic lymph node dissection
PSA	prostate-specific antigen
PtdIns	phosphorylated phosphoinositides
PTEN	phosphatase and tensin homologue
RP	radical prostatectomy
RT-PCR	reverse transcriptase polymerase chain reaction
SPINK1	serine protease inhibitor Kazal type 1
TDRD1	tudor domain containing 1
TMPRSS2	transmembrane protease, serine 2
TNF	tumour necrosis factor
TNM	tumour node metastasis
TRUS	transrectal ultrasound of the prostate
TURP	transurethral resection of the prostate

Chapter I

General Introduction

Prostate cancer

The prostate is a walnut-sized gland that is located caudally from the urinary bladder. It excretes fluid as a part of the semen of men. Prostatic neoplasia is common; in Western developed countries prostate cancer is the most common non-cutaneous malignancy in men and it is the second leading cause of all male cancer deaths (1). The detection of prostate cancer has markedly increased since the introduction of the serum prostate-specific antigen (PSA) in the late eighties (1). Although familial and hereditary prostate cancer occurs, sporadic cancers account for at least 85% of all prostate cancers. Age is the strongest risk factor for developing prostate cancer. From autopsy studies it is known that in the 6th decade already 55% of the male population has the disease, and more than 75% of men older than 85 years have cancer foci in their prostate (2).

Prostate cancer is a very heterogeneous disease that can range from indolent, asymptomatic tumours in many patients to a rapidly fatal malignancy in some. At diagnosis, the majority of tumours are confined to the prostatic gland, named clinical stage cT1 and cT2 (see Table 1 for the Tumour, Node, Metastasis (TNM) classification). Some tumours, however, already grow outside the prostatic capsule (cT3) or even invade the surrounding organs (cT4). Dissemination of the disease usually occurs to the regional pelvic lymph nodes and the axial skeleton. The latter will mainly cause pain, although neurological deficit due to compression of the myelum is also possible in severe cases.

Diagnostic modalities to detect prostate cancer are digital rectal examination (DRE), measurement of the serum prostate-specific antigen (PSA), transrectal ultrasound of the prostate (TRUS) with subsequent ultrasound-guided prostate biopsies, and magnetic resonance imaging (MRI). Computerized tomography (CT) of the pelvis is reserved for detection of enlarged lymph nodes, whereas bone metastases can be detected by nuclear scintigraphy, CT, MRI or conventional X-ray of the skeleton. The serum PSA is a marker for prostate cancer (3). Although PSA lacks specificity, it is currently the most valid biomarker available, not only for diagnostic purposes but also for disease monitoring after treatment with curative intent. Since its introduction, PSA has been widely used as a screening tool to detect prostate cancer in asymptomatic men. Recent findings showed that PSA-based screening for prostate cancer resulted in a relative risk reduction of 20% to die from the disease (4). It must be noted, however, that at the time of the first statistical relevant difference between the intervention and control arm, it was shown that 48 patients had to be treated to prevent one death from prostate cancer. The findings by Schroder *et al.* show the difficulties that remain to correctly identify the indolent prostate tumours from the ones that need curative treatment. Therefore, prognostic markers that facilitate a risk stratification of prostate cancer patients and that are helpful for optimizing treatment decisions are still very much needed.

Table 1 - The 2009 Tumour, node, metastasis (TNM) classification of prostate cancer

Stage T1	**Tumour present, but not detectable clinically or by imaging**
T1a	Tumour incidentally found in less than 5% of prostate tissue resected
T1b	Tumour incidentally found in greater than 5% of prostate tissue resected
T1c	Tumour detected by prostate biopsy performed for elevated PSA
Stage T2	**Organ-confined disease**
T2a	Tumour located in half or less than half of one prostate lobe
T2b	Tumour located in more than half of one prostate lobe
T2c	Tumour located in both prostate lobes
Stage T3	**Extra-prostatic disease**
T3a	Extra capsular extension including microscopic bladder neck involvement
T3b	Tumour invading the seminal vesicle(s)
T4	Tumour invading surrounding organs
N	**Regional lymph nodes**
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Lymph node metastases
M	**Distant metastasis**
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Treatment modalities for organ-confined tumours typically involve surgery (open, laparoscopic or robot-assisted), radiation therapy (external beam, brachytherapy or cyberknife) or active surveillance. Expectant management was initially reserved for patients who had a life expectancy of less than 10 years and suffered from 'insignificant tumours' (5). The vast majority of these tumours will never lead to disease-related symptoms or death. As a result of this, active surveillance is nowadays emerging as a treatment strategy for low-risk prostate cancer with the objective to avoid overtreatment in these patients. In a retrospective study, cancer-specific survival at 10 years was 100% for patients who were suitable for active surveillance (6). A randomized trial, showed a small but significant survival benefit for radical surgery compared with watchful waiting in clinically-detected, localized prostate cancer (7, 8). Therefore, expectant management is only reserved for low-risk prostate cancer, whereas intermediate or high-risk tumours need treatment with curative intent, i.e. surgery or radiation therapy. No prospective studies have been conducted thus far to compare the outcome of curative therapies in prostate cancer. Furthermore, no comparison of open versus laparoscopic or robot-assisted surgery has been carried out.

For locally advanced prostate cancer the combination of external beam radiotherapy and three years of endocrine therapy is the treatment of choice (9). In experienced hands, however, radical prostatectomy seems feasible too for cT3 tumours (10-12). In metastasized patients, endocrine therapy is the cornerstone of treatment, which has the ob-

jective to bring the serum testosterone to castration levels. This can be effectuated by surgical castration, administration of Luteinizing hormone-releasing hormone (LHRH) analogues, anti-androgens or LHRH-antagonists. Recommendations for the timing of endocrine treatment and the mode of castration remain subject to debate (13). Docetaxel-based, cytotoxic chemotherapy is reserved for patients who progress under endocrine therapy. In these patients, chemotherapy does not only improve survival but it also gives relief of pain and other cancer-related symptoms (14).

Androgen signalling in prostate cancer

The prostate is for its development and maintenance of structure and function dependent on androgen signalling (15). Androgen signalling is mediated by the androgen receptor (AR), which is an intracellular steroid hormone receptor. The AR is activated by the androgen 5α-dihydrotestosterone (DHT), which is catalyzed from testosterone by the enzyme 5α-reductase. Binding of DHT results in a rapid migration of the AR to the nucleus where it binds to androgen-responsive elements of target genes and thereby regulating their transcription. *PSA* is considered as the prototype of a tightly androgen-regulated gene. In the normal prostate, these androgen-regulated genes are involved in cellular processes like proliferation, differentiation and cell survival. Malignant prostate cells are also dependent on a functional AR. Already in 1941, Huggins and Hodges described the beneficial effects of androgen ablation by surgical castration in advanced prostate cancer (16). Since then, medical or surgical castration has been the cornerstone for the treatment of metastatic prostate cancer. However, eventually almost all tumours become refractory to castration and so-called castration-resistant prostate cancer (CRPC) emerges. Two mechanisms have been postulated for this phenomenon; first of all it is possible that survival pathways are involved that completely bypass the AR leading to AR-independent growth (17). In these tumours, the AR is downregulated or even inactivated. Secondly, tumour cells in CRPC might adapt in such a way that a functional AR-axis is retained. In this regard, several mechanisms have been described, including *AR* amplification, *AR* mutation, and induced intracrine androgen synthesis (15, 18, 19). *AR* gene amplification was shown to be present in up to 30% of recurrent prostate tumours following orchiectomy but it was absent in hormone-naïve tumours (20). Koivisto *et al.* showed *AR* gene amplification even to be associated with treatment failure in CRPC patients (21). In CRPC, *AR* mutations are less frequent than *AR* gene amplification. *AR* mutation leads to an increase in the types of ligands that can activate the receptor; the 'promiscuous', mutated *AR* might not only be responsive to DHT but also to anti-androgens and low-affinity ligands such as adrenal androgens (22, 23). This mechanism is the main argument for withdrawal of anti-androgens in CRPC patients. *De novo* intratumoral steroidogenesis has recently been identified as a third mechanism of

retaining a functional AR-axis after testosterone suppression (19, 24, 25). Furthermore, there is evidence that not only intratumoral steroidogenesis leads to an increase in testosterone in CRPC but that also adrenal androgens are converted to testosterone (26).

ETS gene fusions in prostate cancer

In 2005, a novel bioinformatics approach lead to the discovery of recurrent genomic rearrangements in prostate cancer (27). In analyzing expression array data for outlier genes, the oncogene v-ets erythroblastosis virus E26 oncogene homolog (avian), *ERG*, and the ETS variant 1 gene, *ETV1*, were identified as overexpressed in several independent prostate cancer sets. *ERG* and *ETV1* that are both transcription factors, were known to be involved in gene fusions in Ewing sarcoma (28-30). By using exon-walking quantitative polymerase chain reaction (PCR), it was shown that only the 3'region of *ERG* RNA was overexpressed in prostate cancer, suggestive of a genetic rearrangement. Characterization of the 5' ends of *ERG* by 5' ligase-mediated rapid amplification of cDNA ends discovered the first exons of the transmembrane protease, serine 2 (*TMPRSS2*) to be fused to *ERG*. *TMPRSS2-ERG* fusion transcripts were then confirmed by PCR and the gene fusions by fluorescence in situ hybridization (FISH). The findings by Tomlins *et al.* were concurred by several other groups (31-33) and nowadays it is known that more than half of all prostate cancers harbour gene fusions (34). It must be noted, however, that the reported prevalence of gene fusions varied and that it depended on the cohorts studied and the techniques used to detect gene fusions. In two expectantly treated cohorts of clinical stage cT1 tumours, the prevalence of *TMPRSS2-ERG*, as detected by FISH, was only 15-30% (35, 36) but in a surgically treated cohort, nested reverse transcriptase (RT) PCR detected more than 70% of the samples to harbour the fusion (37). Furthermore, not only *ERG* and *ETV1* but also the ETS family members *ETV4* and *ETV5* were identified to be involved in recurrent gene fusions (38-40). Whereas *ERG* was predominantly fused to *TMPRSS2*, *ETV1*, *ETV4*, and *ETV5* had multiple fusion partners (41-47).

TMPRSS2 is a prostate-specific and androgen-regulated gene located on chromosome band 21q22 (48). *ERG* is an oncogene that also is located on chromosome 21q22 in the same orientation as *TMPRSS2* approximately 3 megabases (Mb) apart. Before the identification of *TMPRSS2-ERG* gene fusion, Petrovics *et al.* already showed *ERG* to be frequently overexpressed in clinical prostate cancer (49). The identification of gene fusions in prostate cancer clarified this finding by the fact that the androgen-regulated and prostate-specific regulating sequences of *TMPRSS2* were fused to the coding sequences of *ERG*, making the *TMPRSS2-ERG* fusion gene not only prostate-specific but also androgen-regulated. Tomlins *et al.* showed that androgen treatment of the *TMPRSS2-ERG*-positive prostate cancer cell line VCaP induced *ERG* expression, whereas in LNCaP cells, which are androgen-responsive but do not harbour the gene fusion, no induction

of *ERG* expression was noticed (27). Fusion of *TMPRSS2* to *ERG* was shown to occur by two mechanisms: on the one hand the genomic region between both genes can be lost, which is the case in approximately 60% of the fusion-positive tumours, and on the other hand it can be a result of a genomic translocation (32, 50).

A great diversity exists in the structure of *TMPRSS2-ERG* fusion transcripts (37, 51, 52). Many variants are predicted to be non-coding and are probably non-functioning, except two variants that encode almost a full length ERG protein (42). The most common variant involves *TMPRSS2* exon 1 fused to exon 4 of *ERG* (Fig. 1), or less frequently to exons 2, 3 or 5 of *ERG*. Other less frequent combinations are *TMPRSS2* exon 4 or 5 fused to *ERG* exon 4 or 5 (33). The clinical significance of these different fusion transcripts is still largely unknown. *In vitro* it was shown that different splice forms promoted proliferation, invasion and motility with variable activity depending on the N-terminal *ERG* structure (53). Lapointe *et al.* described an alternative first exon mapping 4 kilobases (kb) upstream of the reference sequence, which was exclusively expressed in 10% of the fusions, whereas 40% expressed both variants (54). This alternative first exon was also reported to be involved in *ETV5* fusion transcripts (40). We showed that patients expressing *TMPRSS2-ERG* fusion transcripts containing this alternative first exon had better outcome after radical prostatectomy (55). On the contrary, Wang *et al.* found the expression of another fusion transcript to be correlated with earlier biochemical progression after radical prostatectomy (56).

TMPRSS2-ERG gene fusion is cancer-specific, as it is not found in benign prostatic tissue (31, 57). In the precursor lesion high-grade prostatic intraepithelial neoplasia (PIN), it was reported to be present in up to 20% of the samples (57, 58) and *ERG*-rearranged high-grade PIN was frequently found in close proximity to *ERG*-rearranged tumour (57, 59). Therefore, it is thought that genomic rearrangements are an early event in prostate carcinogenesis. Although there is evidence that ETS gene fusions play a role in the ability of cells to invade, they seem to be insufficient to induce cancer formation on their own. *In vitro* studies showed that overexpression of truncated *ERG* or *ETV1* in immortalized, non-tumorigenic epithelial prostate cells increased cell migration and invasion (44, 46, 53, 60, 61) and that knockdown of *ERG* or *ETV1* in prostate cancer cell lines slowed invasion (46, 53, 61, 62). In mice, expression of *ERG* and *ETV1* resulted in the development of PIN but not of invasive cancer (60, 61). In two other studies, however, *TMPRSS2-ERG* transgenic mice even did not develop PIN, only after crossing with *Pten* knockout mice, PIN and micro invasive cancer was observed (63, 64).

As localized prostate cancer is a multifocal disease, several groups tested for ETS gene fusions in different cancerous foci within one patient. In 41-70% of the cases, individual tumour foci differed according to the presence of ETS rearrangements or fusion mechanism (deletion or translocation) (65, 66). As multiple ETS rearrangements were shown to occur independently in a prostate, this demonstrated that the majority of men develop

multiple cancers in their prostate. Metastatic prostate cancer foci, however, displayed identical ETS rearrangement status, i.e. fusion-positive or -negative, indicating that only one focus was seeding metastatic deposits (67). Furthermore, Mehra *et al.* found in their series on 30 metastasized tumours that all androgen-independent metastases had *ERG* rearrangement as a result of interstitial deletion. They suggested that the loss of sequences 5' to *ERG* was associated with progression to androgen independence and eventually death from the disease. This hypothesis was not supported by the analysis of circulating tumour cells from CRPC patients, which contained either wild-type *ERG*, *ERG* rearrangement without deletion or *ERG* rearrangement with deletion (68).

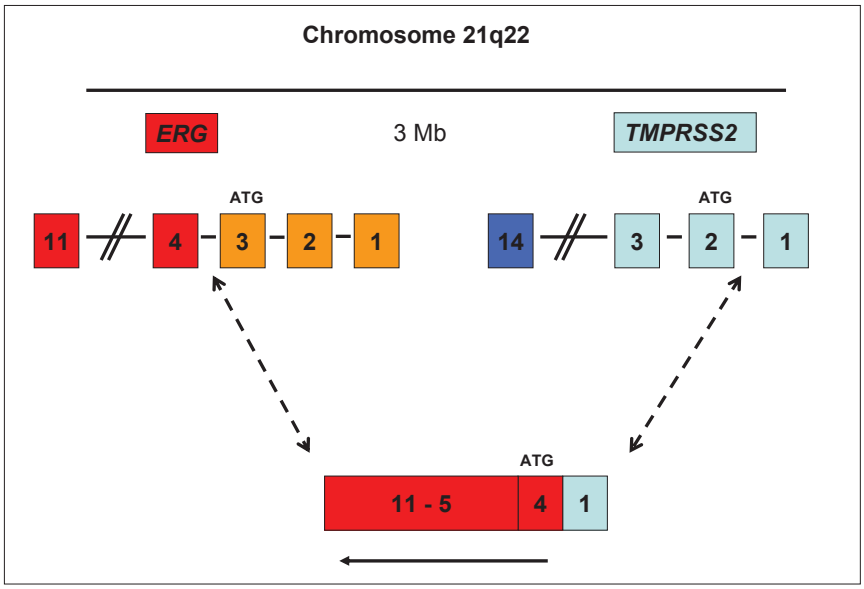

Figure 1. Schematic representation of *TMPRSS2-ERG* gene fusion
TMPRSS2 and *ERG* are both located on chromosome 21q22 in the same orientation approximately 3 Mb apart. *TMPRSS2* counts 14 exons, whereas *ERG* counts 11. *TMPRSS2* is shown in blue; *ERG* is shown in red. The native translation initiation sites are depicted above both genes (ATG). The most common variant of *TMPRSS2-ERG* gene fusion is shown; i.e. exon 1 of *TMPRSS2* linked to *ERG* exon 4. The dashed arrows point to the most common breakpoints in both *TMPRSS2* and *ERG*. The fusion transcript encodes an N-terminal truncated ERG protein. The non-native translation initiation site of the fusion transcript is in exon 4 of *ERG* is indicated (ATG). Light colours indicate noncoding exons; heavy colours indicate coding exons.

As most ETS fusion partners are androgen responsive, the presence of androgens typically stimulates the transcription of ETS gene fusions. This androgen dependence might explain the androgen dependence of prostate cancer and therapeutic strategies that target androgen signalling may function through inhibition of ETS expression. Fusion genes that are normally androgen-regulated, however, can also be expressed in an androgen-

independent fashion: the AR-negative prostate cancer cell line NCI-H660 expresses *TMPRSS2-ERG* (69). In contrast, in xenografts it was shown that androgen-independent xenografts harbouring *TMPRSS2-ERG* did not express a fusion transcript (32). Cai *et al.* found that in CRPC patients *TMPRSS2-ERG* was expressed at levels that were comparable with expression levels in primary tumours (70). Attard *et al.* showed that in CRPC patients who were treated with abiraterone acetate, an inhibitor of testosterone synthesis, *ERG* rearrangement was more frequent in patients who responded well to abiraterone (assessed by decline in PSA-level) than in patients who did not have a good response (defined as a decline in PSA level < 90%) (68). These results suggest that ETS gene fusions remain dependent on androgen signalling, despite the castration-resistant stage of the disease. Whether gene fusion status of the tumour has implications for the timing and the choice of endocrine therapy in daily clinical practice remains to be further clarified.
The analyses of gene expression profiles have implicated different molecular pathways associated with *ERG*-rearranged prostate cancer, although considerable variation has been observed between differentially expressed genes. An 87-gene expression signature that matched a gene profile associated with estradiol signalling was identified using a large patient cohort from Sweden, and the expression of *TMPRSS2-ERG* in the AR-negative cell line NCI-H660 altered by the treatment of estrogen agonists and antagonist (71). Furthermore, Iljin *et al.* showed genes involved in the WNT pathway and the gene Histone deacetylase 1 (*HDAC1*) to be strongly overexpressed in *ERG*-rearranged prostate cancer, whereas expression of tumour necrosis factor (*TNF*) and genes involved in cell death pathways were downregulated in these samples (39). Moreover, the prostate cancer cell lines VCaP and DuCaP were sensitive to the HDAC inhibitor trichostatin A (72) and the transcriptional activation of *TMPRSS2* was inhibited by HDAC inhibitors (73). Barwick *et al.* combined two patient cohorts and identified nine genes co-expressed with *ERG*, including *HDAC1* (74). Sun *et al.* proposed the proto-oncogene *c-MYC* as a potential target as its expression was mediated by *TMPRSS2-ERG* gene fusion (75), whereas Jhavar *et al.* found serine protease inhibitor Kazal type 1 (*SPINK1*) to be overexpressed in tumours lacking *ERG* rearrangement (76) consistent with previous findings by Tomlins *et al.* (77). So far, no studies have been reported that identified genes co-expressed with *ETV1* in clinical prostate cancer.

Prognostic significance of *TMPRSS2-ERG* in prostate cancer

The prognostic significance of *TMPRSS2-ERG* gene fusion is controversial as contradicting results have been reported (Table 2). The discrepancies might be a result of the differences in the populations studied, the techniques used to detect gene fusions and the effect of treatment on the examined tumour samples. Before the identification of gene fusions in prostate cancer, Petrovics *et al.* found that patients with high expression levels

of *ERG* had longer biochemical recurrence-free survival (BRFS) than patients without *ERG* overexpression (49). At the multivariable level, *ERG* overexpression remained a predictor of longer recurrence-free survival, independent of grade, stage and surgical margin status. After the discovery of recurrent gene fusions in prostate cancer, however, *TMPRSS2-ERG* was more frequently correlated with pour outcome. At the population level, a study of 111 men with clinical stage T1a or T1b prostate cancer who were treated conservatively, Demichelis *et al.* found an association between presence of *TMPRSS2-ERG* and cancer-specific death, although after adjusting for Gleason score this association was no longer significant (36). In a larger series of 445 men who were also treated conservatively, a subgroup of patients who had gene fusion with an interstitial deletion of genomic sequences between *TMPRSS2* and *ERG* (so called "class Edel") had poorer cancer-specific and overall survival than gene fusion-negative patients or than patients with *TMPRSS2-ERG* gene fusion without loss of the genomic region between the two genes (35). These findings were not confirmed in two series on surgically treated cohorts; Fitzgerald *et al.* did not find *TMPRSS2-ERG* gene fusion to be correlated with cancer-specific survival at the multivariate level in 214 patients analyzed by FISH (78). Gopalan *et al.* stated that the poor survival associated with copy number increase of chromosome 21 reflected generalized aneuploidy and that it was not due to rearranged *TMPRSS2-ERG* itself (79).
Like the earlier study by Petrovics *et al.* (49), other series correlated *TMPRSS2-ERG* with biochemical progression after radical prostatectomy. Saramäki *et al.* reported similar results in a cohort of 150 patients and showed by FISH-analysis that *TMPRSS2-ERG* fusion (33% of the population) was a predictor of longer BRFS after radical prostatectomy, independent of Gleason score, pathological T-stage and baseline PSA (80). We and others did not find an association between fusion gene expression and outcome, only after stratifying patients according to expression of *TMPRSS2(exon 0)-ERG* fusion transcripts, we found expression of this specific fusion transcript to be a predictor of longer BRFS, independent of stage, grade and surgical margin status (54, 55). Others claimed a negative correlation between *TMPRSS2-ERG* gene fusion and PSA recurrence after prostatectomy. Wang *et al.* characterized several isoforms of *TMPRSS2-ERG* in 59 patients by RT-PCR and showed that isoforms containing the native translation start codon in frame with the ERG protein were associated with early PSA recurrence, although no survival analysis was done (56). Perner *et al.* found in a high-risk cohort of 118 patients (50% Gleason score > 7, 49% pT3b tumours, 72% positive surgical margins, and 56% node-positive disease) by FISH-analysis, *TMPRSS2-ERG* gene fusion to be present in 49% of the population (50). Patients having *TMPRSS2-ERG* rearrangement through deletion showed a statistical trend for higher PSA recurrence rate than patients without fusion but a survival or multivariate analysis was not carried out. In a cohort of 165 patients (49% expressing *TMPRSS2-ERG* as identified by Q-RT-PCR), Nam *et al.* showed that fusion was correlated with earlier biochemical recurrence after prostatectomy, independent of stage and grade, although

the reported median follow-up was short (20 months) (81). Recently, *ERG* rearrangement and another frequent genetic aberration in prostate cancer, loss of the tumour suppressor gene Phosphatase and tensin homologue (*PTEN*), were combined to examine a possible correlation with outcome. Reid *et al.* reported a shorter cancer-specific survival for patients having loss of *PTEN* but lacking *ERG* or *ETV1* rearrangements (82). Their findings were in contrast to an earlier but smaller series, in which loss of *PTEN* together with the presence of an *ERG* rearrangement resulted in a shorter time to biochemical progression after radical prostatectomy (83). It must be noted, however, that the population studied by Reid *et al.* was conservatively managed and that the cohort studied by Yashimoto *et al.* was a surgically treated population.

Table 2 - Studies that reported on the correlation between *TMPRSS2-ERG* fusion gene and clinical outcome of prostate cancer

A. Cancer-specific (CSS) and overall survival (OS)						
Reference	**N**	**Tissue**	**Technique**	***TMPRSS2-ERG***	**Follow-up (median)**	**Fusion-positive versus fusion-negative cases**
Gopalan *et a.l.* (79)	521	RP	FISH	42%	7.9 yr	No difference in OS (univariate level)
Fitzgerald *et al.* (78)	214	RP / TURP	FISH	35.5%	12.3 yr	No difference in CSS (multivariate level)
Attard *et al.* (35)	445	TURP / biopsies	FISH	30%	7.5 yr	Poorer CSS and OS (multivariate level)
Demichelis *et al.* (36)	111	TURP / Millin	FISH	15%	9.1 yr	Poorer CSS (univariate level)
B. Biochemical recurrence-free survival (BRFS)						
Petrovics *et al.* (49)	114	RP	Quantitative RT-PCR	62%*	n.s.	Longer BRFS (univariate level)
Saramäki *et al.* (80)	150	RP	FISH	33%	5.5 yr	Longer BRFS (multivariate level)
Nam *et al.* (81)	165	RP	RT-PCR	42%	1.7 yr	Poorer BRFS (multivariate level)
Perner *et al.* (50)	118	RP	FISH	49%	n.s.	**
Wang *et al.* (56)	59	RP	RT-PCR	59%	n.s.	***
Lapointe *et al.* (54)	63	RP + LN	RT-PCR	70%	2.0 yr	No difference in BRFS
Hermans *et al.* (55)	67	RP	Quantitative RT-PCR	66%	10.2 yr	No difference in BRFS ****

***** *ERG* overexpression

****** Higher recurrence rate, no survival analysis

******* More early recurrences**,** no survival analysis

**** Longer BRFS for *TMPRSS2(exon0)-ERG* (multivariate level)

PI3K-AKT signalling pathway in prostate cancer

The phosphatidylinositol 3-kinase (PI3K) – v-akt murine thymoma viral oncogene homologue (AKT) signalling pathway is involved in cellular processes like cell growth, proliferation, apoptosis, and cytoskeletal rearrangement. Several components of this pathway are deregulated in a wide spectrum of cancers (84). PI3Ks are lipid kinases that are composed of a regulatory subunit (p85α), encoded by the gene *PIK3R1* and a catalytic subunit (p110 α), encoded by the gene *PIK3CA*. PI3Ks are activated by receptor tyrosine kinases (85, 86) and following activation, PI3Ks catalyse the production of phosphorylated phosphoinositides (PtdIns) by synthesizing PtdIns(3,4,5)P3 (PIP3) from PtdIns(4,5) P2 (PIP2) (87). PIP3 acts as a second messenger and activates downstream pathways like the serine-threonine Akt kinase family. PIP3 binds to the pleckstrin homology domain (PHD) of the downstream target v-akt murine thymoma viral oncogene homologue 1 (AKT1). This results in a recruitment of AKT1 to the plasma membrane where the regulatory amino acid residues serine 473 (Ser473) and threonine 308 (Thr308) are phosphorylated (88) (Fig. 2).

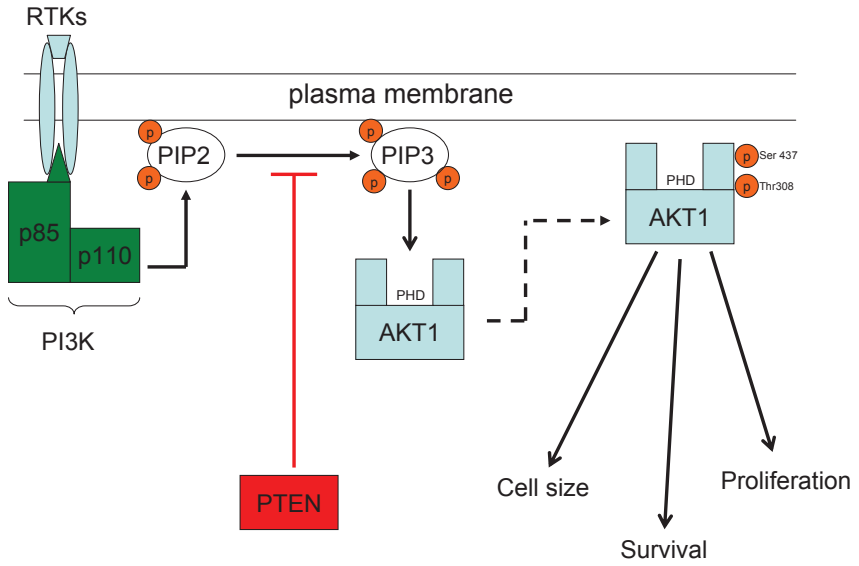

Figure 2. The PI3K-AKT signalling pathway

Phosphoinositol 3-kinase (PI3K) is activated by receptor tyrosine kinases (RTK). PI3K consists of a regulatory and a catalytic subunit (p85 and p110) and activated PI3K catalyses the production of PtdIns(3,4,5)P3 (PIP3) from PtdIns(4,5) P2 (PIP2). PIP3 acts as a second messenger and directly interacts with the pleckstrin homology domain (PHD) of the downstream target AKT1. This results in a recruitment of AKT1 to the plasma membrane where the regulatory amino acid residues serine 473 (Ser473) and threonine 308 (Thr308) are phosphorylated (p), which leads to activation of AKT1. Activated AKT1 is connected to cellular processes like cell size and growth, proliferation, and survival. The tumour suppressor gene *PTEN* has phosphatase activity against the PI3K-AKT pathway by converting PIP3 back to PIP2.

PI3K activity in benign cells is tightly regulated as it is controlled by several phosphatases. The PIP3 phosphatase that is most clearly involved in oncogenesis is encoded by the tumour suppressor gene *PTEN*. *PTEN* inactivation was originally identified in breast and brain cancer (89, 90) and was shown to have phosphatase activity against the PI3K-AKT pathway by converting PIP3 back to PIP2 (91). Germ line mutations of *PTEN* were found in rare hereditary diseases like Bannayan-Zonana syndrome and Cowden disease that are characterized by numerous hamartomas and the predisposition to certain malignancies (92, 93). Absence of PTEN leads to increased phosphorylation of AKT and thereby it stimulates PI3K-AKT signalling connecting PTEN to carcinogenesis (Figure 2). In mice, it was shown that prostate-targeted *Pten* knockout mice developed prostate hyperplasia, intraepithelial neoplasia, and ultimately, invasive cancer (94).

PTEN inactivation is frequent in various sporadic cancers, including glioblastomas, endometrial cancer, and prostate cancer (95). In prostate cancer, *PTEN* inactivation is more common in metastatic disease than in primary tumours. *PTEN* inactivation can be a result of several mechanisms: (homozygous) deletions (96-98), somatic mutations (97-99) or decreased expression (100) have all been described. *PTEN* haplo-insufficiency without inactivation of the second allele (loss of heterozygosity) was detected in up to 68% of primary tumours (83). Hermans *et al.* showed that loss of a small region around the PTEN locus was a frequent event in prostate cancer xenografts and cell lines (101).

Activation of the PI3K-AKT pathway can also take place by other mechanisms than *PTEN* inactivation. Activating mutations in *AKT1* or *PI3K* can lead to stimulation of the PI3K-AKT pathway too. *PIK3CA* has been shown to be mutated in various human cancers (102-104) and mutated *PIK3CA* was found to induce oncogenic transformation (105). *PIK3R1* mutations are less common (106), although recently it was shown that *PIK3R1* was rather frequently mutated in glioblastomas (107, 108) and colon cancer (109). Mutations in either gene leads to a disruption of the interaction between the regulatory and catalytic subunits, which enhances enzymatic activity and thereby activating the PI3K-AKT pathway (110). In prostate cancer, rare mutations in *PIK3CA* were only very recently discovered (111), whereas in *PIK3R1* no mutations have been reported thus far (112, 113). In addition, a unique but rare mutation in the PHD of *AKT1* has been identified in several cancers (114). This mutation results in a lysine substitution for glutamate (E17K) and it leads to a PI3K-independent activation of AKT1. The mutation was mutually exclusive with respect to mutations in *PI3K* and loss of PTEN protein expression. Others confirmed the mutation in breast and colorectal tumours (115) and incidental cases were identified in bladder, endometrial, lung and skin cancer (116-121). We detected the first case of the E17K substitution in AKT1 in a prostate cancer patient (122). A further characterization of AKT1(E17K) in prostate cancer revealed that the substitution was not associated with a specific growth pattern of prostate tumours but it seemed to be correlated with a favourable clinical course (123).

Outline of the thesis

The aim of this thesis is to investigate whether certain histopathologic parameters and important genetic alterations in prostate cancer carry prognostic significance. In **Chapter 2**, a large cohort of hormone-naïve, metastasized prostate cancer patients is analysed. Histopathological characteristics of lymph node metastases are collected and the Gleason score, which originally was reserved to grade primary prostate cancer (124), is applied to the lymph node metastases of the patients. All characteristics are correlated with cancer-specific survival. In **Chapter 3**, a subset of the lymph node metastases is selected and RNA is isolated from the paraffin-embedded tissue samples. The expression of *TMPRSS2-ERG* is examined and the expression of the fusion gene is correlated with the histology of the lymph nodes and patient outcome. The major objective is to evaluate whether the expression of the androgen-regulated *TMPRSS2-ERG* fusion gene predicts the duration of response to endocrine therapy.
As *TMPRSS2* transcription can start at two alternative first exons, exon 0 and exon 1, **Chapter 4** describes the specific characteristics of both transcripts in normal tissue, in prostate cancer cell lines, and prostate cancer xenografts. Furthermore, we examine the expression of *TMPRSS2(exon 0)-ERG* and *TMPRSS2(exon 1)-ERG* fusion transcripts in prostate cancer cell lines, xenografts, and clinical prostate cancer samples (both primary and recurrent tumours). In the primary prostate tumours, expression of *TMPRSS2(exon 0)-ERG* is correlated with time to PSA progression after radical prostatectomy.
In **Chapter 5**, RNA expression analysis using the GeneChip Human Exon 1.0 ST array is carried out to test for genes that are correlated with *ERG* overexpression in a cohort of primary and late-stage prostate tumours. Quantitative PCR is used to confirm the expression array data and to validate the expression of genes that are strongly co-expressed with *ERG* overexpression in a second cohort of primary prostate tumours. Expression of Tudor domain containing 1 (*TDRD1*) showed the strongest correlation with *ERG* expression in primary prostate tumours. To obtain more insight in the function of *ERG*, expression profiling and Q-PCR experiments were completed by analysis of late-stage prostate cancer and mouse prostate development.
Chapter 6 and **7** address the prognostic value of somatic mutations in the PI3K-AKT pathway. In **Chapter 6**, the first identification of the E17K substitution in AKT1 in prostate cancer is described. In **Chapter 7**, the exact prevalence of AKT1(E17K) is assessed and an association of AKT1(E17K) with a specific growth pattern of prostate cancer and clinical outcome is evaluated. In addition, a mutational profiling of the mutational hotspots in the catalytic and regulatory subunits of *PI3K* is carried out.
Finally, the results of this thesis are discussed in **Chapter 8** and suggestions for further research are mentioned.

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Part I

Prognostic implications of histopathological characteristics and molecular alterations in lymph-node positive prostate cancer

Chapter II

Histopathological characteristics of lymph node metastases predict cancer-specific survival in node-positive prostate cancer

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SUMMARY

Objective: To correlate the histopathological characteristics of lymph node metastases in prostate cancer with cancer-specific survival (CSS).

Patients and Methods: The histopathological slides from 142 patients who had had a pelvic lymph node dissection for node-positive prostate cancer were reviewed. For each patient we recorded the number of lymph nodes removed, the number of positive nodes, the diameter of the largest metastasis and extra nodal extension (ENE). The lymph node metastases were graded according to the Gleason system. The variables were correlated with CSS.

Results: The mean age of the population was 62.4 years and the mean preoperative prostate-specific antigen level was 40.2 ng/ml. The median follow-up was 77.5 months, and the median overall and CSS were 91 and 112 months, respectively. On univariable analysis the following variables correlated with poor CSS: a nodal Gleason score of >7 (hazard ratio 2.4, P < 0.001), a diameter of the largest metastasis of >3 mm (2.2, P = 0.025), more than two lymph node metastases (2.0, P = 0.003), and ENE in more than one lymph node (1.9, P = 0.014). Multivariable analysis showed only the nodal Gleason score and the diameter of the largest metastasis to be independent predictors of CSS (1.8, P = 0.021, and 2.2, P = 0.046, respectively).

Conclusion: The histopathological characteristics of lymph node metastases in prostate cancer have predictive value for the clinical outcome. The nodal Gleason score and the diameter of the largest metastasis are independent predictors of survival.

Key words: lymph node metastases; mortality; prognosis; prostate cancer

1. INTRODUCTION

According to the European Association of Urology Guidelines on Prostate Cancer, pelvic lymph node dissection (PLND) is the reference standard for N-staging in prostate cancer [1]. The involvement of regional lymph nodes in prostate cancer is regarded as a poor prognostic factor and can be a reason why a patient is not selected for radical prostatectomy (RP). However, several reports show that long-term survival after RP is possible for patients with node-positive disease [2-7]. Although various series have reported on the prognostic significance of the number of positive lymph nodes or nodal tumour burden, the 2002 TNM system for prostate cancer does not have different subclasses for node-positive disease (N0 vs N1). To draw general conclusions from previous reports is rather difficult, as study cohorts and primary endpoints differ [6-9]. In most series almost all patients were treated with both RP and PLND, with time to progression and recurrence-free survival as primary endpoints, and not cancer-specific survival.
In the present series we analysed the histopathological features of lymph node metastases from 142 patients, of whom >80% were initially treated with PLND only. We applied the Gleason score to the nodal tumours to see whether there was a correlation with CSS. Furthermore, the number of metastases removed, presence of extranodal extension (ENE), and the nodal tumour diameter, were correlated with CSS. Our objective was to identify the histopathological characteristics in lymph-node-positive prostate cancer that have prognostic significance.

2. METHODS

In all, 146 patients with histological proven node-positive prostate cancer were analysed: all had had a PLND at the authors'institution between February 1987 and April 2005, either before RP as a synchronous procedure or, in high-risk patients, as a single procedure to confirm the involvement of the pelvic lymph nodes. At the time of surgery none of the patients had clinical signs of distant metastases or had a positive bone scintigraphy. Four patients who had had endocrine treatment or radiotherapy before PLND were excluded, leaving a total of 142 patients eligible for the analysis.
Almost all patients had a limited and not an extended PLND, in the vast majority by an open procedure. Using a sub-umbilical midline incision, exposing the obturator fossa, all lymph nodes present between the external iliac vein, the obturator nerve, the bladder and the lateral pelvic wall were removed. Craniocaudal boundaries were the bifurcation of the common iliac artery and the deep circumflex iliac vessels. In all patients, lymph nodes were examined by frozen section during PLND, and routine histological examination after PLND. If the frozen section analysis showed nodal metastases the RP

was aborted, according to our institution's protocols. The study was performed in accordance with the regulations of the institution's ethical committee.
The reviewers of the histopathological slides (JB, GvL) were unaware of the follow-up data. Retrospectively, the following histological characteristics were scored for each patient: the total number of lymph nodes removed, the number of positive lymph nodes, the diameter of the largest metastasis, and ENE, defined as penetration of tumour cells beyond the lymph-node capsule into the perinodal fatty tissue. Although originally designed only for the primary tumour, we graded the lymph node tumours according to the system described by Gleason [10,11].
The histological characteristics of the lymph node metastases were correlated with the primary endpoint, CSS, calculated as the time from PLND to death from prostate cancer. Patients who were still alive or who died from other causes were censored at the date of last contact. Survival curves were calculated using the Kaplan-Meier method. The log-rank test was used to assess univariate associations with CSS. The effect of various clinical and pathological factors on patient outcome was analysed using Cox proportional regression analysis (forward stepwise selection). The model included: clinical T-stage, pretreatment PSA level, RP or not, the Gleason score of the nodal metastases, ENE, the number of positive nodes, and diameter of the largest metastasis. For the statistical tests we set a significance level of 0.05 (two-tailed probability).

3. RESULTS

The mean (range) age of the 142 patients included was 62.4 (42-76) years, the median (range) follow-up was 77.5 (3-212) months and the mean (range) PSA level before treatment was 40.2 (1.9-252.5) ng/mL. For 13 patients the pretreatment PSA levels were not known, as they were from the period before PSA testing. The other clinical characteristics of the patients are shown in Table 1. Twenty-six patients (18.3 %) had both RP and PLND, while the other 116 patients had PLND only. During the follow-up, 125 patients (88.1%) received endocrine treatment; immediate endocrine treatment (i.e. ≤90 days after PLND) and delayed endocrine treatment (i.e. >90 days after PLND) were equally distributed among the patients, at 65 vs 60. Almost 60% of the patients (83) developed distant metastases during the follow-up, 49 (34.5%) did not, and for six (4.2%) it was not known. The mean time to metastases after PLND was 50 months. Table 1 also shows the characteristics of the lymph nodes; each lymph-node metastasis was assigned a Gleason score (Fig. 1).
The median (95% CI) overall and cancer-specific survival was 91 (78-104) and 112 (90-134) months, respectively; 72 patients (50.7%) died from prostate cancer during the follow-up, while 59 (41.5%) died from other causes, or were still alive at the end of the follow-up, and for 11 patients (7.7%) these data were not available. Patients who re-

ceived endocrine treatment after surgery had poorer CSS than those who had not, but the difference was not statistically significant (P = 0.071). Within the group of patients who had received endocrine treatment, there was no difference in CSS between those who had immediate and those who had delayed endocrine treatment (P = 0.47).
The variables associated with CSS were the nodal Gleason score, the diameter of the largest metastasis, the number of metastases and ENE. Median CSS was less in patients with lymph node metastases with a nodal Gleason score of >7 than those with a nodal Gleason score of ≤7 (P < 0.001; Fig. 2a). Patients in whom the diameter of the largest metastasis was >3 mm had a shorter median CSS than those in whom the diameter was ≤3 mm (P = 0.025; Fig. 2b). The median CSS was longer for patients with two or fewer vs more than two metastases (P = 0.003). For patients with ENE in more than one lymph node, the median CSS was lower than for those with ENE in no more than one lymph node (P = 0.014). In patients who had had RP, the median CSS was longer than in those who had not (P = 0.024).

Table 1 - Characteristics of the patients and the lymph nodes

Characteristic	Mean (range) or n (%)
Age, years	62.4 (42.0-76.0)
PSA before treatment, ng/mL	40.2 (1.9-252.5)
T stage	
T1b	5 (3.5)
T1c	7 (5.8)
cT2	64 (45.1)
cT3	53 (37.3)
cT4	8 (5.6)
cTx	5 (3.5)
RP done	26 (18.3)
Endocrine therapy	125 (88.1)
Number of nodes removed	10.0 (1-27)
Number of positive nodes	2.4 (1-10)
Diameter of largest metastasis, mm	6.5 (1.0-23.5)
Gleason score	
≤6	9 (6.3)
7	76 (53.5)
8	34 (23.9)
>8	23 (16.2)
ENE	63 (44.4)

In the multivariable analysis (Cox regression) with the model including clinical T-stage, pretreatment PSA level, RP or not, nodal Gleason score, ENE, number of positive lymph nodes, and diameter of the largest metastasis, the nodal Gleason score and the diameter of the largest metastasis were independent predictors of clinical outcome (Table 2).

Table 2 - Results of univariable and multivariable analysis (forward stepwise selection)

			Univariable		Multivariable	
Variable	**No. of patients**	**Median (95% CI) CSS**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Clinical T stage						
T1b	5	114.0 (79.6-148.4)	1.000			
T1c	7	*	0.954 (0.134-6.786)	0.962		
cT2	60	127.0 (93.0-161.0)	1.010 (0.237-4.314)	0.989		
cT3	51	103.0 (70.2-135.8)	1.776 (0.425-7.417)	0.431		
cT4	7	114.0 (93.8-134.2)	3.336 (0.669-16.641)	0.142		
Pretreatment PSA level, ng/mL	131	-	1.004	0.072		
RP						
No	108	95.0 (74.8-115.2)	1.000		1.000	
Yes	23	127.0 (117.7-136.3)	0.405 (0.185-0.888)	0.024	0.466 (0.208-1.045)	0.064
Gleason score						
≤7	77	127.0 (110.9-143.1)	1.000		1.000	
>7	54	112.0 (91.8-132.2)	2.412 (1.508-3.861)	< 0.001	1.847 (1.098-3.106)	0.021
Number of metastases						
≤2	81	127.0 (115.4-138.6)	1.000			
>2	50	88.0 (74.9-101.1)	2.038 (1.280-3.246)	0.003		
ENE						
≤1	60	128.0 (109.8-146.2)	1.000			
>1	71	91.0 (91.8-132.2)	1.850 (1.130-3.021)	0.014		
Diameter of largest metastasis, mm	34	123.0 (75.0-171.0)	1.000		1.000	
≤3	97	91.0 (76.4-105.6)	2.231 (1.107-4.496)	0.025	2.173 (1.013-4.660)	0.046
>3						

* Median survival not reached

4. DISCUSSION

Lymph-node-positive prostate cancer is a serious condition that leads to death from prostate cancer in many patients, although a subset will have long overall CSS. Several reports in which RP and PLND were combined with hormonal treatment show long overall and disease-specific survival [2-5, 7]. In the present study of 142 lymph-node-positive patients, who had either had a PLND before RP with curative intent, or who had a PLND as a staging procedure, about half died from prostate cancer. Within these patients we were able to identify several histopathological characteristics of lymph node metastases that have prognostic significance.

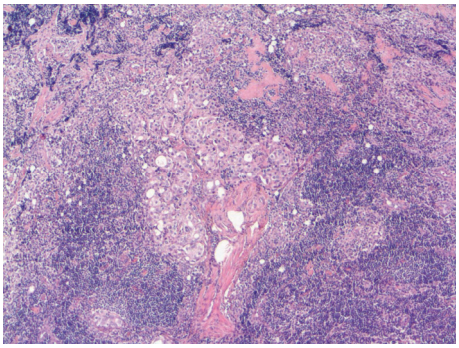

Figure 1a.

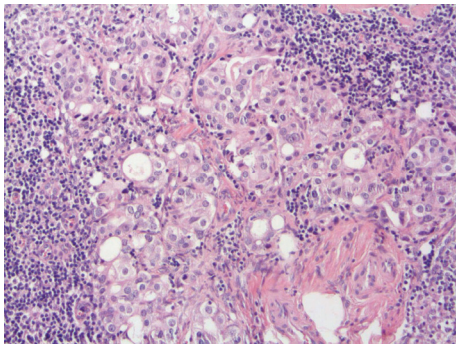

Figure 1b.

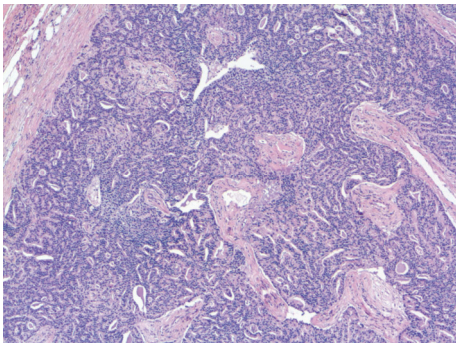

Figure 1c.

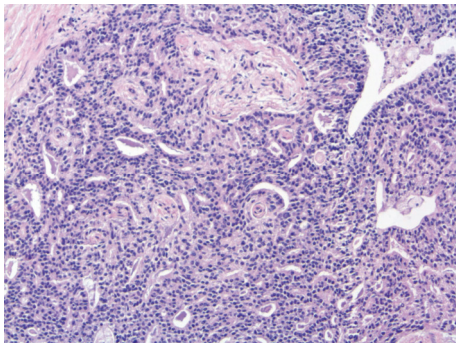

Figure 1d.

Figure 1. Lymph node metastases from two prostate cancer patients
In **a** and **b**, a small focus of a nodal metastasis is shown, nodal Gleason score 3+4=7 (magnification x40 and x100). In **c** and **d** a poorly differentiated lymph node metastasis is shown with a nodal Gleason score of 4+4=8 (magnification x40 and x100). Both haemotoxylin and eosin.

First, all metastases were graded according to the system originally described by Gleason, and the nodal Gleason scores were correlated with CSS. Higher Gleason scores were associated with poorer CSS and on multivariable analysis a nodal Gleason score of >7 was an independent predictor of clinical outcome (hazard ratio, HR 1.85, P = 0.021). Although originally designed for grading the primary tumour, the Gleason system has also been used to grade nodal metastases [12-15]. Cheng *et al.* [14] graded the primary tumours and lymph node metastases of 242 patients according to the Gleason system. In almost half of their patients (45%), the nodal Gleason score was higher than the Gleason score of the primary tumour. Patients with this so-called 'histologic dedifferentiation' (higher Gleason scores in lymph node metastases than in the primary tumour) had a significantly higher risk of disease progression than those with no dedifferentiation. Hofer *et al.* [15] evaluated 201 node-positive patients who were treated with RP, PLND, and

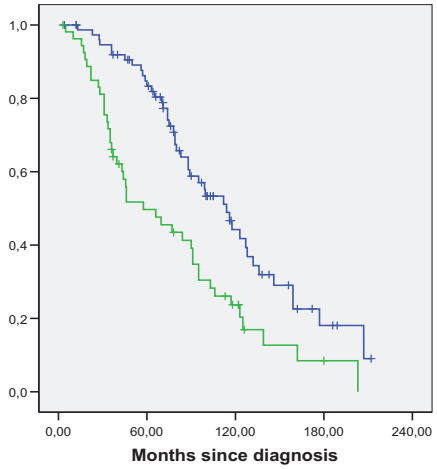

Figure 2a.

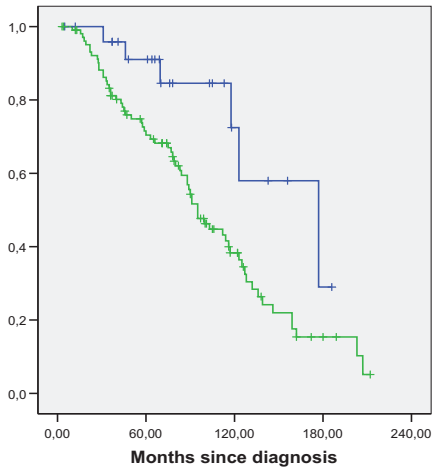

Figure 2b.

Figure 2. (a) Kaplan-Meier curves for CSS in patients with lymph node metastases with a nodal Gleason score of ≤7 (blue line) vs >7 (green line) and (b) Kaplan-Meier curves for CSS in patients with a diameter of the largest metastasis of ≤3 mm (blue line) vs > 3 mm (green line).

early hormonal deprivation, and found that higher Gleason patterns (4 and 5) in lymph node metastases were associated with an increased risk of PSA progression. However, multivariable analysis showed only lymphovascular invasion of the lymph nodes and nuclear grade of the primary to be associated with PSA recurrence.

We then estimated nodal cancer volume by measuring the diameter of the largest metastasis. A diameter threshold of 3 mm gave the highest risk of death from prostate cancer. At the multivariable level this remained an independent predictor of CSS (HR 2.22, P = 0.046). An early series from Johns Hopkins Medical Centre showed already that nodal cancer volume had prognostic significance [16]. In these series, lymph node metastases of >2 mm were associated with a poor clinical outcome. A later report by Cheng *et al.* [8] (median follow-up 6.1 years) showed that the nodal cancer volume was the strongest predictor of systemic progression (defined as the presence of distant metastases documented by biopsies or radiography). A series by Sgrignoli *et al.* [17] did not confirm this, but median follow-up was only 33 months and only 18% of their study population reached the study endpoint (distant metastasis on radiography).

We also investigated whether the number of metastases was associated with CSS, finding that a threshold of two metastases was the strongest predictor for CSS. At the multivariable level the total number of lymph node metastases was no longer an independent predictor of patient outcome. In previous reports the influence of the number of nodal metastases on clinical outcome is not clear. In a series on 3463 patients treated with both

RP and PLND, of whom 322 had node-positive disease, Cheng *et al.* [18] reported an insignificant HR of 1.5 for death from prostate cancer for patients with one nodal metastasis, vs those who were node-negative. In that population the presence of two or more than three positive lymph nodes gave a significant increase in the risk of death from prostate cancer (HR 6.1 and 4.3, respectively). Daneshmand *et al.* [6] found a 10-year clinical recurrence-free survival of 70% and 73% for patients with one and two lymph node metastases present, vs 49% for those with five or more lymph-node metastases (P = 0.003). In 507 node-positive patients treated with RP, PLND and early endocrine treatment Boorjian *et al.* [7] reported a doubling of the risk of death from prostate cancer for patients with two or more positive nodes vs those with only one metastasis (HR 2.2, P = 0.001). The median (range) number of lymph nodes removed in the present patients was 10 (range 1-27). This is in concordance with former protocols of our institution to use only a limited PLND. That the minimum of lymph nodes removed was only one showes that there were patients in whom a PLND was used only to confirm the diagnosis of node-positive prostate cancer.

Of the present patients, 44% had ENE in one or more lymph node metastasis, and there was a correlation between ENE and CSS on univariable but not on multivariable analysis. There are no reports showing a consensus on the impact of ENE on clinical outcome. Hofer *et al.* [15] did not show an association between ENE (56% from 119 patients) and PSA recurrence. Cheng *et al.* [19] found no correlation between ENE and metastasis-free or CSS, but a series by Griebling *et al.* [20] who reviewed pathology slides of 60 patients with node-positive prostate cancer (70% having ENE), found that on univariable and multivariable anaylsis ENE was an independent predictor of overall survival.

Finally, in the present series, patients who had had RP had better CSS than those who had not. As stated, RP was aborted when the frozen-section analysis showed metastases; 26 patients (18.3%) still had both RP and PLND. In these patients, the frozen-section analysis was negative, but definitive histological examination revealed metastases. The 18% of false-negative frozen-section results is consistent with other series [21,22]. However, the role of occult metastases remains unclear. Pagliarulo *et al.* [21] reported that occult metastases were an independent predictor of time to recurrence and death in patients with pT3 prostate cancer who were treated with both RP and PLND (relative risks 1.92 and 2.24, respectively). Furthermore, overall survival in their series was similar for patients with occult and regular node-positive disease. Not surprisingly, in the present series, patients who had negative frozen-section analysis (and thus had RP) had fewer and smaller metastases than patients with positive frozen sections on histological examination after surgery. However, the multivariable analysis, which corrects for such confounders, showed that RP was not an independent predictor of CSS (HR 0.47; P = 0.064).

Of the present patients, >85% had endocrine therapy during the follow-up. The timing of endocrine therapy depended on the doctor's and patient's preferences, the participa-

tion of some patients in a trial randomizing between immediate and delayed endocrine treatment, and the biological behaviour of the tumour. There was no difference in CSS between the groups. In terms of overall survival, the results of the 30846 protocol of the European Organisation for the Research and Treatment of Cancer suggested an advantage for early treatment over delayed treatment for patients with node-positive disease with no local treatment of the primary tumour (7.8 vs 6.2 year) [23]. This difference was not statistically significant, and the authors reported that the series was under-powered, so the superiority of early treatment was not confirmed. Messing *et al.* [4, 5] reported an advantage for early over delayed androgen deprivation therapy in node-positive disease (median overall survival 13.9 vs 11.3 years). It is difficult to compare both reports, because patients in the latter were treated with both RP and PLND in combination with endocrine treatment. Moreover, the size of the study population in the series of Messing *et al.* was limited (98 patients).

In conclusion, although various prognostic factors have been described for node-positive prostate cancer, the current TNM staging system for prostate cancer has no subclasses for involvement of the regional lymph nodes. Due to patient selection, different treatments and various endpoints, it is difficult to compare published results. In the present series, we analysed 142 node-positive patients who had had a PLND and of whom most had not had a RP. Histopathological characterization of the lymph node metastases identified a nodal Gleason score of >7 and a diameter of the largest metastasis of >3 mm as independent predictors of clinical outcome.

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Chapter III

Expression of the androgen-regulated fusion gene *TMPRSS2-ERG* does not predict response to endocrine treatment in hormone-naïve, node-positive prostate cancer

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ABSTRACT

Background: Fusion of the androgen-regulated gene transmembrane protease, serine 2, *TMPRSS2* to the v-ets erythroblastosis virus E26 oncogene homolog (avian), *ERG*, of the erythroblast transformation-specific (ETS) family is the most common genetic alteration in prostate cancer (PCa).

Objectives: To determine whether expression of androgen-regulated *TMPRSS2-ERG* predicts response to endocrine treatment in hormone-naïve, node-positive PCa.

Design, Setting, and Participants: Eighty-five patients with histologically confirmed, node-positive PCa who were without treatment at the moment of lymph node dissection were analysed. RNA was isolated from the paraffin-embedded lymph node metastases and complementary DNA (cDNA) was made. The quality of cDNA was tested by polymerase chain reaction (PCR) analysis of the expression of the housekeeping gene hydroxymethylbilane synthase, *HMBS* (formerly *PBGD*). *TMPRSS2-ERG* expression was analyzed by PCR using a forward primer in *TMPRSS2* exon 1 and a reverse primer in *ERG* exon 4.

Measurements: The primary end point was time from start of endocrine therapy to the occurrence of three consecutive rises in prostate-specific antigen (PSA) that were at least 2 wk apart and resulted in two 50% increases over the PSA nadir. Secondary end points were time to PSA nadir after start of endocrine treatment and cancer-specific and overall survival.

Results and Limitations: *TMPRSS2-ERG* was expressed in 59% of the 71 patients who could be analysed. Median duration of response to endocrine therapy was 20.9 versus 24.1 mo for gene fusion-positive versus gene fusion-negative patients (95% confidence intervals: 18.6-23.1 vs. 18.9-29.4, p=0.70). Furthermore, no significant differences were seen between the two groups for the secondary end points.

Conclusions: Expression of *TMPRSS2-ERG* is frequent in lymph node metastases of patients with untreated PCa; however, expression of this androgen-regulated fusion gene did not correspond with duration of response to endocrine therapy. Our results suggest that expression of *TMPRSS2-ERG* is not a candidate marker to select for metastatic PCa patients who will benefit more from endocrine treatment.

1. INTRODUCTION

Prostate cancer (PCa) is the second leading cause of male cancer deaths in the Western world [1]. The majority of PCa deaths are a result of metastatic dissemination of the primary tumour. Although endocrine treatment initially is effective in metastatic disease, eventually the vast majority of patients develop progression of disease and so-called castration-resistant prostate cancer (CRPC) emerges. Recently, fusion of the androgen-regulated and prostate-specific gene transmembrane protease, serine 2, *TMPRSS2* to the v-ets erythroblastosis virus E26 oncogene homolog (avian), *ERG*, of the erythroblast transformation-specific (ETS) family was identified as a common molecular event in PCa [2]. The fusion gene *TMPRSS2-ERG* is present in 40-70% of primary PCa. In the precursor lesion high-grade prostatic intraepithelial neoplasia (PIN), *TMPRSS2-ERG* is also detected in approximately 20% of the cases but no fusion is seen in benign prostatic tissue [3]. *TMPRSS2* maps to the chromosome band 21q22 and codes for a membrane-bound serine protease. *ERG* is also located on band 21q22, approximately 3 Mb proximal to *TMPRSS2*. Fusion of the 5'-part of *TMPRSS2* to the 3'-part of *ERG* is a result of either a chromosomal rearrangement or an interstitial deletion of genomic sequences between *TMPRSS2* and *ERG* [4,5]. As a result of this fusion, *ERG* becomes androgen regulated, leading to overexpression of N-truncated ERG in *TMPRSS2-ERG*-positive cancers. Tomlins et al have shown that stimulation of the *TMPRSS2-ERG*-positive PCa cell line VCaP with the synthetic androgen R1881 resulted in an increased expression of *TMPRSS2-ERG* [2]. Downregulation of *TMPRSS2-ERG* in VCaP cells inhibited the tumourigenic capacity of these cells [6,7]. Previous work from our group on PCa xenografts indicated a key role for *TMPRSS2-ERG* fusion in androgen-dependent PCa; however, it was bypassed in late-stage, androgen-receptor (AR)-negative disease [4].

The objective of the present study was to explore the relationship between *TMPRSS2-ERG* fusion gene and the response to endocrine treatment in clinical PCa. We hypothesized that expression of androgen-regulated *TMPRSS2-ERG* in lymph node metastases might correspond with a prolonged response to endocrine treatment in PCa patients who were without treatment at the moment of pelvic lymph node dissection (PLND). Moreover, it could be that these patients responded more quickly to androgen deprivation therapy (ADT), resulting in a shorter interval between start of endocrine therapy and the prostate-specific antigen (PSA) nadir. We also investigated whether there was a correlation between expression of *TMPRSS2-ERG* fusion and cancer-specific or overall survival.

2. MATERIAL AND METHODS

2.1 Patient samples

A total of 85 patients with histologically proven, node-positive PCa were analysed. Forty-six patients participated in European Organisation for Research and Treatment of Cancer (EORTC) study 30846. All lymph node metastases were collected by PLND. PLND was performed either before radical prostatectomy (RP) as a synchronous procedure or, in high-risk patients, as a single procedure to confirm the involvement of the pelvic lymph nodes. Using a subumbilical midline incision, exposing the obturator fossa, all lymph nodes present between the external iliac vein, the obturator nerve, the bladder and the lateral pelvic wall were removed. Craniocaudal boundaries were the bifurcation of the common iliac artery and the deep circumflex iliac vessels. In all patients, lymph nodes were examined by frozen-section analysis during PLND and routine histological examination after PLND. If the frozen-section analysis showed nodal metastases, the RP was aborted, according to our institution's protocols. At the time of surgery, none of the patients had clinical signs of distant metastases or had a positive bone scintigraphy.
The paraffin-embedded lymph node metastases were reviewed by a specialized uropathologist (GvL) and were graded according to the system originally described by Gleason [8]. Additional paraffin-embedded prostate tumour samples were available for 19 patients who had also had a transurethral resection of the prostate (TURP). TURP in these patients was performed for reasons of bladder outlet obstruction that had occurred during follow-up after PLND. The study was performed in accordance with the regulations of the institution's ethical committee.

2.2 RNA extraction and complementary DNA synthesis

From the cancerous regions of the formalin-fixed, paraffin-embedded lymph node metastases and TURP samples, 1-mm core biopsies were taken (Beecher Instruments, Silver Spring, MD, USA). RNA was isolated using the High Pure RNA Paraffin Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the cores were deparaffinised, and the samples were treated overnight with ProteinaseK. Genomic DNA was removed by DNase treatment for 45 min at 37 °C. A second round of ProteinaseK was then performed for 1 hr at 55 °C. Finally, RNA was eluted in elution buffer in an end volume of 50 µl.
Complementary DNA was synthesised by reverse transcribing 1 µg messenger RNA with 200 units M-MLVT RT (Invitrogen Life Technologies, Carlsbad, CA, USA) and hexamer primers. Next, standard polymerase chain reaction (PCR) was done for the housekeeping gene hydroxymethylbilane synthase, *HMBS* (formerly *PBGD*). PCR products were identi-

fied by 2% agarose gel electrophoresis and ethidium bromide staining. If amplification of *HMBS* was found, reverse transcription PCR (RT-PCR) was done for *TMPRSS2-ERG* using a forward primer in *TMPRSS2* exon 1 and a reverse primer in *ERG* exon 4. The following primer combinations were used: *HMBS*-F (5′-CAAAGATGAGAGTGATTCGC-3′), *HMBS*-R (5′-CACACTGTCCGTCTGTATGC-3′), *TMPRSS2(exon 1)-ERG(exon 4)*-F (5′-CCGCCTGGAGC-GCGGCA-3′) and *TMPRSS2(exon 1)-ERG(exon 4)*-R 5′- GTAGGCACACTCAAACAACGACT-GG-3′), yielding fragments of 67 and 69 base pairs, respectively. Other less frequent fusion partners like ets variant 1, *ETV1*; ets variant 4, *ETV4*; and ets variant 5, *ETV5*, which are known to occur in approximately 10% of all PCa patients [9], were not studied because RNA yield from the paraffin-embedded tissue samples was too limited to test for all rare fusion partners.

2.3 Statistical Analysis

Expression of *TMPRSS2-ERG* was correlated with the primary end point, duration of response to endocrine treatment, defined as the time of start of endocrine treatment after PLND to the occurrence of three consecutive rises of PSA that were at least 2 wk apart and resulted in two 50% increases over the nadir. The date of the third PSA measurement was taken as the end of response. Secondary endpoints were time to PSA nadir after start of endocrine treatment, cancer-specific survival, and overall survival. Survival was calculated as the time from PLND to death from PCa. Patients who were still alive or who died from other causes were censored at the date of last contact or death. For calculation of survival curves, the Kaplan-Meier method was used. To assess univariate associations with duration of response to endocrine treatment, time to PSA nadir, or survival, the log-rank test or the Breslow method was used, as appropriate. Associations between clinical and histopathological variables and expression of the *TMPRSS2-ERG* were evaluated by the Pearson χ^2 test or the Mann-Whitney test, as appropriate. Statistical analyses were performed using the Statistical Package for Social Sciences, v.15.0 (SPSS, Chicago, IL, USA), with a significance level of 0.05 (two-tailed probability).

3. RESULTS

3.1 Patient demographics

Eight patients who were treated before PLND, either by endocrine therapy (n = 5) or radiotherapy (n = 3), were excluded, as were four other cases in which RNA isolation was unsuccessful. Furthermore, two patients that were known to harbor *ETV1* gene fusions were also excluded. The mean age of the remaining 71 patients was 62.7 yr (standard

deviation: ± 6.6). With a median follow-up of 71.1 mo (range 6.9-216.1), 63% of the population (45 patients) died from PCa. The clinicopathological characteristics of the population are summarised in Table 1.

Table 1 - Clinical and pathological characteristics of the population (71 patients)

Characteristic	
Mean age (SD), yr	62.7 ± 6.6
Mean pre-treatment PSA (SD), ng/ml	56.0 ± 72.6
cT-stage, *n (%)*	
≤cT2	22 (31.0)
≥cT3	38 (53.5)
Unknown	11 (15.5)
Death from prostate cancer, *n (%)*	45 (63)
Endocrine treatment modality, *n (%)*	
None	1 (1.4)
LHRH-agonists	53 (74.6)
Anti-androgens	6 (8.5)
Surgical castration	7 (9.9)
Unknown	4 (5.6)
Mean number of lymph nodes removed (SD)	8.1 ± 5.3
Mean number of positive lymph nodes (SD)	3.0 ± 2.2
Mean diameter of metastases (SD)	8.1 mm ± 4.1
Nodal Gleason score, *n (%)*	
< 8	35 (49.3)
≥ 8	36 (50.7)
Extra nodal extension, *n (%)*	
Yes	44 (62.0)
No	27 (38.0)

SD = standard deviation; PSA = prostate-specific antigen; cT-stage = clinical T stage; LHRH = luteinising hormone-releasing hormone.

3.2 *TMPRSS2-ERG* and clinical outcome

TMPRSS2-ERG was expressed in 42 out of 71 patients (59%). Notably, in the 19 patients for whom additional TUR-P samples were available, fusion-gene status of the lymph node metastases and TUR-P samples was in full agreement: In tumours from 13 patients, expression of *TMPRSS2-ERG* was detected, whereas 6 patient samples did not express the fusion gene. There were no significant differences in clinical and pathological characteristics between the *TMPRSS2-ERG*-positive and *TMPRSS2-ERG*-negative groups (Table 2). Four patients had had both an RP and a PLND. In these patients (three in the gene fusion-negative and one in the gene fusion-positive group), the frozen-section analysis during PLND was negative; however, histological examination after PLND showed pres-

ence of nodal metastases. All patients (except one who died from other causes within 1 yr after PLND) received endocrine treatment after PLND. Endocrine treatment consisted of a depot of luteinizing hormone-releasing-hormone (LHRH) agonists and 1 mo of an antiandrogen (n = 53), antiandrogen monotherapy (n = 6), or surgical castration (n = 7). For four patients, the endocrine treatment modality could not be retrieved. Timing of endocrine therapy depended on participation of patients in EORTC study 30846, which randomised subjects between immediate and delayed endocrine treatment, or on the doctor's and the patient's preferences. Immediate endocrine treatment (ie ≤90 d of PLND) and delayed endocrine treatment (ie, >90 days after PLND) were equally distributed among the gene fusion-positive and gene fusion-negative groups (61% versus 59%, respectively, for immediate treatment).

Table 2 - Clinical and pathological characteristics of 71 patients according to expression of *TMPRSS2-ERG*†

Characteristic	*TMPRSS2-ERG* positive (n=42)	*TMPRSS2-ERG* negative (n=29)	p-value
Mean age (SD), yr	62.2 ± 6.7	63.3 ± 6.6	p=0.69*
Mean pre-treatment PSA (SD), ng/ml	44.8 ± 33.5	72.6 ± 106.0	p=0.94*
RP performed, *n (%)*	1 (2.4)	3 (10.4)	p=0.16***
cT-stage *n (%)*			
≤cT2	15 (44.1)	7 (26.9)	p=0.17**
≥cT3	19 (55.9)	19 (73.1)	
Endocrine treatment modality, *n (%)*			
None	0 (0)	1 (3.8)	p=0.51***
LHRH-agonists	32 (78.0)	21 (80.8)	
Anti-androgens	4 (9.8)	2 (7.7)	
Surgical castration	5 (12.2)	2 (7.7)	
Mean number of positive lymph nodes (SD), mm	2.7 ± 1.7	3.5 ± 2.7	p=0.25*
Mean diameter of metastases (SD)	8.9 ± 4.6	6.8 ± 3.0	p=0.092*
Nodal Gleason Score, *n (%)*			
< 8	20 (47.1)	15 (51.7)	p=0.73**
≥ 8	22 (52.9)	14 (48.3)	
Extra nodal extension, *n (%)*			
Yes	25 (59.5)	19 (65.5)	p=0.61**
No	17 (40.5)	10 (34.5)	

SD = standard deviation; RP = radical prostatectomy; cT-stage = clinical T stage; LHRH = luteinising hormone-releasing hormone; PSA = prostate-specific antigen.

* = Mann-Whitney U test.

** = χ^2 test.

*** = Likelihood Ratio.

† Values that were not known were not included in this table.

We did not observe a significant difference in median duration of response to endocrine therapy for *TMPRSS2-ERG*-positive versus *TMPRSS2-ERG*-negative patients: 20.9 versus 24.1 mo (95% confidence intervals (CIs): 18.6-23.1 vs 18.9-29.4, $p = 0.70$) (Fig. 1). The median time to PSA nadir after start of endocrine treatment was 9.0 mo for gene fusion-negative patients versus 9.6 mo for gene fusion-positive patients (95% CI: 8.2-11.1 vs 5.8-12.1, $p = 0.074$) (Fig. 1b). Furthermore, no significant differences were observed in cancer-specific or overall survival between gene fusion-positive and gene fusion-negative patients. The median cancer-specific survival for gene fusion-positive and gene fusion-negative patients was 83.9 versus 103.2 mo (95% CI: 73.8-94.0 vs 79.6-126.7, $p = 0.22$) (Fig.2a) and the median overall survival was 73.4 versus 75.3 mo (95% CI 56.7-90.0 vs 49.9-100.7, $p = 0.71$) (Fig. 2b).

4. DISCUSSION

The identification of fusion of *TMPRSS2* to the ETS family genes *ERG* and *ETV1* is one of the most important breakthroughs in recent PCa research. *TMPRSS2* is a prostate-specific and androgen-regulated gene; thus, it appears that *TMPRSS2-ERG* acts as a novel androgen-responsive oncogene. We studied the possibility that hormone-naïve PCa patients who express the androgen-regulated *TMPRSS2-ERG* might have a prolonged response to endocrine treatment or that such patients will respond more quickly to ADT, resulting in a shorter interval between start of endocrine treatment and PSA nadir. We did not, however, find a correlation between expression of *TMPRSS2-ERG* and duration of response to endocrine treatment in a group of 71 node-positive PCa patients who were without treatment at the moment of diagnosis (median follow-up: 6 yr). Additionally, we did not observe a difference in time to PSA nadir after start of endocrine therapy between gene fusion-positive and gene fusion-negative patients. As far as we know, this report is the first to explore the relationship between expression of *TMPRSS2-ERG* and endocrine therapy in clinical PCa.

Huggins et al were the first to describe the benefits of androgen ablation in the treatment of PCa [10]. Since then, chemical or surgical castration has been the cornerstone in the treatment of advanced PCa. The various treatment modalities (LHRH agonists, antiandrogens, or surgical castration) seem to be equally effective in terms of cancer-specific and overall survival [11]. The timing of endocrine therapy in node-positive PCa, however, remains matter of debate. There is evidence from a randomised controlled trial that there is a survival benefit for immediate therapy in patients with node-positive disease treated by RP and PLND [12]. EORTC 30846, the trial in which more than half of the current patients participated, also suggested an advantage for early treatment in terms

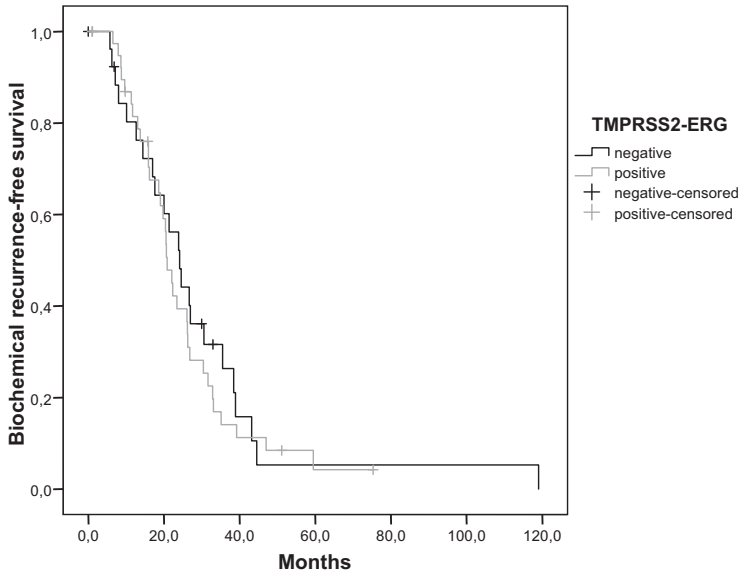

Figure 1a.

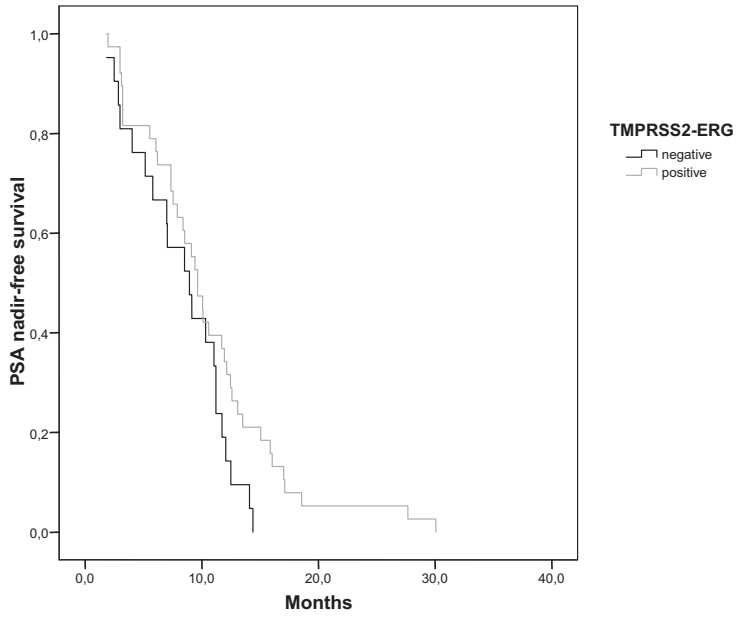

Figure 1b.

Figure 1. Kaplan-Meier curves (A) for duration of response to endocrine therapy and (B) for time to PSA nadir after start of endocrine treatment in prostate cancer patients with lymph node metastases expressing *TMPRSS2-ERG* (grey line) versus prostate cancer patients with lymph node metastases not expressing *TMPRSS2-ERG* (black line).

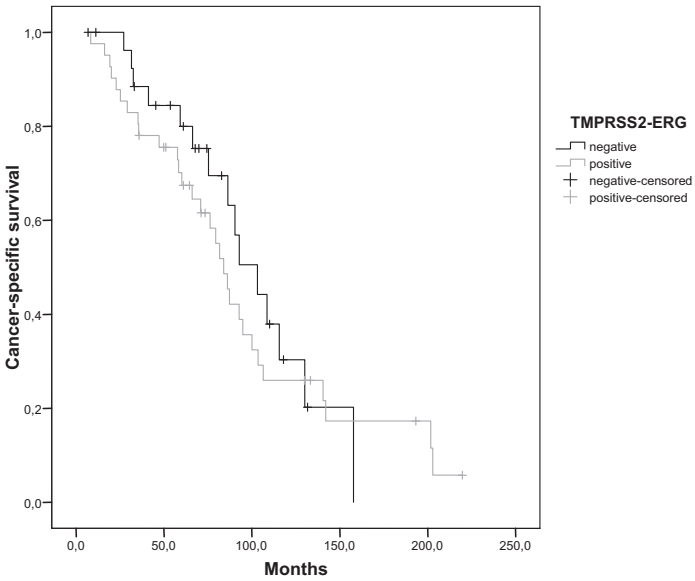

Figure 2a.

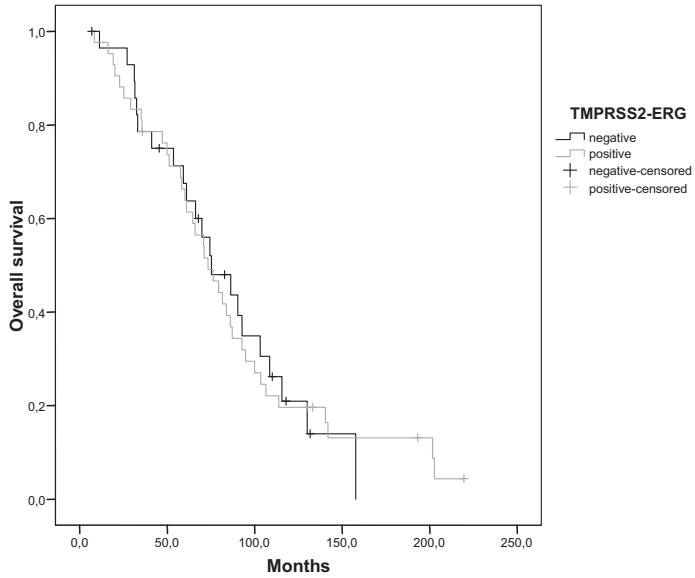

Figure 2. Kaplan-Meier curves (A) for cancer-specific survival and (B) for overall survival in prostate cancer patients with lymph node metastases expressing *TMPRSS2-ERG* (grey line) versus prostate cancer patients with lymph node metastases not expressing *TMPRSS2-ERG*

of overall survival (7.8 vs 6.2 yr), although the difference was not statistically significant and, in these patients, the primary tumour was left untreated [13].

Because patients who are on ADT can suffer from serious side-effects, a marker that can select for metastatic patients who will benefit more from (early) endocrine treatment would be relevant. Although it has been shown that the in vitro invasive properties and in vivo growth of *TMPRSS2-ERG*-positive VCaP cells depends on expression of the fusion gene [6,7], the results of the present study suggest that *TMPRSS2-ERG* is not a predictive marker for endocrine therapy in clinical PCa.

During endocrine therapy, the tumour cells adapt their properties in such a way that the androgen-AR axis is still functional. These molecular changes can even cause treatment failure in CRPC patients. In this regard, several mechanisms have been described, including *AR* mutation, *AR* amplification, and induced androgen synthesis, that are mutually not exclusive [14-16]. In fact, it has been observed that expression of *TMPRSS2-ERG* is retained in recurrent AR-positive tumours [KG Hermans, unpublished data) [17]. Only in very late stage, AR-negative tumours fusion gene expression is downregulated [4].

Questions remain why certain *TMPRSS2-ERG*-positive tumours are less responsive than others and why some *TMPRSS2-ERG*-negative tumours respond better to the endocrine therapy than others. There might be several explanations for these observations. The mechanisms of the tumour to retain a functional androgen-AR axis (and not the presence or absence of the fusion gene itself) might determine the sensitivity to the therapy, as is known for example for *AR* gene amplification [18]. It can also not be excluded that the presence of other tumour-growth-promoting pathways contributes to tumour growth to different extents, making the contribution of *TMPRSS2-ERG* less relevant. It has recently been shown that transgenic *TMPRSS2-ERG* mice develop PIN that can progress to invasive cancer but only in the context of concomitant loss of the tumour-suppressor gene phosphatase and tensin homolog, *PTEN* [19,20]. In PCa patients, this combination of *TMPRSS2-ERG* and *PTEN* loss might lead to earlier biochemical recurrence after RP [21]. Furthermore, it remains also possible that other, as yet unidentified androgen-regulated mechanisms determine the response to endocrine therapy in the *TMPRSS2-ERG*-negative group.

The present study has some limitations that might have contributed to the absence of correlation between expression of *TMPRSS2-ERG* and response to endocrine therapy. First, the sample size of 71 is rather limited, and the samples used were not suitable for a quantitative assessment of *TMPRSS2-ERG* expression because they were all paraffin-embedded lymph node metastases, some of which some dated back to the late 1980s. This resulted in varying RNA quality and quantity. Still, for part of the samples, RNA also was available from frozen tissue sections. In all cases, the data obtained from frozen samples were identical to the data obtained from the paraffin-embedded tissues. Second, duration of response to endocrine treatment was based on rising PSA levels, according to the definition of CRPC [22]. It is known that PSA is only a surrogate marker which has important limitations [23], so rising PSA levels might not represent the end

of response to endocrine therapy. A correlation with more robust end points (cancer-specific survival and overall survival), however, could also not be established. Finally, we only studied *TMPRSS2-ERG* fusion in our population. In the gene fusion-negative group, some patients might express other androgen-regulated (fusion) genes that also could have responded to ADT. However, because *TMPRSS2-ERG* was expressed in almost 60% of the population, it is unlikely that addressing other ETS gene fusions, which are known to be relatively rare events [24], would have altered the conclusions.

5. CONCLUSIONS

Taken together, we show that *TMPRSS2-ERG* is frequently expressed in lymph node metastases of previously untreated PCa patients. Although this oncogenic fusion gene is androgen-regulated, expression of *TMPRSS2-ERG* did not correspond with duration of response to endocrine therapy in hormone-naïve PCa patients. Our results suggest that *TMPRSS2-ERG* is not a candidate marker that can select metastasised patients who will benefit more from ADT.

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Part II

TMPRSS2-ERG fusion gene in prostate cancer

Chapter IV

Overexpression of prostate-specific *TMPRSS2(exon 0)-ERG* fusion transcripts corresponds with a favourable prognosis of prostate cancer

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STATEMENT OF TRANSLATIONAL RELEVANCE

The *TMPRSS2-ERG* gene fusion is the major genetic alteration in prostate cancer. The prognostic value of *TMPRSS2-ERG* in prostate cancer is still a subject to debate. We describe novel findings of a *TMPRSSS2* transcript starting at an alternative exon, denoted exon 0. We show that this transcript is much more prostate-specific than the generally studied transcript starting at *TMPRSS2* exon 1. Furthermore, we provide important evidence that the expression of the *TMPRSS2(exon 0)-ERG* fusion transcript correlates with a good prognosis of prostate cancer, whereas *TMPRSS2(exon 1)-ERG* transcripts do not show such a correlation. Our findings urge further investigation of the heterogeneity of *TMPRSS2-ERG* in prostate cancer. More systematic identification of specific fusion transcripts like *TMPRSS2(exon 0)-ERG* or alternatively spliced mRNAs might assist in a molecular classification of prostate cancers, which can be integrated in therapeutic decision making in the future.

ABSTRACT

Purpose: To gain insight in the mechanism and clinical relevance of *TMPRSS2-ERG* expression in prostate cancer, we determined the specific characteristics of fusion transcripts starting at *TMPRSS2* exon 1 and at a more upstream and less characterized exon 0.
Experimental design: We used quantitative PCR analysis to investigate expression of wild-type *TMPRSS2(exon 0)* and *TMPRSS2(exon 1)* and of *ERG* fusion transcripts. Expression was tested in normal tissue samples, in prostate cancer cell lines and xenografts, and in fresh-frozen clinical prostate cancer samples (primary tumors and recurrences). Expression in clinical samples was correlated with disease progression.
Results: *TMPRSS2(exon 0)* and *TMPRSS2(exon 1)* transcripts were similarly androgen regulated in prostate cancer cell lines, but the expression levels of *TMPRSS2(exon 1)* were much higher. Comparison of expression in different tissues showed *TMPRSS2(exon 0)* expression to be much more prostate specific. In androgen receptor-positive prostate cancer xenografts, *TMPRSS2(exon 1)* transcripts were expressed at similar levels, but *TMPRSS2(exon 0)* transcripts were expressed at very variable levels. The same phenomenon was observed for *TMPRSS2-ERG* fusion transcripts. In clinical prostate cancers, the expression of *TMPRSS2(exon 0)-ERG* was even more variable. Expression of *TMPRSS2(exon 0)-ERG* transcripts was detected in 55% (24 of 44) of gene fusion-positive primary tumors but only in 15% (4 of 27) of gene fusion-positive recurrences and at much lower levels. Furthermore, in primary tumors, expression of *TMPRSS2(exon 0)-ERG* transcripts was an independent predictor of biochemical progression-free survival.
Conclusion: The expression of *TMPRSS2(exon 0)-ERG* fusion transcripts in prostate cancer is associated with a less-aggressive biological behavior.

INTRODUCTION

Recently, recurrent fusions of prostate-specific and androgen-regulated *TMPRSS2* to the ETS genes *ERG*, *ETV1*, *ETV4,* and *ETV5* have been reported as the most frequent genetic alterations in clinical prostate cancer (1-7). *TMPRSS2-ERG* fusion is detected in 40-70% of clinical prostate cancers. Fusion of *ETV1*, *ETV4,* and *ETV5* to *TMPRSS2* are much less frequent, but *ETV1*, *ETV4,* and *ETV5* have multiple fusion partners. Expression of most of these partner genes is prostate specific and androgen regulated (1-5). Some clinical studies have shown *TMPRSS2-ERG* to be associated with a more aggressive prostate cancer phenotype (8-12). However, other studies did not find an association with outcome in patients treated by radical prostatectomy (13,14) or even described *TMPRSS2-ERG* to be correlated with a more favorable outcome (15,16).
TMPRSS2 has more than one first exon (UCSC Genome Browser, genome.ucsc.edu). Not only fusion transcripts starting at the well known *TMPRSS2* exon 1 but also transcripts that start from a more upstream and less well characterized alternative first exon, here denoted exon 0, have been identified (14; Hermans, unpublished).
In the present study, we determined the specific characteristics of *TMPRSS2* transcripts starting at exons 1 and 0 in benign prostatic tissue and in prostate cancer. Moreover, we investigated clinical prostate cancer samples (primary tumors and recurrences) for expression of *TMPRSS2(exon 0)-ERG* and *TMPRSS2(exon 1)-ERG* fusion transcripts. In the primary tumors, we correlated fusion gene expression with time to biochemical progression after radical prostatectomy. Our data show different expression patterns of *TMPRSS2(exon 0)* and *TMPRSS2(exon 1)* transcripts. Furthermore, our findings indicate a more favorable prognosis of tumors with *TMPRSS2(exon 0)-ERG* expression.

MATERIALS AND METHODS

Prostate cancer cell lines and xenografts

Prostate cancer cell lines LNCaP and DuCaP were grown in RPMI-1640 supplemented with 5% fetal calf serum and antibiotics. Androgen receptor-positive prostate cancer xenografts PCEW, PC82, PC295, PC310, PC329, PC346, and PC374, and androgen receptor-negative xenografts PC133, PC135, PC324, and PC339, were propagated by serial transplantation on male nude mice, as described (17,18).

Clinical samples

Primary prostate tumors were obtained by radical prostatectomy and recurrent tumors by transurethral resection of the prostate. Hematoxilin/eosin stained tissue sections were histologically evaluated by two pathologists (T. van der Kwast; G.J.L.H. van Leenders). Although introducing a bias, only samples that contained at least 70% tumor were selected for analysis. The clinical and pathological demographics of the patients with primary prostate tumors included in the statistical analysis (N=67) are given in Supplementary Table S1. Tissues were snap frozen and stored in liquid nitrogen. Use of the samples for research purposes was approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act (MEC-2004-261).

RNA isolation

RNAs from the prostate cancer cell lines LNCaP and DuCaP cultured in the absence or the presence of 10^{-9} mol/L R1881 were isolated using the RNeasy RNA extraction kit (Qiagen). RNA from clinical prostate cancer samples was isolated from frozen tissue sections using RNA-Bee (Campro Scientific). Xenograft RNA was isolated according to the LiCl protocol.

Quantitative PCR (Q-RT-PCR)

Total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen) and an oligo dT12 primer. cDNAs of 16 different tissues were purchased from Clontech. Quantitative RT-PCRs were done in Power SYBR Green PCR Master Mix (25 µl), containing 0.33 µmol/L forward and reverse primer in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Amplified products were quantified relative to porphobilinogen deaminase (*PBGD*). Primer sequences of the primers used are summarized in Supplementary Table S2.

Statistical analysis

Associations between clinical and histopathological variables and expression of *TMPRSS2-ERG* transcripts were evaluated by the Pearson's χ^2 test, the Mann-Whitney U test, or Kruskal-Wallis test, where appropriate. Expression of *TMPRSS2-ERG* transcripts was correlated with the primary end point, biochemical progression-free survival, defined as time from radical prostatectomy to date of biochemical recurrence. Biochemical recurrence was defined as (*a*) a prostate-specific antigen level of >0.2 ng/ml at two consecutive measurements with a 3-month interval if the prostate-specific antigen nadir

was <0.1 ng/ml or (*b*) a prostate-specific antigen nadir of ≥0.2 ng/ml. Patients that died from causes other than prostate cancer or that were lost to follow-up were censored at the date of last prostate-specific antigen test. Patients were routinely followed thrice monthly during the first year after radical prostatectomy, semiannually during the second year, and subsequently at 12-month intervals. In case of progression, patients were followed every 3 months. Kaplan-Meier curves were constructed to assess the probability of remaining free of biochemical recurrence as a function of time after surgery. The differences between the survival curves of the groups were tested using the log-rank test or Breslow method if appropriate. A Cox proportional regression analysis with forward stepwise elimination was done to assess the impact of various parameters on time to recurrence. In the multivariate analysis, the model included pathological T stage, surgical margin status, the Gleason score of the primary tumor, and expression of indicated *TMPRSS2-ERG* fusion transcripts. Patients with unknown parameters were excluded from the analysis. Statistical analyses were done using the Statistical Package for Social Sciences, version 15.0 (SPSS), with a significance level of 0.05 (two-tailed probability).

RESULTS

TMPRSS2-ERG gene fusion is present in 40-70% of primary prostate tumors. *ERG* and *TMPRSS2* are located ~3 Mbp apart on chromosome 21q22.3 in the same orientation (Fig. 1A). The most common *TMPRSS2-ERG* fusion transcripts are composed of *TMPRRS2* exon 1 or exons 1 and 2 linked to exon 4 of *ERG*. Less frequently, fusion of *TMPRSS2* exon 1 or 2 to other *ERG* exons have been detected (12). Genomic databases describe that *TMPRSS2* transcripts might also contain an alternative first exon, here denoted exon 0, which maps ~4-kbp upstream of exon 1 (Fig. 1A). *TMPRSS-ERG* fusion transcripts might also contain *TMPRSS2* exon 0 (14; Hermans, unpublished).

We determined the specific characteristics of transcripts starting at either exon 0 or exon 1 of *TMPRSS2*. First, the expression of both transcripts was investigated on a panel of cDNA from 16 different normal tissues. Although expression of *TMPRSS2(exon 1)* mRNA was highest in the prostate, these transcripts were also detected in lung, kidney, pancreas and colon samples. In contrast, *TMPRSS2(exon 0)* mRNA had a completely prostate-specific expression pattern (Fig. 1B). Subsequently, we did a quantitative RT-PCR analysis on RNA from prostate cancer cell lines DuCaP and LNCaP cultured in absence or presence of the synthetic androgen R1881. Both *TMPRSS2* transcripts were induced by androgens (Fig. 1C). Notably, in the cell lines, expression of *TMPRSS2(exon 0)* transcripts was much lower than expression of *TMPRSS2(exon 1)*.

Testing of RNAs from eleven human prostate cancer xenografts for expression of *TMPRSS2* starting at either exon 0 or 1 showed that six androgen receptor-positive xenografts, PCEW, PC82, PC295, PC329, PC346, and PC374, expressed *TMPRSS2(exon 1)* at less-variable levels (Fig. 1D). Xenograft PC310 showed no expression of *TMPRSS2* because of a deletion of the wild-type allele (17). Five xenografts expressed *TMPRSS2(exon 0)* with a much more variable level of expression. None of the androgen receptor-negative xenografts expressed *TMPRSS2*.

Previously, we have shown that five androgen-dependent xenografts, PCEW, PC82, PC295, PC310, and PC329, contained *TMPRSS2(exon 1)-ERG* mRNA (17). Quantitative RT-PCR analysis for [*TMPRSS2(exon 1)-ERG* and *TMPRSS2(exon 0)-ERG*] fusion transcripts showed that three xenografts, PC82, PC295, and PC329, expressed *TMPRSS2(exon 0)-ERG* at different levels (Fig. 1D). However, the other two xenografts that expressed *TMPRSS2-ERG* transcripts, PCEW and PC310, did not express the *TMPRSS2(exon 0)-ERG* fusion transcript at all. The expression levels of *TMPRSS2(exon 1)-ERG* transcripts in the five xenografts did not show a large variation (Fig. 1D). The difference in *TMPRSS2(exon 0)-ERG* and *TMPRSS2(exon 1)-ERG* expression was similar to that observed for wild-type *TMPRSS2(exon 0)* and *(exon 1)* transcripts.

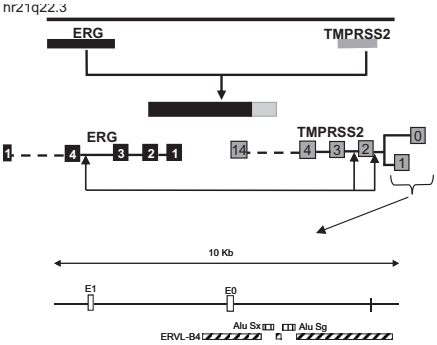


Figure 1a.

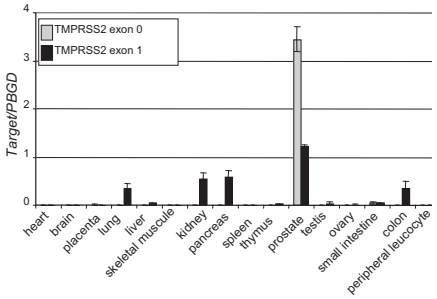


Figure 1b.

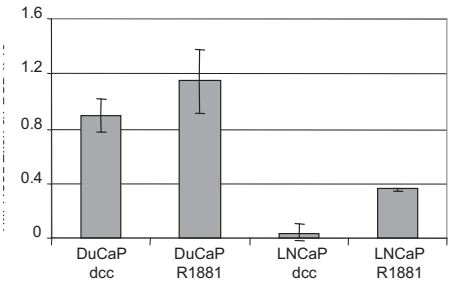
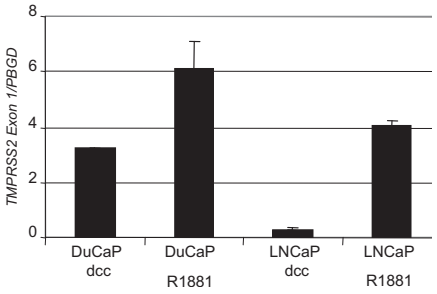
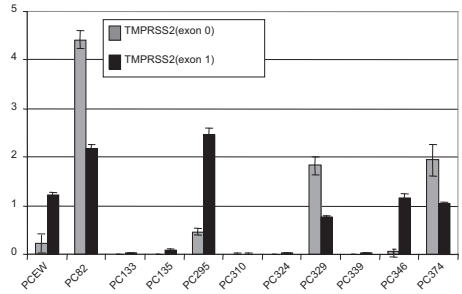


Figure 1c.





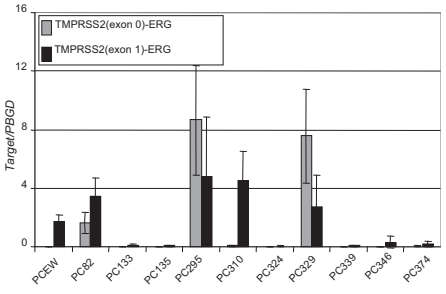


Figure 1d.

Figure 1. Characterization of *TMPRSS2* and *TMPRSS2-ERG* transcripts starting at exon 0 or exon 1. *A,* schematic representation of the *TMPRSS2-ERG* locus on chromosome band 21q22.3. The most frequent gene fusion events are indicated. The enlarged genomic region containing *TMPRSS2* shows exon 0 and 1 and repeat sequences. *B,* tissue-specific expression of *TMPRSS2(exon 0)* and *TMPRSS2(exon 1)* mRNA assessed by quantitative RT-PCR analysis on a cDNA panel from 16 different normal tissues. Mean of duplicate experiment relative to *PBGD* with SD. *C,* androgen-regulated expression of *TMPRSS2(exon 0)* (*left*) and *TMPRSS2(exon 1)* (*right*) mRNA in androgen receptor-positive prostate cancer cell lines LNCaP and DuCaP. LNCaP and DuCaP cells were grown in the absence and presence of synthetic androgen R1881 (10^{-9} mol/L) for 24h. Mean of duplicate experiments relative to *PBGD* with SD are depicted. Note that the level of *TMPRSS2(exon 0)* expression is much lower in the cell lines than in the normal prostatic tissue (*B*). *D,* quantitative RT-PCR analysis of *TMPRSS2(exon 0)* and *TMPRSS2(exon 1)* (*left*) and *TMPRSS2(exon 0)-ERG* and *TMPRSS2(exon 1)-ERG* (*right*) transcripts in 11 human prostate cancer xenografts. Mean of duplicate experiments relative to *PBGD* with SD.

Next, we determined the expression of *TMPRSS2(exon 0)-ERG* and *TMPRSS2(exon 1)-ERG* transcripts in a cohort of 126 fresh-frozen clinical prostate cancer samples (81 primary tumors and 45 recurrent tumors; Fig. 2A). *TMPRSS2-ERG* transcripts were detected in 54% (44 of81) of the primary tumors and in 60% (27 of 45) of the recurrences. In the primary tumors, 20 (25%) of 81 of the cases exclusively expressed *TMPRSS2(exon 1)-ERG* transcripts, three samples (4%) exclusively expressed *TMPRSS2(exon 0)-ERG* and 21 (26%) expressed both transcripts. Analysis of wild-type *TMPRSS2* in benign prostatic tissue excluded preferential expression of *TMPRSS2(exon 1)* or *TMPRSS2(exon 0)* transcripts in one of the different prostate zones (data not shown). In the recurrent tumors, exclusive expression of *TMPRSS2(exon 1)-ERG* was detected in 23 (51%) of 45 of the cases, whereas none expressed exclusively *TMPRRS2(exon 0)-ERG* and only four cases (9%) expressed both transcripts. Expression levels of *TMPRSS2(exon 0)-ERG* transcripts were significantly higher in primary tumors than in recurrent tumors (p=0.015), and variation in expression was much larger in the primary tumors than in the recurrences (Fig. 2B). In contrast, the percentage of tumors expressing *TMPRSS2(exon 1)-ERG* transcripts was in the same range for primary and recurrent tumors, and the expression levels of these transcripts did not differ between both tumor types (p=0.74).

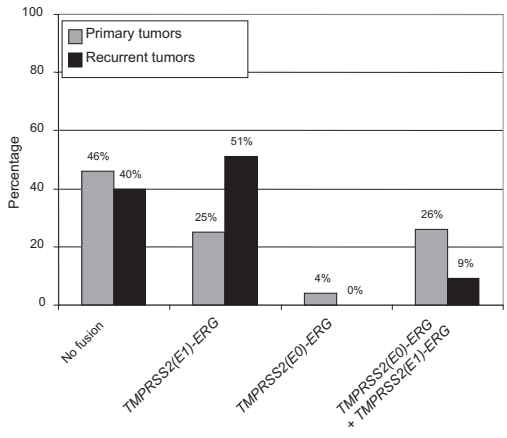


Figure 2a.

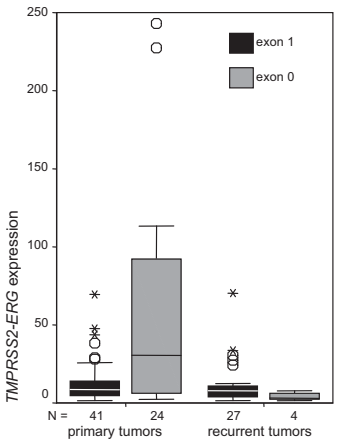


Figure 2b.

Figure 2. Expression of *TMPRSS2(exon 0)-ERG* and *TMPRSS2(exon 1)-ERG* transcripts in clinical prostate cancer samples. *A*, distribution of both *TMPRSS2-ERG* fusion transcripts in primary and recurrent tumors. Primary tumors, n = 81; recurrent tumors, n = 45. *B,* box plot of *TMPRSS2(exon 0)-ERG* and *TMPRSS2(exon 1)-ERG* mRNA expression levels in primary tumors and recurrences. Open circles, outliers; asterisk, extremes.

We correlated expression of *TMPRSS2(exon 0)-ERG* with clinical outcome in the primary prostate cancer cohort (n = 81) to see whether it was of prognostic value. We excluded from the analysis 10 patients that were known to harbor fusion or overexpression of other *ETS* genes and four patients whose primary treatment was not a radical prostatectomy. Despite the very long follow-up available (median, >10 years), only 11 of the remaining 67 patients died from prostate cancer, precluding statistical analysis. Instead, we used time to prostate-specific antigen recurrence after radical prostatectomy as an end point. The patients' demographics are summarized in Supplementary Table S1. No differences were seen in clinical and histopathological characteristics between patients expressing *TMPRSS2-ERG* and gene fusion-negative patients, although *TMPRSS2-ERG*-negative patients had higher Gleason scores with borderline significance (P = 0.053; Supplementary Table S3). The median time to prostate-specific antigen progression was not significantly different between the two groups: 73.2 versus 122.1 months [95% confidence interval (95% CI), 32.7-113.7 versus 70.6-173.6; P = 0.45; Fig. 3A].

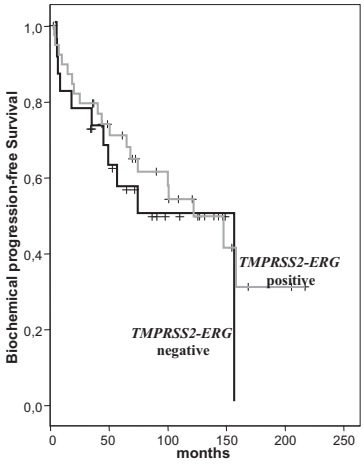


Figure 3a.

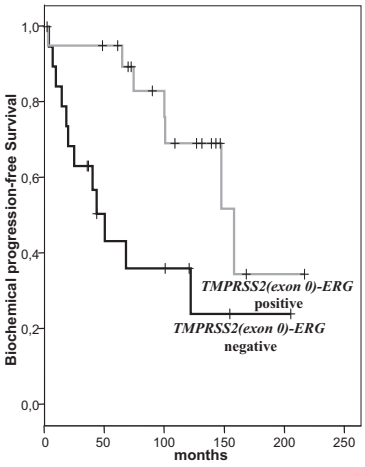


Figure 3b.

Figure 3. Kaplan-Meier curves for time to prostate-specific antigen recurrence after radical prostatectomy defined by *TMPRSS2-ERG* fusion transcript status. *A*, biochemical progression-free survival curves for prostate cancer patients with or without expression of *TMPRSS2-ERG* transcripts. *B*, biochemical progression-free survival curves for the *TMPRSS2-ERG*-positive group, which was stratified in patients with and without expression of *TMPRSS2(exon 0)-ERG* transcripts.

Within the *TMPRSS2-ERG*-positive population the only difference between patients that exclusively expressed *TMPRSS2(exon 1)-ERG* transcripts and patients that expressed the *TMPRSS2(exon 0)-ERG* subtype was that the former had higher pathological stages than

the latter (P = 0.009; Supplementary Table S4). The median time to prostate-specific antigen progression for patients expressing *TMPRSS2(exon 0)-ERG* transcripts was significantly longer than for patients that exclusively expressed *TMPRSS2(exon 1)-ERG* transcripts: 158.2 versus 50.5 months (95% CI: 98.9-217.5 versus 32.6-68.4; P = 0.012; Fig. 3B). Using a Cox proportional hazards model, positive surgical margins, Gleason score of ≥7, pathological stage of ≥pT3a, and absence of *TMPRSS2(exon 0)-ERG* transcripts were all associated with a worse biochemical progression-free survival. Importantly, multivariate analysis with forward stepwise selection showed expression of *TMPRSS2(exon 0)-ERG* fusion transcripts to be an independent predictor of progression-free survival (hazard ratio, 0.34; 95% CI, 0.14-0.84; P = 0.019; Table 1).

Table 1 - Results of univariate and multivariate analyses

		Univariate					Multivariate	
Variable	***n***	**Median time to PSA recurrence (mo)**	**95% CI**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Surgical margins								
positive	30	43.9	31.0-56.8	< 0.001	7.5 (3.0-18.5)	< 0.001	7.7 (3.0-19.4)	0.001
negative	31	122.1	49.5-194.7		1.0			
Gleason Score								
≥ 7	26	68.2	41.5-94.9	0.037	2.1 (1.0-4.5)	0.041		
< 7	35	155.4	117.7-192.2		1.0			
pT-stage								
extraprostatic	43	65.0	37.4-92.5	< 0.001	5.7 (1.9-16.8)	0.002		
organ confined	18	158.2	146.7-169.7		1.0			
***TMPRSS2 (exon 0)-ERG* expression**								
Yes	2140	158.2	98.9-217.5	0.015	0.36 (0.15-0.85)	0.02	0.34 (0.14-0.84)	0.019
No		68.2	36.8-99.6		1.0			

Abbreviations: HR, hazard ratio; pT-stage, pathological T-stage; PSA, prostate-specific antigen.

DISCUSSION

This study addresses two important aspects of *TMPRSS2-ERG* expression in prostate cancer. First of all, a remarkable difference in expression characteristics was detected between *TMPRSS2(exon 1)* and *TMPRSS2(exon 1)-ERG* transcripts on the one hand and *TMPRSS2(exon 0)* and *TMPRSS2(exon 0)-ERG* transcripts on the other hand. Secondly, the clinical data indicated a more favorable prognosis for prostate cancer patients expressing *TMPRSS2(exon 0)-ERG* transcripts.

It is estimated that almost half of all genes in the human genome contain more than one first exon, as an important mechanism to regulate gene expression (19). Here, we showed

that *TMPRSS2* transcripts starting at exon 0 were much more prostate specific than those starting at exon 1 (Fig. 1B) and that the expression level of transcripts containing exon 0 was much more variable (Fig. 1D). *TMPRSS2* exon 0 is located in a retroviral repeat element, ERVL-B4 (Fig. 1A). This repeat does not contain a standard long terminal repeat promoter element; however, other retroviral repeat sequences might function as cryptic promoters (19, 20). Within the same retroviral repeat, the *TMPRSS2* sequence present in a *TMPRSS2-ETV4* fusion transcript is located (6). Although a different 5'-untranslated region might affect translation efficacy, the major proteins translated from the fusion transcripts seem identical N-truncated ERG proteins, which are translated from an ATG codon in the *ERG* exon 4 part of the fusion transcripts.

It could be speculated that the prostate-specific *TMPRSS2(exon 0)* transcripts are expressed in tumors with a more differentiated phenotype. Recurrent tumors represent late-stage prostate cancer that normally has a less differentiated phenotype. In our study, 55% of the recurrences had a Gleason score of ≥8 compared with 15% of the primary tumors. An alternative explanation is that expression from exon 0 is stimulated by the stromal compartment, which will be different in primary tumors and recurrences. Obviously, there are no stromal cells present in *in vitro* cultures of prostate cell lines, which showed very low expression levels of *TMPRSS2(exon 0)* (Fig. 1).

The prognostic significance of *TMPRSS2-ERG* gene fusion remains subject of debate, although a growing number of studies have been published on this matter (8-13, 16). Because technology used to investigate *TMPRSS2-ERG* varies (FISH or quantitative RT-PCR) and compositions of patient cohorts differ considerably, it is difficult to draw general conclusions from available data. In the present study on a selected and rather limited group of well-defined patients with a very long median follow-up, there was no difference in time to prostate-specific antigen recurrence after radical prostatectomy between patients that expressed *TMPRSS2-ERG* and patients without expression of the fusion gene.

In two studies on watchful waiting cohorts, it was shown that patients having *TMPRSS2-ERG* fusion had a higher incidence of metastases or cancer-specific death than gene fusion-negative patients (8,9); however, this was not confirmed in a large surgically treated cohort (13). Attard et al. (8) showed by FISH analysis that patients with an interstitial deletion of genomic sequences between *TMPRSS2* and *ERG* (so called "class Edel") had poorer cancer-specific and overall survival than gene fusion-negative patients or than patients with *TMPRSS2-ERG* fusion without loss of the genomic region between the two genes. Other studies have correlated *TMPRSS2-ERG* with biochemical progression after radical prostatectomy, like in the present study. Before the identification of *TMPRSS2-ERG*, Petrovics et al. (15) found that patients with high expression levels of *ERG* had longer prostate-specific antigen recurrence-free survival than patients without *ERG* overexpression. Recently, similar results were reported by Saramaki et al. (16), using FISH analysis of *TMPRSS2-ERG*. However, other studies claimed a negative correlation

between *TMPRSS2-ERG* and prostate-specific antigen recurrence (10-12). Perner et al. indicated that patients with *TMPRSS2-ERG* rearrangement through deletion showed a trend for higher prostate-specific antigen recurrence rate than patients without fusion (11). Wang et al. (12) provided evidence that specific *TMPRSS2-ERG* splice variants were associated with early prostate-specific antigen recurrence.

Information on *TMPRSS2(exon 0)-ERG* transcripts in prostate cancer is still scarce. Thus far, this transcript was only identified by Lapointe et al. (14). However, the clinical implications of this specific fusion transcript subtype were not investigated. The low expression frequency of *TMPRSS2(exon 0)-ERG* transcripts in late-stage prostate cancer and the favorable prognosis for patients expressing this fusion transcript in primary tumors, as shown in our study, urges further investigation of the heterogeneity of *TMPRSS2-ERG* in prostate cancer. More systematic identification of specific fusion transcripts like *TMPRSS2(exon 0)-ERG* or alternatively spliced mRNAs (12) might assist in a molecular classification of prostate cancers, which can be integrated in therapeutic decision making in the future.

ACKNOWLEDGMENTS

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Supplementary Table S1 - Clinical and pathological characteristics of 67 patients with primary prostate cancer

Characteristic		
Mean age (± SD)	62.5	± 5.4 years
Median follow-up (± SD)	127.0	± 48.3 months
Mean PSA (± SD)	15.6	± 22.8 ng/ml
cT-stage		
organ confined	45	67.2%
extraprostatic	13	19.4%
unknown	9	13.4%
pT-stage		
pT2a	2	3.0%
pT2b	3	4.5%
pT2c	13	19.4%
pT2x	1	1.5%
pT3a	24	35.8%
pT3b	16	23.9%
pT4	5	7.5%
unknown	3	4.5%
Surgical margins		
positive	31	46.3%
negative	32	47.8%
unknown	4	6.0%
Gleason score		
< 7	38	56.7%
= 7	19	28.3%
> 7	10	15.0%
Occult metastases at RP		
Yes	7	10.4%
No	56	83.6%
unknown	4	6.0%
PSA recurrence		
Yes	33	49.3%
No	33	49.3%
Unknown	1	1.5%
Local recurrence		
Yes	9	13.4%
No	55	82.1%
Unknown	3	4.5%
Distant metastases at follow-up		
Yes	11	16.4%
No	55	82.1%
Unknown	1	1.5%
Death		
Yes	27	40.3%
No	40	59.7%

Supplementary Table S1 - continued

Characteristic		
Prostate cancer death		
Yes	11	16.4%
No	54	80.6%
Unknown	2	3.0%

Abbreviations: SD, standard deviation; cT-stage, clinical T-stage; pT-stage, pathological T-stage; RP, radical prostatectomy; PSA, prostate-specific antigen.

Supplementary Table S2 – Primer sequences of primers used for quantitative RT-PCR analysis

Target	Forward 5'->3'	Reverse 5'->3'
PBGD	catgtctggtaacggcaatg	gtacgaggctttcaatgttg
TMPRSS2 Exon 1 – Exon 3	gagctaagcaggaggcgga	aggggttttccggttggtatc
TMPRSS2 Exon 0 – Exon 3	gactacttctactccaccag	aggggttttccggttggtatc
TMPRSS2(exon 0)-ERG	gactacttctactccaccag	catcaggagagttccttgag
TMPRSS2(exon 1)-ERG	gagctaagcaggaggcgga	catcaggagagttccttgag

Supplementary Table S3 - Clinical and pathological characteristics of patients expressing *TMPRSS2-ERG* versus patients not expressing *TMPRSS2-ERG*

	***TMPRSS2-ERG* positive (n = 44)**	***TMPRSS2-ERG* negative (n = 23)**	**p-value**	**Test**
Mean age (± SD)	62.2 (± 5.6)	63.0 (± 5.1)	0.57	MWU
Mean PSA (± SD)	16.1 (± 26.7)	14.8 (± 12.5)	0.30	MWU
Gleason Score				
< 7	29 (65.9%)	9 (40.9%)	0.053	χ^2
≥ 7	15 (34.1%)	13 (59.1%)		
pT-stage				
organ confined	13 (31.0%)	6 (27.3%)	0.76	χ^2
extraprostatic	29 (69.0%)	16 (72.7%)		
Surgical margins				
Positive	21 (50.0%)	10 (47.6%)	0.86	χ^2
Negative	21 (50.0%)	11 (52.4%)		

Abbreviations: SD, standard deviation; MWU, Mann-Whitney U test; PSA, prostate-specific antigen; pT-stage, pathological T-stage

*Patients with unknown parameters were not included in the analysis.

Supplementary Table S4 - Clinical and pathological characteristics of patients expressing *TMPRSS2(exon 0)-ERG* fusion transcripts versus patients exclusively expressing *TMPRSS2(exon 1)-ERG* fusion transcripts

	TMPRSS2(exon 0)-ERG expression (n = 24)	Exclusive *TMPRSS2(exon 1)-ERG* expression (n = 20)	p-value	Test
Mean age (± SD)	61.5 (± 4.8)	63.1 (± 6.4)	0.40	MWU
Mean PSA (± SD)	19.7 (± 35.2)	11.9 (± 10.3)	0.63	MWU
Gleason Score				
< 7	15 (62.5%)	14 (70.0%)	0.60	χ^2
≥ 7	9 (37.5%)	6 (30.0%)		
pT-stage				
organ confined	11 (47.8%)	2 (10.5%)	0.009	χ^2
extraprostatic	12 (52.2%)	17 (89.5%)		
Surgical margins				
Positive	9 (39.1%)	12 (63.2%)	0.12	χ^2
Negative	14 (60.9%)	7 (36.8%)		

Abbreviations: SD, standard deviation; MWU, Mann-Whitney U test; PSA, prostate-specific antigen; pT-stage, pathological T-stage

*Patients with unknown parameters were not included in the analysis.

Chapter V

Strong correlation between expression of *TDRD1* and *ERG* but not with *ETV1* overexpression in primary prostate cancer

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To be submitted

INTRODUCTION

ETS transcription factors regulate the expression of genes that are important to cancer-related processes like cell growth, differentiation, and transformation (1). It is well known that deregulated expression of erythroblast transformation-specific (ETS) family members or formation of chimeric fusion proteins involving these genes due to chromosomal translocations is associated with leukaemia and Ewing's sarcoma (1-3). In prostate cancer, gene fusions involving ETS transcription factors, including fusions of the v-ets erythroblastosis virus E26 oncogene homolog (avian), *ERG*, and the ETS variant gene 1, *ETV1*, were only recently detected (4). Fusion of the androgen-regulated and prostate-specific gene transmembrane protease, serine 2, *TMPRSS2*, to *ERG* is the most frequent genomic rearrangement in prostate cancer with a reported frequency of 40-70%, whereas fusions involving *ETV1* are present in 5-10% (5, 6). The significance of fusion genes involving ETS family members in the development and progression of prostate cancer is still largely unknown. Transgenic mice with targeted, prostate-specific overexpression of *ERG* were shown to develop prostate intra-epithelial neoplasia (PIN) (7, 8), although others reported the absence of a phenotype in such mice (9). Progression to invasive cancer in transgenic *ERG* mice was described in a phosphatase and tensin homologue (*Pten*) haploinsufficient background (10, 11). Like observed in *ERG* transgenic mice, mice with targeted, prostate-specific overexpression of *ETV1* developed PIN, but invasive prostate cancer was not observed (12, 13). These data indicate that overexpression of *ERG* or *ETV1* is insufficient for prostate cancer development and that additional molecular events are necessary for neoplastic transformation.

In the present study, we used RNA expression profiles to identify genes that were co-expressed with *ERG* and *ETV1* rearrangement in primary and late-stage prostate cancer, and in prostate cancer cell lines and xenografts. In primary prostate tumours, the gene Tudor domain containing 1 (*TDRD1*) showed the strongest correlation with *ERG* overexpression. During mouse prostate development, *Erg* and *Tdrd1* also showed co-expression. In late-stage prostate cancer samples and in prostate cancer cell lines and xenografts, *TDRD1* was not only expressed in *ERG*-positive, but also in a proportion of *ERG*-negative tumour samples. Interestingly, *TDRD1* was hardly expressed in primary prostate tumours overexpressing *ETV1* and the expression of *TDRD1* even seemed to be downregulated in these tumours.

MATERIAL AND METHODS

Human prostate cancer cell lines and xenografts and mouse tumour samples

Prostate cancer cell lines LNCaP, VCaP, PC346C, LAPC4, 22Rv1, and MDAPCa2B were grown in RPMI-1640 supplemented with 5% fetal calf serum and antibiotics. Androgen receptor-positive prostate cancer xenografts PCEW, PC82, PC295, PC310, PC329, PC346B, and PC374, and androgen receptor-negative xenografts PC133, PC135, PC324, and PC339, were propagated by serial transplantation on male nude mice, as described (14, 15).
Balb/c mouse prostate tissues were collected at different developmental stages (16.5 and 18.5 embryonal days and postnatal days 3, 9, 15, and 50).

Clinical samples

Primary prostate tumours were obtained by radical prostatectomy, late-stage tumours by transurethral resection of the prostate, and lymph node metastases by pelvic lymphadenectomy. Primary tumours whose initial treatment was not a radical prostatectomy were excluded form the analysis. Hematoxilin/eosin stained tissue sections were histologically evaluated by two uropathologists (T.H. van der Kwast; G.J.L.H. van Leenders). All samples contained at least 70% tumour. Tissues were snap-frozen and stored in liquid nitrogen. Use of the samples for research purposes was approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act (MEC-2004-261).

RNA isolation

RNA from clinical prostate cancer samples was isolated from frozen tissue sections using RNA-Bee (Campro Scientific, Berlin, Germany). The RNeasy RNA extraction kit (Qiagen, Valencia, CA, USA) was used to isolate RNA from human prostate cancer cell lines and mouse prostates of different developmental stages. Xenograft RNA was isolated according to the LiCl prototcol.

Hybridization and analysis of exon array

Expression profiles were determined using GeneChip Human Exon 1.0 ST (Affymetrix, Santa Clara, CA, USA) at the Center for Biomics, Erasmus MC, Rotterdam and at ServiceXS, Leiden, The Netherlands, according to the manufacturer's instructions. Microarray data were processed and RMA quantile normalized using Partek Genomics Suite (St. Louis, MO, USA). To study the expression of genes differentially expressed between

prostate tumours with and without *ERG* rearrangement, the expression values of the different probe sets of a transcript were processed by Partek on gene level. By significance of microarrays (SAM) genes co-expressed with *ERG* were identified (16). The q-value (false discovery rate) for genes identified by SAM was set to zero. The programs Cluster and Treeview were used to visualize genes with the highest differential expression according to SAM (17).

Quantitative RT-PCR (Q-RT-PCR)

Total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen, Carslbad, CA, USA) and an oligo dT12 primer. Q-RT-PCR was performed using Power SYBR Green PCR Master Mix (25 µl), containing 0.33 µM forward and reverse primer in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Amplified products were quantified relative to Hydroxymethylbilane synthase (*HMBS*, formerly *PBGD*; human RNAs), or Hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt*; mouse RNAs) by the Standard curve method (Applied Biosystems). Primer sequences are summarized in Supplementary Table S1.

Statistical analysis

A Perl implementation of Pearson's correlation (correlation factors > 0.5 or ≤ 0.5) was used to identify genes that were most closely co-expressed with *ERG*. Associations between clinicopathological variables and the expression of different transcripts were evaluated by the Pearson's χ^2 test or the Mann-Whitney U (MWU) test, where appropriate. Linear regression analysis was used to model the relationship between the expression of *ERG* and that of other genes.
Statistical analyses were done using the Statistical Package for Social Sciences, version 15.0 (SPSS) with a significance level of 0.05 (two-tailed probability).

RESULTS

Strong co-expression of *TDRD1* and *ERG* in primary prostate tumours

A cohort of 48 primary prostate tumours was analyzed for genes that were co-expressed with *ERG* overexpression using the Affymetrix GeneChip Human Exon 1.0 ST array. The clinicopathological characteristics of the patient cohort (cohort A) are summarized in Supplementary Table S2. As previously analyzed by Q-RT-PCR, 33 out of 48 tumour samples (69%) harboured *ERG* overexpression, which was confirmed in the expression array

experiment. SAM identified 209 genes that were significantly differentially expressed between *ERG*-positive and *ERG*-negative tumour samples (see Supplementary Table S3). Two hundred genes were positively correlated with *ERG* overexpression, whereas the expression of nine genes was inverse correlated with *ERG* overexpression. *TDRD1* was by far the strongest correlated gene with *ERG* overexpression (Score(d) = 10.3 and Fold Change = 8.55) (Fig. 1a and Supplementary Table S3).
In Fig. 1b, a heat map of the twenty genes with the most significant positive correlation with *ERG* overexpression is shown. The function of these genes is variable. The gene abbreviations, full gene names and accession numbers are summarized in Supplementary Table S4. Only one tumour (G275) had low *ERG* and high *TDRD1* expression. For validation purposes, Q-RT-PCR was performed for *TDRD1* in the cohort of 48 primary tumours. The results were consistent with the expression array data with two exceptions (G165 and G124; Supplementary Figure S1). The median level of expression of *TDRD1* in tumours with *ERG* overexpression was significantly higher than in tumours that did not overexpress *ERG* ($p < 0.001$, MWU test; Fig. 1c).
Next, the expression of *ERG* and *TDRD1* was analyzed in an additional set of 31 primary prostate tumours (cohort B) to validate the results obtained in cohort A. The clinicopathological characteristics of the validation cohort are summarized in Supplementary Table S2. As previously analyzed by Q-RT-PCR, 10 out of the 31 samples (32%) overexpressed *ERG*. In cohort B, *TDRD1* expression was also strongly correlated with *ERG* overexpression (Fig. 2a) and the median expression level of *TDRD1* was significantly higher in *ERG*-positive than in *ERG*-negative samples ($p < 0.001$, MWU test, data not shown). Linear regression analysis confirmed the significant correlation between *TDRD1* and *ERG* expression for both cohort A and B ($p < 0.001$ and $p < 0.001$, Fig. 2b).

To extend the evidence that *TDRD1* was the gene most correlated with *ERG* overexpression in primary tumours, the expression of two additional annotated genes that correlated with *ERG* overexpression in the expression arrays was analyzed by Q-RT-PCR in both cohorts A and B. Na+/K+ transporting ATPase interacting 1 (*NKAIN1*) and Calcium channel, voltage-dependent, L type alpha 1D subunit (*CACNA1D*) were selected, because of their significant co-expression with *ERG* (Score(d) = 7.08 and 6.56, respectively) (Fig. 1b). The median expression level of *NKAIN1* was significantly higher in *ERG*-positive than in *ERG*-negative tumours (cohort A: $p < 0.001$ and cohort B: $p = 0.017$, MWU test), but for *CACNA1D* this was only seen in cohort A and not in the validation cohort, cohort B ($p = 0.011$ and $p = 0.21$, MWU test). By linear regression analysis, the correlation between *NKAIN1* and *ERG* expression, and between *CACNA1D* and *ERG* expression was confirmed in cohort A ($p < 0.001$ and $p < 0.001$, Supplementary Figure S2), but in cohort B no significant correlation was observed ($p = 0.40$ and $p = 0.21$, Supplementary Figure S2). These data indicate that in our patient cohorts, *TDRD1* indeed showed the strongest correlation with *ERG* overexpression.

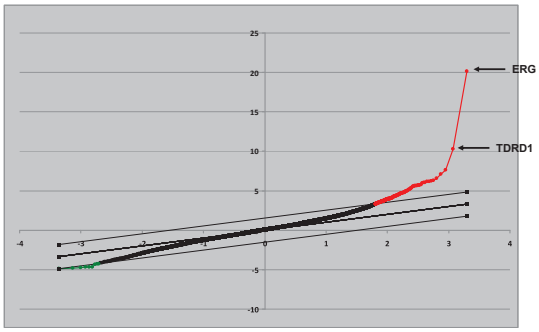


Figure 1a.

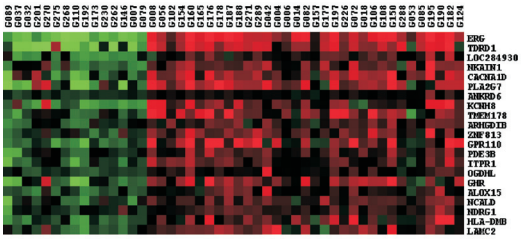
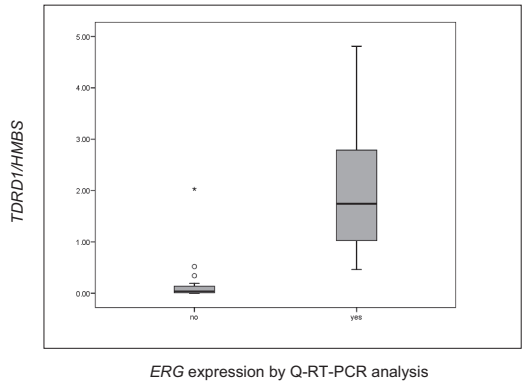


Figure 1b.



ERG expression by Q-RT-PCR analysis

Figure 1c.

Figure 1. Identification of genes co-expressed with *ERG* in a cohort of 48 primary prostate tumours (Cohort A)

a) Scatter plot of the observed relative difference score (*X*-axis) versus the expected relative difference score (*Y*-axis) from the comparison of primary prostate tumours that overexpress *ERG* (N=33) versus tumours without *ERG* overexpression (N=15) by SAM. *ERG* and *TDRD1* are indicated.

b) Heat map shows the 20 most correlated genes with *ERG* overexpression. Each cell in the image shows the $\log^2$ expression ratio for the particular gene divided by the median expression of that gene in all the samples. *Red*, expression above the median; *green*, expression below the median.

c) Box plot showing median expression of *TDRD1* relative to *HMBS*, as detected by Q-RT-PCR, in 48 primary prostate tumours, of which 33 overexpressed *ERG* and 15 did not overexpress *ERG*, $p < 0.001$ (MWU test). Outliers are depicted by an open circle (°) and extremes are depicted by an asterisk (*).

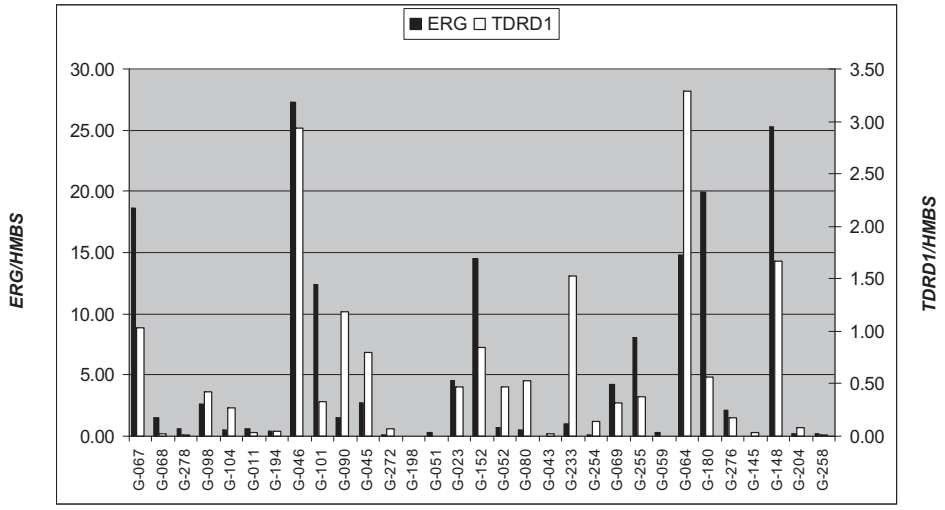


Figure 2a.

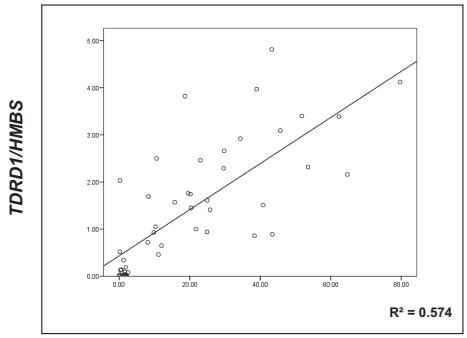


Figure 2b.

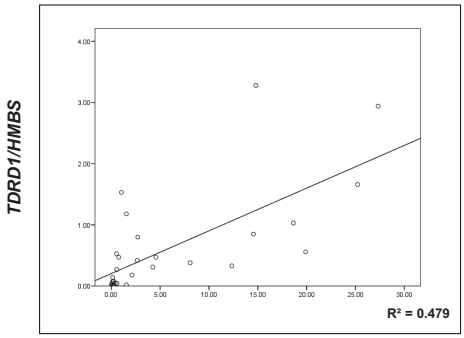


Figure 2c.

Figure 2. *TDRD1* co-expression with *ERG* overexpression in a validation cohort of 31 primary prostate tumours (Cohort B)

a) Q-RT-PCR analysis of *TDRD1* and *ERG* transcripts in a validation cohort of primary prostate tumours (N=31). *ERG* and *TDRD1* expression relative to *HMBS* is shown. The relative expression of *ERG* is depicted on the primary *Y*-axis, whereas the relative expression of *TDRD1* is depicted on the secondary *Y*-axis.

b) Correlation plot showing co-expression of *ERG* and *TDRD1* in the primary prostate cancer samples of both cohort A (N=48, left panel) and the cohort B (validation cohort, N=31, right panel). *ERG* and *TDRD1* expression relative to *HMBS*, as detected by Q-RT-PCR, is shown. The trend line and *R* squared change (R^2) are indicated.

TDRD1 and *ERG* co-expression in late-stage prostate cancer

Next, we investigated whether the strong correlation between *TDRD1* and *ERG* expression could also be found in late-stage prostate cancer. Analysis of the expression array data of a small set of nine late-stage prostate tumours (three *ERG*-positive and six *ERG*-negative tumours) indicated that *TDRD1* was not only expressed in the *ERG*-positive but also in several *ERG*-negative tumours (data not shown). This preliminary finding was validated and extended by Q-RT-PCR in a larger cohort of 51 late-stage prostate tumours and 11 prostate cancer lymph node metastases. Like observed in primary tumours, *TDRD1* was co-expressed with *ERG*, however, not only *ERG*-positive tumours but also a proportion of *ERG*-negative, late-stage tumour samples and metastases showed expression of *TDRD1* (Fig. 3a). In addition, in the samples with *TDRD1* overexpression, the median expression level of *TDRD1* in late-stage tumours and lymph node metastases (N=32 and N=9, respectively) was significantly higher than in the primary tumours (N=48) ($p = 0.005$, MWU test) (Fig. 3b), but in samples that overexpressed *ERG* (45 primary tumours versus 41 late-stage tumours and metastases (N=31 and N=10, respectively), the median expression level of *ERG* was comparable ($p = 0.73$, MWU test). In line with these data, high *TDRD1* expression was observed in the prostate cancer cell line VCaP, which overexpresses *ERG*, and xenografts with *ERG* overexpression, but here too several *ERG*-negative samples showed *TDRD1* expression (PC346C, PC133, PC324, and PC346B, Supplementary Figure S3).

TDRD1 and *ERG* are co-expressed during mouse prostate development

Next, we addressed the question whether *ERG* and *TDRD1* were also co-expressed in normal prostate samples with high *ERG* expression. In the normal adult human prostate both *ERG* and *TDRD1* expression are low (data not shown). During mouse prostate development, Q-RT-PCR analysis showed high expression of *Erg* in the urogenital sinus at the time point that prostate development starts, which is embryonal day 16.5 (16.5 e.d.) (Supplementary Figure S4). During later stages of mouse prostate development, *Erg* was expressed at a much lower level. *Tdrd1* expression was high during the early stages of mouse prostate development, whereas in the adult mouse prostate (50 postnatal days, 50 p.d.), *Tdrd1* expression was very low.

Co-expression of *TDRD1* and *ERG* is not associated with overexpression of prostate cancer markers or other Tudor family members

TDRD1 belongs to a large family of Tudor domain containing proteins. To investigate whether the observed correlation between *ERG* and *TDRD1* expression was specific for

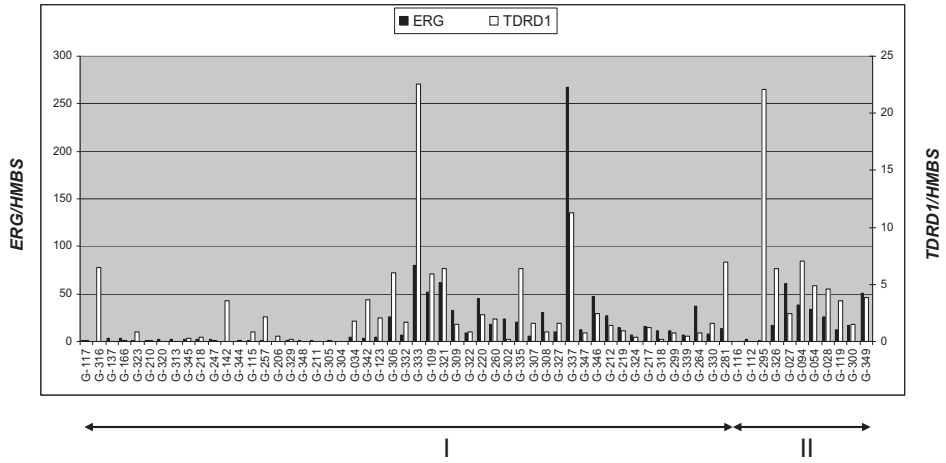


Figure 3a.

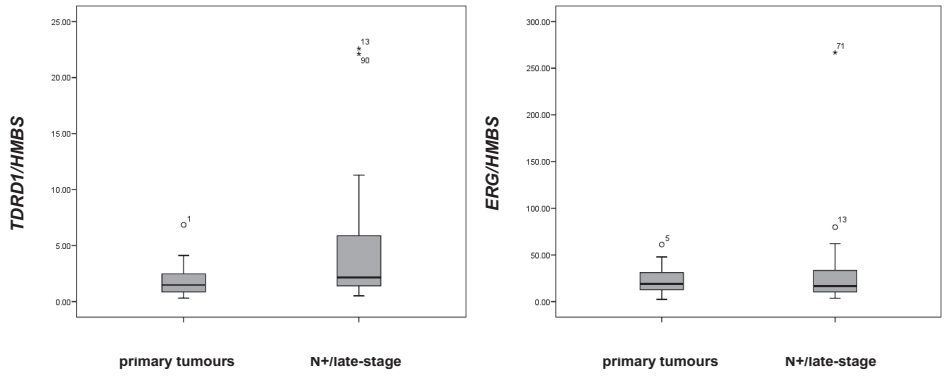


Figure 3b.

Figure 3. *TDRD1* co-expression with *ERG* overexpression in late-stage prostate cancer

a) Q-RT-PCR analysis of *ERG* and *TDRD1* transcripts in a cohort of late-stage prostate tumours and prostate cancer lymph node metastases. Late-stage tumour samples are indicated by I (N=51), whereas the lymph node metastases are indicated by II (N=11). *ERG* (black bars) and *TDRD1* (blue bars) expression relative to *HMBS* is shown. The relative expression of *ERG* is depicted on the primary *Y*-axis, whereas the relative expression of *TDRD1* is depicted on the secondary *Y*-axis.

b) Box plot showing median expression of *TDRD1* (left panel) and *ERG* (right panel) relative to *HMBS*, as detected by Q-RT-PCR in primary versus late-stage prostate tumours and prostate cancer lymph node metastases. Only samples with *TDRD1* (left panel) or *ERG* (right panel) overexpression are included in the analysis. Outliers are depicted by an open circle (°), and extremes by an asterisk (*).

TDRD1, the expression of other Tudor family members on the Human Exon 1.0 ST array was analyzed in cohort A (Supplementary Figure S5). In contrast to the *TDRD1* expression, expression of other Tudor family members showed no correlation with *ERG* overexpression.

Previously, an inverse correlation was observed between *ERG* overexpression and expression of the Serine protease inhibitor Kazal type 1 (*SPINK1*) in primary prostate cancer (18). To investigate the relationship between *TDRD1* and *SPINK1* expression, we compared the expression of exon array data in cohort A. Furthermore, we compared the expression of *TDRD1* with the expression of prostate cancer markers Homo sapiens non-coding RNA DD3 sequence (*PCA3*) and Alpha-methylacyl-CoA racemase isoform 1 (*AMACR*). We did not find a correlation between expression of *TDRD1* and *SPINK1* in the primary tumours. The expression of *SPINK1* seemed to be low in tumour samples expressing *TDRD1*, however, multiple *TDRD1*-negative tumours also did not express *SPINK1* (Supplementary Figure S6). Furthermore, no correlation between the expression of *PCA3*, *AMACR*, and *TDRD1* was seen; *PCA3* and *AMACR* were not only expressed in *TDRD1*-positive samples, but also in multiple *TDRD1*-negative samples (Supplementary Figure S6).

Low expression of *TDRD1* in primary prostate tumours with *ETV1* overexpression

Finally, we analyzed *TDRD1* expression in correlation with the expression of the *ERG*-related ETS transcription factors *ETV1*, *ETV4*, and *ETV5*. *ETV1* is known to be involved in 5-10% of prostate cancer gene fusions, whereas *ETV4* and *ETV5* are involved in 1% or less (5, 6). Analysis of expression array data identified five out of 48 primary prostate tumours to harbour *ETV1* overexpression, whereas *ETV4* or *ETV5* overexpression was not found. None of the five tumour samples overexpressing *ETV1* showed *TDRD1* overexpression (Fig. 4a). Next, we carried out Q-RT-PCR for *ETV1* and *TDRD1* in a larger cohort of primary prostate tumours, which were without treatment at the time of radical prostatectomy (N=79). Nine out of the 79 tumours (11%) showed *ETV1* overexpression. Remarkably, the median expression of *TDRD1* was significantly lower in samples with *ETV1* overexpression than in samples that did not overexpress *ETV1* ($p = 0.002$, MWU test, data not shown). Three out of nine samples with *ETV1* overexpression showed *TDRD1* expression at very low levels, whereas in six samples *TDRD1* expression was undetectable (Fig. 4b). These data suggest that, in contrast to the strong positive correlation with *ERG* overexpression, *TDRD1* expression is inversely correlated with *ETV1* overexpression in primary prostate cancer.

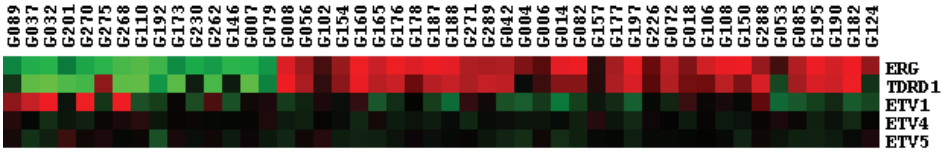


Figure 4a.

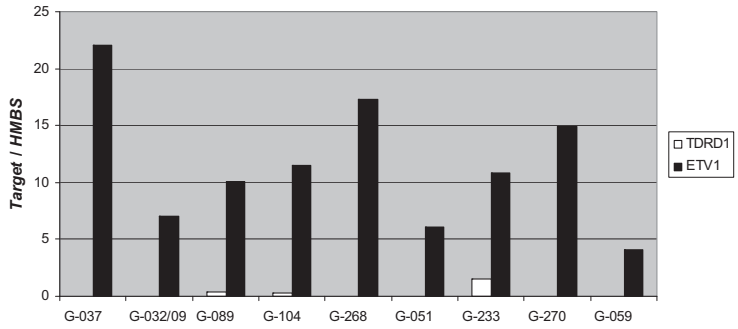


Figure 4b.

Figure 4. Low expression of *TDRD1* in primary prostate tumours with *ETV1* overexpression

a) Heat map shows the correlation between the expression of ETS family members *ETV1*, *ETV4*, and *ETV5*, and the expression of *ERG* and *TDRD1*. Each cell in the image shows the log² expression ratio for the particular gene divided by the median expression of that gene in all the samples. *Red*, expression above the median; *green*, expression below the median.

b) Bar chart showing expression levels of *TDRD1* and *ETV1* relative to *HMBS* for nine primary prostate tumours with *ETV1* overexpression.

DISCUSSION

Gene fusions are important genomic alterations in prostate cancer. Fusion of the androgen-regulated and prostate-specific gene *TMPRSS2* and the oncogene *ERG*, resulting in *ERG* overexpression, is the most frequent gene fusion. The role of the *TMPRSS2-ERG* fusion gene in prostate cancer is still largely unknown. In the present study, we analyzed gene expression in various stages of prostate cancer using both the GeneChip Human Exon 1.0 ST array and Q-RT-PCR to identify genes that co-expressed with *ERG* overexpression. In two independent cohorts of primary prostate tumours, expression of *TDRD1* showed by far the strongest correlation with *ERG* overexpression. In addition, during mouse prostate development, *Tdrd1* showed similar kinetics as *Erg*. In late-stage prostate cancer, however, *TDRD1* was not only expressed in all *ERG*-positive prostate cancers but also in a proportion of *ERG*-negative tumours. *TDRD1* expression seemed inversely correlated with *ETV1* overexpression in primary prostate tumours.

Gene expression profiling is nowadays the method of choice to identify genes differentially expressed in tumours with different biological behaviour and prognosis. Several gene expression signatures predicting clinical outcome in prostate cancer have been proposed by transcriptome analysis (19-24). The high frequency of *ERG* overexpression in prostate cancer enabled transcriptional profiling to be used for the characterization of this subgroup of prostate tumours and for the identification of potential direct or indirect targets of the ERG transcription factor. *In vitro* studies in the prostate cancer cell line VCaP that overexpresses *ERG*, and in prostate cells with forced *ERG* overexpression suggested several genes and molecular pathways to be differentially expressed in *ERG*-rearranged prostate cancer (7, 25-28). Tomlins *et al.* found that overexpression of *ERG* in immortalized, non-tumourigenic prostate cells induced the expression of genes in the plasminogen pathway, which were involved in invasion (7). Downregulation of *ERG* expression VCaP cells inhibited the invasive capacity of the cells and induced the expression of genes expressed in differentiated luminal epithelial cells, including NK3 homeobox 1 (*NKX3.1*), Prostate specific antigen (*PSA*), Solute carrier family 45 member 3 (*SLC45A3*), and *TMPRSS2*. Sun *et al.* showed that knockdown of *ERG* in VCaP downregulated the expression of the proto-oncogene *c-MYC* (29). These data suggest that *ERG* may keep prostate cancer cells in a less differentiated state.

Studies addressing gene expression profiles in clinical prostate cancers that overexpress *ERG* are limited. So far, published manuscripts and database information were obtained in small patient cohorts or for a limited number of genes. The strength of our study is that we analyzed genome-wide gene expression in a set of 48 well-defined primary prostate tumours and that we confirmed and validated expression array data by Q-RT-PCR. We found *ERG* overexpression in 33 samples (Fig. 1b) and approximately 200 genes showed significant overexpression in *ERG*-positive tumours. Previously, genes involved in the WNT pathway and Histone deacetylase 1 (*HDAC1*) were shown to be overexpressed in *ERG*-rearranged prostate cancer, whereas expression of Tumour necrosis factor (*TNF*) and genes involved in cell death pathways were downregulated in these tumours (25). Furthermore, a 87-gene expression signature involving estrogen signalling was proposed in *TMPRSS2-ERG* positive prostate cancer in a patient cohort from Sweden (26). This signature included the genes *CACNA1D* and Phospholipase A2, Group VII (*PLA2G7*), which were also present in the top-20 genes overexpressed in our *ERG*-positive cohort (Fig. 1B). Barwick *et al.* combined the Swedish patient cohort and a second cohort from Canada and identified nine genes co-expressed with *ERG1*, including *HDAC1* (27). Jhavar and co-workers analyzed gene expression profiles in a small cohort of 28 primary prostate cancers (28, 30). *ERG* was overexpressed in over 50% of the samples. Although the number of genes presented that co-expressed with *ERG* was limited, like in our cohort, high expression of *TDRD1*, *CACNAD1*, Neurocalcin δ (*NCALD*), and Potassium voltage-gated channel subfamily H (*KCNH8*) was detected among the genes that showed high expres-

sion in *ERG*-positive tumours. The partial correlation between these findings and our observations strengthens the robustness of our data.

We validated the co-expression of *TDRD1* with *ERG* overexpression in a second cohort of independent primary prostate tumours and confirmed the very strong correlated expression between both genes. A possible explanation why most previous series did not find *TDRD1* overexpression in *ERG*-rearranged prostate cancer might be that the expression arrays previously used did not contain a *TDRD1* probe. Differences might also be due to the composition of patient cohorts. If the samples analyzed consisted of many late-stage tumours, the correlation between expression of *TDRD1* and *ERG* becomes less clear. Finally, primary tumours whose initial treatment was not a radical prostatectomy were excluded from our analysis, because endocrine treatment or radiotherapy prior to sample collection might influence gene expression.

As discussed above, in our series, *CACNA1D*, *PLA2G7*, *KCNH8*, and *NCALD* genes previously reported to be co-expressed with *ERG* (25, 26, 30), showed also co-expression with *ERG* (Fig. 1b). However, these genes had a much lower significance score (Score(d) of 6.65, 6.28, 6.19, and 5.63, respectively) and a lower fold change in expression (3.52, 5.09, 3.36, and 2.09, respectively) than *TDRD1* (Supplementary Table S3). SAM also identified the potential prostate cancer marker Cysteine-rich secretory protein 3 (*CRISP3*) (31) to be positively correlated with *ERG* overexpression (Fold Change = 8.8), although the significance score was much lower than that of *TDRD1* (Score(d) = 4.3). *CRISP3* was overexpressed in ~70% of the *ERG*-positive primary tumours but 20% of tumours without *ERG* overexpression also showed very high expression of *CRISP3* (see Supplementary Figure S6). Among the nine genes with lower expression in *ERG*-positive primary prostate tumours, two candidate tumour suppressor genes, Hydroxyprostaglandin dehydrogenase 15-(NAD) (*HPGD*) and Glycine N-methyltransferase (*GNMT*), were identified. *HPGD* is known to be lower expressed in bladder, gastric and colorectal cancer (32-34) and *GNMT* was suggested to have a tumour suppressor function in hepatocellular carcinoma and prostate cancer (35, 36). Remarkably, expression of Trefoil factor 3 (*TFF3*), a gene higher expressed in various tumours including prostate cancer (37-40), was inversely correlated with *ERG*-positive primary prostate tumours. The significance of these findings remains to be investigated.

In late-stage tumours, *TDRD1* was co-expressed with *ERG* overexpression; however, multiple *ERG*-negative late-stage tumour samples also expressed *TDRD1*. We propose that other pathways that regulate *TDRD1* expression become activated in late-stage tumours. In addition, the effect of medical or surgical castration should be taken into account, as the vast majority of late-stage tumour samples were castrated at the moment of sample collection. Castration might lead to altered gene expression levels of *TDRD1*-related pathways. High *TDRD1* expression in late-stage prostate cancer supports an important role of TDRD1 in tumour progression.

Our results suggest that only *TDRD1* and not other Tudor family members play a role in *ERG*-rearranged prostate cancer. So far, the function of TDRD1 in prostate cancer is unknown. Tudor domain containing proteins, including TDRD1, were previously identified as binding partners of Piwi family proteins (41). Piwi family proteins interact with a specific class of noncoding RNAs (piRNAs) that are involved in post-transcriptional gene regulation. The formation of Piwi-TDRD1 complexes is critical for the subcellular localization of these proteins, which is essential for spermatogenesis (42). Sterility in mice as a result of mutant *TDRD1* further stressed the importance of *TDRD1* in normal spermatogenesis (43). Recently, it has been published that loss of *TDRD1* activates transposons (44). Further functional studies, including forced *TDRD1* overexpression and specific *TDRD1* downregulation in cell lines will be instrumental in clarifying the role of TDRD1 in prostate cancer. The strong correlation between *ERG* and *TDRD1* expression suggests that *TDRD1* is a direct *ERG* target gene and in the *TDRD1* promoter several candidate *ERG* binding sites can be identified (data not shown). Again, functional studies remain to be done to prove this direct correlation.
In contrast to high expression of *TDRD1* in *ERG*-positive tumours, *TDRD1* was hardly expressed in primary tumours with *ETV1* overexpression. SAM of the Affymetrix GeneChip Human Exon 1.0 ST array data indicated 33 genes that were negatively correlated with *ETV1* overexpression, including *TDRD1* (data not shown). Other genes that showed co-expression with *ERG* also lacked high expression in *ETV1*-positive tumours (Fig. 1b). These observations argue in favour of a different concept of *ERG*- versus *ETV1*-rearranged prostate cancer. In concordance, differences in cell growth, proliferation and migration were previously described for both types of rearranged prostate cancers (8, 45). Wei *et al.* recently showed that, although ERG and ETV1 possess a similar DNA-binding domain, even small differences in DNA-binding preferences can contribute to *in vivo* targeting specificities (46). So far, gene expression profiling in a larger cohort of prostate cancers to identify genes co-expressed with *ETV1* overexpression have not been reported, although *in vitro* studies suggested matrix metalloproteinases and integrins as potential targets of *ETV1* (45, 47).

In our opinion, the data presented here on the one hand add to the complex nature of prostate cancer and the apparent confusing role of ETS fusion genes in the disease. On the other hand, gene expression profiles are an important tool in the description of the molecular alterations in *ERG*-positive and *ERG*-negative tumour development, allowing the elucidation of the differential function of ETS transcription factors in the prostate.

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Supplementary Table S1 - Sequences of primers used for Q-RT-PCR analysis

Target	Forward 5'->3'	Reverse 5'->3'
HMBS	catgtctggtaacggcaatg	gtacgaggctttcaatgttg
CACNA1D	ggactttcacaccagccagc	cacaggcatcagagatttcg
ERG	tgctcaaccatctccttcca	tgggtttgctcttccgctct
Erg	tgctcaaccatctccttcca	tgggtttgctcttccgctct
ETV1	cataccaacggcgaggatca	tggagaaaagggcttctgga
Hprt	tccctggttaagcagtacag	ttccagtttcactaatgacac
NKAIN1	ggcagaggctaccccgac	cttcgtgttcgcctgctac
TDRD1	accaccctatagaccaagaat	tttcaatgtttccatagtctgca
Tdrd1	gagaagctgtgtgtgttag	tccgacgtttccagaacaatg
TMPRSS2-ERG	gagctaagcaggaggcgga	catcaggagagttccttgag

Supplementary Table S2 - Clinicopathological characteristics of two cohorts of patients with primary prostate cancer; cohort A and the validation cohort (cohort B).

Characteristic	Cohort A (N=48)	Cohort B (N=31)	p-value
Mean age (± SD)	61.8 ± 5.9 years	63.1 ± 5.1 years	p = 0.30*
Mean PSA (± SD)	16.6 ± 25.5 ng/ml	15.9 ± 14.0 ng/ml	p = 0.56**
pT-stage			
≤ pT2c	16 (33.3%)	8 (28.6%)	p = 0.57***
≥ pT3a	32 (66.7%)	20 (71.4%)	
Surgical margins			
positive	24 (50.0%)	13 (48.1%)	p = 0.67***
negative	24 (50.0%)	14 (51.9%)	
Gleason score			
< 7	23 (47.9%)	22 (73.3%)	p = 0.028***
≥ 7	25 (52.1%)	8 (26.7%)	
Occult metastases at RP			
Yes	4 (8.7%)	5 (21.7%)	p = 0.24***
No	42 (91.3%)	23 (78.3%)	

Abbreviations: SD, standard deviation; PSA, prostate-specific antigen; pT-stage, pathological T-stage; RP, radical prostatectomy

* Student's T-test

** Mann-Whitney U test

*** Pearson's χ^2 test

Patients with unknown values were not included in the analysis.

Supplementary Table S3 - Significantly differentially expressed genes between primary prostate tumours with *ERG* rearrangement versus those lacking *ERG* rearrangement as assayed by SAM.

Significantly upregulated genes

Affymetrix ID	Score(d)	Fold Change	Gene Name
3931765	20.11815351	12.56928284	*ERG*
3265175	10.31880671	8.548930766	*TDRD1*
3949431	7.654239186	2.75881225	*LOC284930*
2404344	7.083695342	2.916805869	*NKAIN1*
2624385	6.562973646	3.515014739	*CACNA1D*
2955827	6.284218276	5.094809046	*PLA2G7*
2916825	6.239813812	1.510350802	*ANKRD6*
2613293	6.190598291	3.364139375	*KCNH8*
2478269	6.159897026	3.345057544	*TMEM178*
3445786	6.048628694	2.090047161	*ARHGDIB*
3840883	6.033349426	2.43219119	*ZNF813*
2955999	5.967458475	4.380334029	*GPR110*
3321512	5.798619393	2.137256133	*PDE3B*
2608469	5.725527815	2.30793309	*ITPR1*
3288803	5.701459382	1.804776118	*OGDHL*
2807949	5.699595264	3.139162528	*GHR*
3742212	5.652896818	2.040232386	*ALOX15*
3147173	5.626123781	2.088663486	*NCALD*
3154317	5.610370053	1.822068523	*NDRG1*
2950263	5.56894717	2.643441127	*HLA-DMB*
2371139	5.556637999	2.455049535	*LAMC2*
2710599	5.522741838	2.811994064	*CLDN1*
2471384	5.376457822	1.679835213	*KCNS3*
2400027	5.326569239	3.691847969	*PLA2G2A*
3320169	5.2844448	1.657733612	*AMPD3*
3279154	5.188297225	1.62425659	*C10orf38*
2955932	5.179257518	2.646192195	*GPR110*
3126504	5.100278577	2.246074912	*CSGALNACT1*
3160175	5.025259167	1.934213066	*VLDLR*
3200648	5.003535755	1.406835715	*ADFP*
2387606	4.998395716	2.760392738	*CHRM3*
3291281	4.952526048	2.986098026	*TMEM26*
2764192	4.884880425	1.919841897	*KIAA0746*
2793137	4.880417952	2.017299002	*SH3RF1*
3095313	4.820305763	2.808387509	*C8orf4*
2658595	4.79828303	1.712748518	*HES1*
2920085	4.786303861	1.445044654	*SOBP*
2638077	4.780924706	2.669008213	*PLA1A*
2739308	4.739927836	1.876999137	*EGF*

Supplementary Table S3 - continued

Significantly upregulated genes			
2420642	4.717546409	1.637377438	*MCOLN2*
2443370	4.695895595	3.866672489	*F5*
2522094	4.686100955	1.460226116	*LOC26010*
2520138	4.679949915	1.529052135	*FLJ20160*
3898796	4.677369825	1.770275826	*KIF16B*
3502156	4.644714636	1.800081131	*ATP11A*
3735847	4.627355977	1.451229804	*40065*
3840949	4.627111716	2.64826626	*ZNF765*
3179706	4.56713944	1.455687789	*WNK2*
3483468	4.547871607	1.399702817	*KIAA0774*
2748923	4.52922682	1.696402387	*GUCY1B3*
3081501	4.515340825	2.068810651	*LOC285889*
2397025	4.494232495	1.375398004	*DHRS3*
2970897	4.482979889	2.066092599	*FRK*
3986168	4.46409298	1.928715879	*MUM1L1*
2582124	4.446282167	1.708768373	*NR4A2*
3616894	4.406652066	1.994699698	*FMN1*
3863669	4.399789835	1.86038205	*CEACAM1*
3399678	4.382631111	1.463053387	*B3GAT1*
3792273	4.36321453	1.7373645	*CDH7*
3000342	4.360552372	1.521137276	*ADCY1*
3081511	4.352991478	1.879197713	*LOC100134747*
3943234	4.307140978	2.43918501	*SLC5A1*
3137120	4.29892628	1.583290823	*CA8*
2937144	4.287936637	1.854446861	*SMOC2*
2964231	4.285443353	1.660154612	*RRAGD*
2975741	4.283998041	1.496451081	*MAP7*
2956563	4.276122612	8.834620938	*CRISP3*
3775334	4.253594045	1.554865308	*ZNF750*
3060332	4.236117045	2.703018138	*STEAP4*
2694001	4.235766382	1.36425711	*MGLL*
2748830	4.217080423	1.986026953	*GUCY1A3*
4054204	4.187278008	2.316394533	*APOD*
3577666	4.147469963	1.725208593	*SERPINA11*
3474372	4.125236881	1.30367961	*PXN*
3337516	4.124455486	1.360880436	*LRP5*
3717034	4.116761564	2.056348763	*LOC400590*
2828146	4.103578225	1.549152214	*CDC42SE2*
3285119	4.095545782	1.38600811	*FZD8*
3371114	4.089525239	1.66876887	*SYT13*
3189932	4.071245082	1.490306837	*STXBP1*
2916716	4.055421691	1.426159206	*PNRC1*
2967276	4.052242689	2.254050278	*POPDC3*
3352948	4.04866979	1.614764196	*SORL1*

Supplementary Table S3 - continued

Significantly upregulated genes			
2700365	4.042865344	1.874200124	*TM4SF1*
2467066	4.032138103	1.590334278	*PXDN*
2458513	4.018399386	1.521583341	*TMEM63A*
2453006	4.003630497	4.260013754	*PIGR*
2596763	4.002496831	1.826955077	*FZD5*
2474071	3.999224813	1.339510232	*MAPRE3*
3908149	3.994629098	1.547991608	*ZMYND8*
3802980	3.983226526	1.728043642	*DSC2*
3822723	3.982844451	1.374481371	*PKN1*
2991395	3.980750483	1.802373017	*HDAC9*
2420681	3.974350721	1.434130754	*MCOLN3*
3754797	3.964140209	1.729582229	*HNF1B*
3057370	3.960367175	1.515672812	*HIP1*
3971806	3.940405211	1.775739568	*SAT1*
3453120	3.932887602	1.290759991	*ZNF641*
3635776	3.930874745	1.444531761	*EFTUD1*
4035833	3.929207818	1.797029902	*CD24*
3751058	3.928555417	1.333999396	*C17orf63*
3790479	3.920415705	2.020758563	*SEC11C*
2796951	3.912745135	1.959066525	*PDLIM3*
2671728	3.902339079	1.615674209	*CDCP1*
3696317	3.900166063	1.457318566	*SMPD3*
2328868	3.899598663	1.375801232	*HDAC1*
2550175	3.898590786	1.875675153	*KCNG3*
2656837	3.892646409	1.936854559	*ST6GAL1*
2353337	3.8505693	1.399889162	*SLC22A15*
3353441	3.846655707	1.297024844	*C11orf63*
2350489	3.841816602	2.16548607	*KIAA1324*
3835777	3.830684949	1.582777226	*BCAM*
3126625	3.81875319	1.79799529	*LOC100130604*
3781980	3.80492526	1.420933424	*C18orf17*
2976113	3.785671393	1.661191802	*IFNGR1*
3820443	3.782711948	1.623599342	*ICAM1*
3131741	3.78082806	1.451970357	*RAB11FIP1*
2468811	3.765167583	1.298364093	*DDEF2*
4010461	3.763385321	1.547059667	*SPIN4*
3980867	3.763240429	1.471490267	*GJB1*
2339995	3.745008365	1.541343736	*ROR1*
2443537	3.737885278	1.428894196	*SCYL3*
2903782	3.730675319	1.526479176	*ITPR3*
2899022	3.724634574	1.609330178	*TRIM38*
3614534	3.723644743	1.450647121	*GABRB3*
3723687	3.719107624	1.416058926	*MAPT*

Supplementary Table S3 - continued

Significantly upregulated genes			
3858852	3.713427579	1.579943843	*RHPN2*
2353396	3.713275166	1.360298159	*C1orf161*
2395177	3.704340292	1.64092129	*ERRFI1*
3365136	3.692520899	1.422734318	*SERGEF*
2412624	3.689483365	1.953094331	*RAB3B*
3864646	3.680145655	1.771210239	*KCNN4*
3444252	3.670669778	1.634248292	*CSDA*
2749011	3.657047123	2.977329188	*TDO2*
3616796	3.654830853	1.481868871	*FMN1*
2777070	3.64431845	1.766456164	*HSD17B11*
3955357	3.635452093	1.24483905	*C22orf36*
2811145	3.635066984	1.671184886	*PART1*
2749611	3.632380478	1.688781496	*FNIP2*
3695867	3.624532481	1.283714077	*RANBP10*
3273870	3.621964145	1.911906512	*LOC642384*
2857204	3.619257703	1.915833592	*PPAP2A*
3445108	3.617308071	1.584433688	*GPRC5D*
2623662	3.612402949	1.18719986	*DNAH1*
2824581	3.610276326	1.89574887	*KCNN2*
2867836	3.604394636	1.575296503	*GLRX*
3001479	3.600356851	1.375611397	*IKZF1*
2468622	3.593341965	1.288577329	*ID2*
3835751	3.581201134	1.291915074	*CBLC*
3907830	3.562971082	1.430525851	*ELMO2*
3209623	3.562020796	1.476760961	*ZFAND5*
3435192	3.552606731	1.373290392	*MLXIP*
2353477	3.551918339	1.40823953	*ATP1A1*
3464405	3.546554978	1.659432084	*RASSF9*
3464000	3.545001838	1.283685147	*CCDC59*
3628832	3.536542168	1.215934591	*DAPK2*
4021341	3.536169905	1.424082766	*ZDHHC9*
3587015	3.53526787	1.257521875	*KLF13*
2872848	3.527310093	1.711440292	*LOX*
3107342	3.520658739	1.480168231	*PPM2C*
3081205	3.515316576	1.391751666	*SHH*
3220846	3.509322522	1.323586639	*SUSD1*
2980290	3.50425814	1.56239401	*RGS17*
3747399	3.502795197	1.425098608	*KRT14*
2963313	3.499296777	1.45709902	*SNX14*
3869379	3.498439045	1.684610201	*ZNF614*
2327677	3.491184055	1.315659605	*EPB41*
3272736	3.484256312	1.386691951	*ZNF511*
3205659	3.478042927	1.234079329	*SHB*
2806091	3.476487583	1.538899582	*RAI14*

Supplementary Table S3 - continued

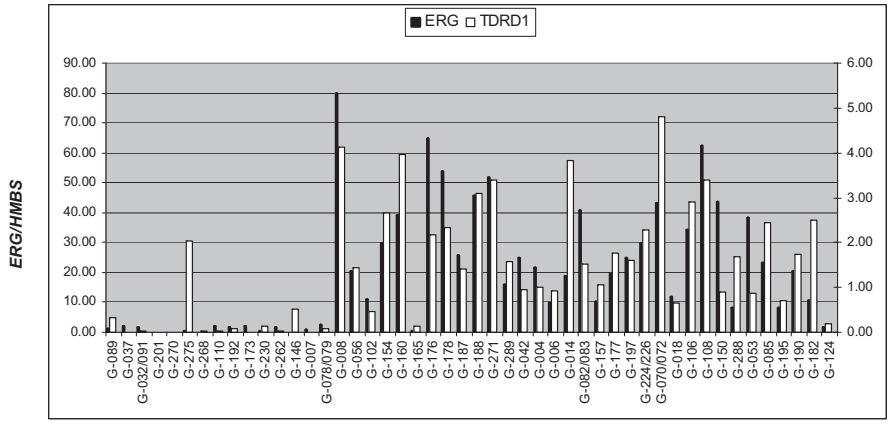
Significantly upregulated genes			
2955863	3.473740078	2.134974473	*GPR116*
3706439	3.466729369	1.454694181	*GARNL4*
3933863	3.454344062	2.275794469	*C21orf105*
2461531	3.452527119	1.405667821	*IRF2BP2*
2515471	3.445555967	1.310739192	*DLX1*
3567050	3.437908973	1.52212833	*RTN1*
2899051	3.437435152	1.92004995	*TRIM38*
3509473	3.43045929	1.516630856	*DCLK1*
2679406	3.422880112	1.731536325	*CADPS*
3400730	3.418955127	1.33544904	*CACNA1C*
3877265	3.410319797	1.236065017	*MACROD2*
3473727	3.40181891	1.464717754	*WSB2*
2835300	3.39979255	1.610155759	*SLC26A2*
3844470	3.39901199	1.378599617	*PPAP2C*
2322389	3.396312919	1.289602636	*NECAP2*
3181240	3.378710688	1.38746325	*TMOD1*
2400518	3.373323472	1.443457902	*ECE1*
2921296	3.365389801	1.868899546	*AMD1*
3900091	3.364981704	1.594094208	*C20orf74*
2510713	3.364879555	1.41786028	*FMNL2*
2344464	3.360364928	1.347543626	*SAMD13*
2408681	3.349902322	1.192359105	*HIVEP3*
3384321	3.346401513	1.54282711	*RAB30*
3628432	3.343449591	1.565482856	*LOC100128979*
3493579	3.341394097	2.111674481	*NMD3*
2832423	3.340532568	1.690935206	*PCDHB10*
3363091	3.333025052	1.398984356	*GALNTL4*
3058991	3.330778902	1.76828498	*CACNA2D1*
3335952	3.327007836	1.310104224	*PACS1*
3590709	3.325950346	1.231756358	*LOC100137047-PLA2G4B*

Significantly downregulated genes

Affymetrix ID	**Score(d)**	**Fold Change**	**Gene Name**
2794408	-4.86256937	0.331226614	*HPGD*
2907513	-4.833486317	0.527619446	*GNMT*
3916686	-4.746720159	0.583033121	*C21orf118*
2398287	-4.707438607	0.627350674	*NBPF16*
2632778	-4.6991188	0.396396476	*EPHA6*
3933536	-4.663890297	0.578068262	*TFF3*
2384401	-4.366122845	0.532071017	*RHOU*
3612739	-4.277834169	0.234330422	*CXADR*
3937943	-4.247219376	0.526048307	*FLJ42953*

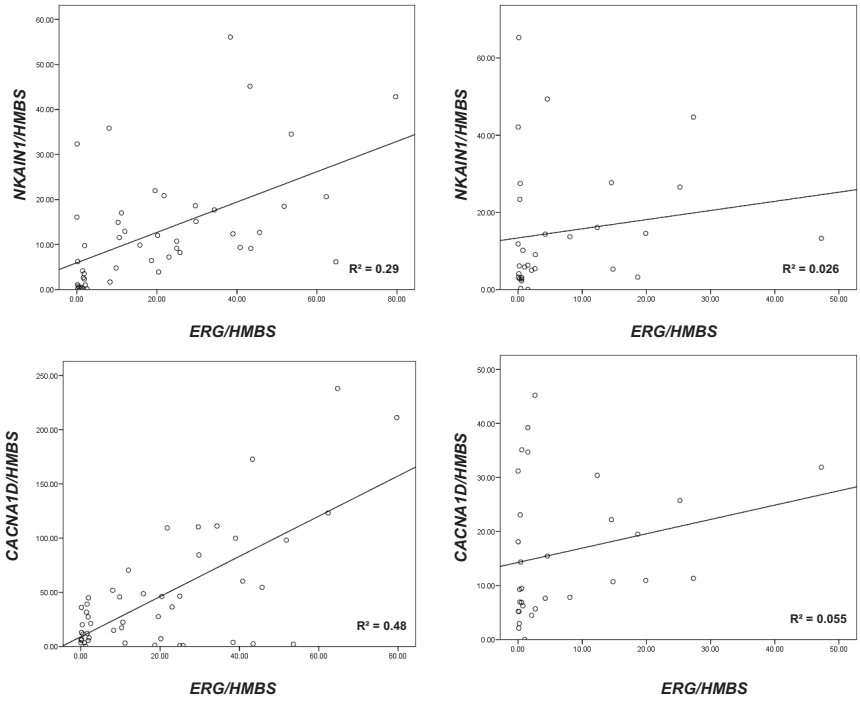
Supplementary Table S4 - Full names, abbreviations, and accession numbers of the 20 most correlated genes with *ERG* overexpression in primary prostate tumours as shown in Figure 1b.

Abbreviation	Full gene name	Accession number
ERG	V-ets erythoblastosis virus E26 oncogene homolog (avian)	NM182918 (isoform 1) NM004449 (isoform 2) NM001136154 (isoform 3) NM001136155 (isoform 4)
TDRD1	Tudor domain-containing protein 1	NM198895
LOC284930	--	AK093107 BC039485
NKAIN1	Na+/K+ transporting ATPase interacting 1	NM024522
CACNA1D	Calcium channel, voltage-dependent, L type, alpha unit 1D	NM000720 (isoform 1) NM001128840 (isoform 2) NM001128839 (isoform 3)
PLA2G7	Phospholipase A2, Group VII (platelet-activating factor acetylhydrolase, plasma)	NM005084 (isoform 1) NM001168357 (isoform 2)
ANKRD6	Ankyrin repeat domain 6	NM014942
KCNH8	Potassium voltage-gated channel, subfamiliy H (eag-related), member 8	NM144633
TMEM178	Transmembrane protein 178	NM152390 (isoform 1) NM001167959 (isoform 2)
ARHGDIB	Rho GDP dissociation inhibitor (GDI), beta	NM001175
ZNF813	Zinc finger protein 813	NM001004301
GPR110	G protein-coupled receptor 110	NM153840 (isoform 1) NM025048 (isoform 2)
PDE3B	Phosphodiesterase 3B, cGMP-inhibited	NM000922
ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	NM001099952 (isoform 1) NM002222 (isoform 2) NM001168272 (isoform 3)
OGDHL	Oxyglutarate dehydrogenase-like	NM018245 (isoform 1) NM001143996 (isoform 2) NM001143997 (isoform 3)
GHR	Growth hormone receptor	NM000163
ALOX15	Arachidonate 15-lipoxygenase	NM001140
NCALD	Neurocalcin delta	NM001040624 (isoform 1) NM001040625 (isoform 2) NM001040626 (isoform 3) NM001040627 (isoform 4) NM001040628 (isoform 5) NM001040629 (isoform 6) NM001040630 (isoform 7) NM032041 (isoform 8)
NDRG1	N-myc downstream regulated 1	NM001135242 (isoform 1) NM006096 (isoform 2)
HLA-DMB	Major histocompatibility complex, class II, DM beta	NM002118



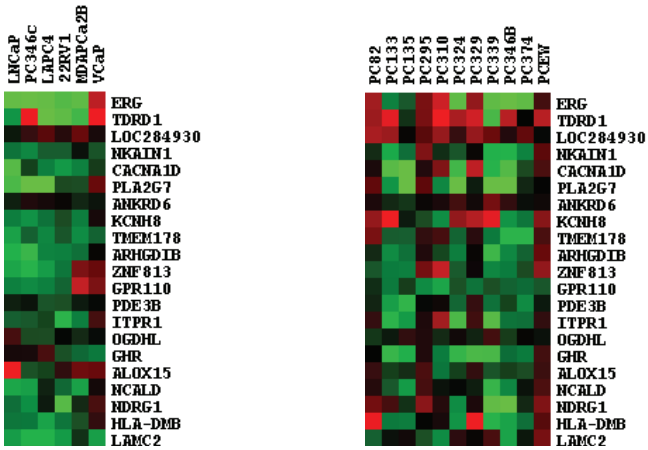
Supplemental Figure S1

Q-RT-PCR analysis of *TDRD1* and *ERG* expression in primary prostate tumours cohort A (N=48). *ERG* and *TDRD1* expression relative to *HMBS* are shown. The relative expression of *ERG* is depicted on the primary *Y*-axis, whereas the relative expression of *TDRD1* is depicted on the secondary *Y*-axis.



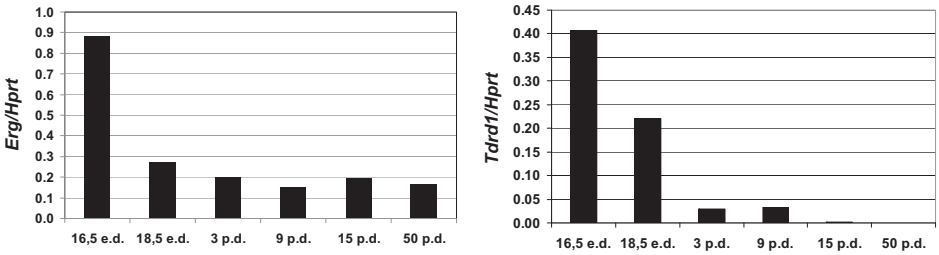
Supplemental Figure S2

Correlation plots showing co-expression of *ERG* and *NKAIN1*, and of *ERG* and *CACNA1D* in primary prostate tumours of both cohort A (N=48, left panels) and cohort B (validation cohort, N=31, right panels). Expression of *ERG, NKAIN1*, and *CACNA1D* relative to *HMBS*, as detected by Q-RT-PCR, are shown. The trend lines and *R* squared changes (R^2) are indicated.



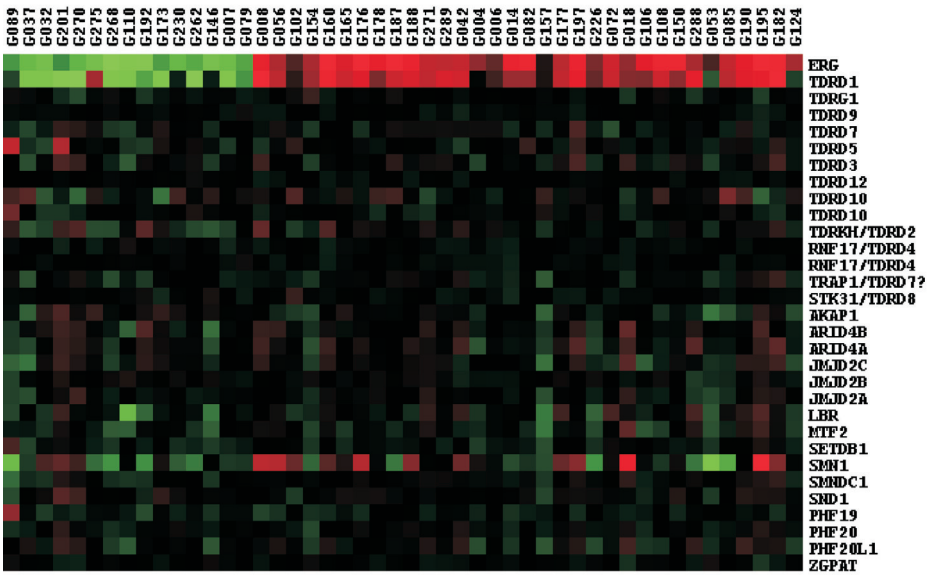
Supplemental Figure S3

Heat maps showing the correlation of the expression of the 20 genes, which were most significantly co-expressed with *ERG* overexpression in primary prostate tumours as determined by SAM, in six prostate cancer cell lines (left panel) and 11 prostate cancer xenografts (right panel). Each cell in the image shows the $\log^2$ expression ratio for the particular gene divided by the median expression of that gene in all the samples. *Red*, expression above the median; *green*, expression below the median.



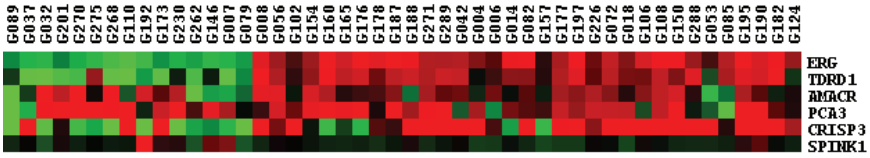
Supplemental Figure S4

The expression of *Erg* and *Tdrd1* during mouse prostate development. The expression of *Erg* (left panel) and *Tdrd1* (right panel) relative to *Hprt* expression is presented on the *Y*-axis. Time points of RNA isolation are indicated on the *X*-axis. E.d. = embryonal day; p.d. = postnatal day.



Supplemental Figure S5

Heat map showing the correlation of the expression of Tudor family members with *ERG* expression. Each cell in the image shows the $\log^2$ expression ratio for the particular gene divided by the median expression of that gene in all the samples. *Red*, expression above the median; *green*, expression below the median.



Supplemental Figure S6

Heat map showing the correlation of the expression of prostate cancer markers *PCA3*, *AMACR*, *CRISP3*, and *SPINK1* with *ERG* and *TDRD1* expression. Each cell in the image shows the $\log^2$ expression ratio for the particular gene divided by the median expression of that gene in all the samples. *Red*, expression above the median; *green*, expression below the median.

Part III

Prognostic implications of genetic alterations of the PI3K-AKT signalling pathway in prostate cancer

Chapter VI

Letter to the editor
An Activating Mutation in *AKT1* in Human Prostate Cancer

Joost L. Boormans[1], Karin G. Hermans[2], Geert J.L.H. van Leenders[2], Jan Trapman[2], Paul C.M.S. Verhagen[1]

[1] Department of Urology, Erasmus Medical Centre, Rotterdam, the Netherlands.
[2] Department of Pathology, Erasmus Medical Centre, Rotterdam, the Netherlands.

Int J Cancer 2008;123:2725-6

Dear Sir,

The phosphatidylinositol 3-kinase (PI3K) AKT pathway is frequently activated in human cancers [1]. PI3K catalyzes the synthesis of phosphorylated phosphoinositides, resulting in AKT1 activation. PI3K is counteracted by the phosphatase PTEN, which is often inactivated in prostate cancer. In prostate cancer, inactivation of *PTEN* is associated with advanced stage and grade, and appears to be more common in late-stage disease [2,3]. Recently, a transforming mutation in the pleckstrin homology domain of *AKT1* was described in human breast, colon and ovarian cancer [4]. The G > A mutation at nucleotide 49 results in a glutamic acid to lysine substitution at amino acid 17 (E17K) and leads to a PI3K-independent activation of AKT1. The E17 K substitution in *AKT1* has been confirmed by others in breast cancer, and recently it has also been identified in squamous cell carcinoma of the lung [5,6].

We identified the E17K substitution in 1 out of 92 clinical prostate cancer samples (Fig. 1*a*). The prostate cancer samples sequenced were lymph node metastases (n = 13), or transurethral resections of the prostate (TURP) (n = 79). All lymph node metastases were without prior treatment. Over 60% of the TURP samples were taken under endocrine therapy, indicating that the majority of patients had late-stage disease.

Array-based comparative genomic hybridization of the tumor with the E17K substitution showed a chromosome composition characteristic for prostate cancer, including deletion of 8p and 16q (Fig. 1*b*). *PTEN* was not inactivated by deletion (Fig 1B) or point mutation, and the sequences of the mutational hotspots in the catalytic subunit of *PI3K* were wild-type. Moreover, like the majority of prostate cancers, the tumor expressed the *TMPRSS2-ERG* fusion gene, as measured by quantitative polymerase chain reaction.

Histological examination of the tumor (TURP) revealed a prostatic ductal adenocarcinoma with extensive growth within pre-existent glandulae. Immunohistochemical staining showed overexpression of p-AKT in cancer cells, suggesting activation of the AKT pathway (Fig. 1*c*).

The patient was diagnosed with prostate cancer at the age of 74. His initial serum PSA was 8.8 ng/ml and a watchful waiting policy was initiated. For reasons of local progression, a TURP was done twice, five and eight years after diagnosis, respectively. Endocrine treatment was started after the second TURP. Currently, 17 years after diagnosis the patient is still alive, although PSA levels are rising under endocrine treatment, indicating that the tumor has reached a hormone-independent state (Fig. 1*d*).

In conclusion, we have identified and characterized the E17K substitution in *AKT1* for the first time in human prostate cancer. The tumor was a characteristic prostatic ductal

adenocarcinoma and the mutation was mutually exclusive with respect to *PTEN* inactivation and *PI3K* activation. The patient has a very long survival, which is in agreement with the suggestion that, in contrast to *PTEN* inactivation, tumors carrying the *AKT1* mutation may follow a more favorable clinical course [7].

Yours Sincerely,

Joost Boormans, Karin Hermans, Geert van Leenders, Jan Trapman and Paul Verhagen

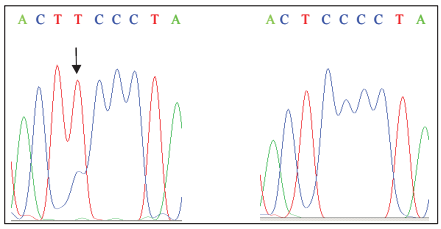


Figure 1a.

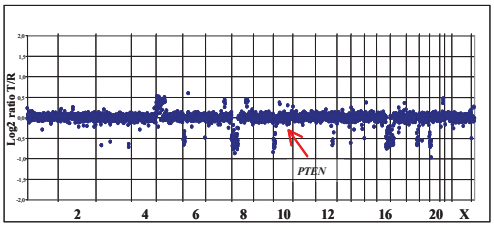
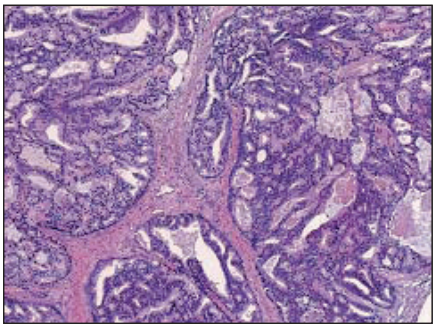


Figure 1b.



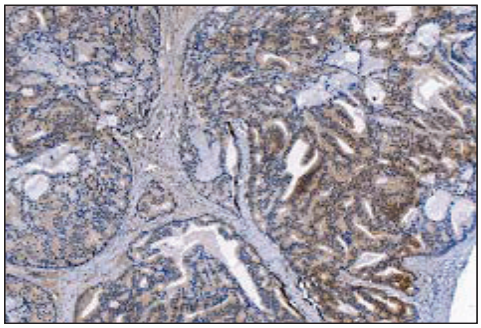


Figure 1c.

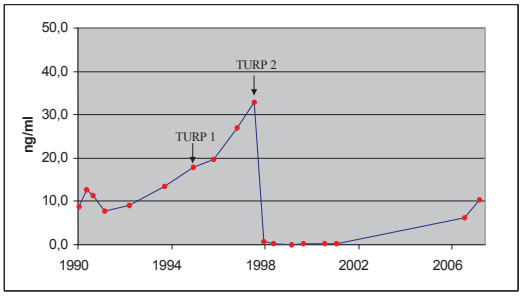


Figure 1d.

Figure 1. Genetic, morphological and immunohistochemical characterization of the prostate tumor with E17K substitution in *AKT1*.

A. Sequence of mutated *AKT1* (left panel), illustrating the G > A point mutation at nucleotide 49 (arrow), compared with sequence of wild-type *AKT1* (right panel). The second *AKT1* allele in the tumor is wild-type. Sequences depicted are reverse complementary.

B. Panel *b* shows genome-wide comparative genomic hybridization (CGH) profile of DNA from the prostate tumor with mutant *AKT1*. There is no loss at the *PTEN* locus (Chr. 10q23) (red arrow).

C. Left panel: Hematoxylin & Eosin section of the patient's prostate tumor (transurethral resection of the prostate) showing a prostatic ductal adenocarcinoma characterized by cribriform glands with extensive slit-like lumina, and presence of columnar cells with oval nuclei (x50 magnification). Right panel: Immunohistochemical staining for p-AKT (Ser473), showing overexpression in the cytoplasm of the same prostatic ductal adenocarcinoma (brown staining; x50 magnification).

D. The patient's PSA levels (in ng/ml) from moment of diagnosis in 1990 till 2007. The arrows indicate both transurethral resections of the prostate (TURP). Endocrine treatment was started after the second TURP.

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Chapter VII

E17K substitution in AKT1 in prostate cancer

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Br J Cancer 2010;102:1491-4

STRUCTURED ABSTRACT

Background: The PI3K-AKT pathway is activated in many cancers. Mutational hotspots in *AKT1* and in the regulatory and catalytic subunits of *PI3K* have been detected in multiple tumour types. In AKT1, the E17K substitution leads to a PI3K-independent activation of AKT1.

Methods: A mutational profiling of *AKT1* and of the mutational hotspots in *PIK3CA* and *PIK3R1* was carried out in samples from primary and recurrent prostate tumours.

Results: We show that in prostate cancer, AKT1(E17K) had a prevalence of 1.4%. The mutation seemed to be associated with a favourable clinical course but it was not associated with a specific tumour growth pattern. Activating mutations in *PIK3CA* or *PIK3R1* were not found in prostate cancer.

Conclusion: The E17K substitution in AKT1 is rare in prostate cancer. It seems associated with a favourable clinical outcome but not with a specific histology of the tumour.

INTRODUCTION

The phosphatidylinositol 3-kinase (PI3K) – v-akt murine thymoma viral oncogene homologue (AKT) signalling pathway is involved in cellular processes such as cell growth, proliferation, apoptosis, and cytoskeletal rearrangement. PI3K functions by catalysing the production of phosphorylated phosphoinositides (PtdIns). PI3K phosphorylates PtdIns(4,5)P2 (PIP2) into PtdIns(3,4,5)P3 (PIP3), which binds to the pleckstrin homology domain of the downstream target, v-akt murine thymoma viral oncogene homologue 1 (AKT1). This results in a recruitment of AKT1 to the plasma membrane in which regulatory amino-acid residues serine 473 (Ser473) and threonine 308 (Thr308) are phosphorylated and activated (Vivanco and Sawyers, 2002).

The tumour-suppressor gene phosphatase and tensin homologue (*PTEN*) directly antagonizes the PI3K-AKT pathway by converting PIP3 back to PIP2. Absence of PTEN leads to increased phosphorylation of AKT (Cantley and Neel, 1999), thereby stimulating PI3K-AKT signalling. *PTEN* inactivation is frequent in various cancers, including prostate cancer (Li *et al*, 1997). It can occur by deletion (Cairns *et al*, 1997; Vlietstra *et al*, 1998; Verhagen *et al*, 2006), mutation (Suzuki *et al*, 1998; Vlietstra *et al*, 1998; Verhagen *et al*, 2006), or by decreased expression (Whang *et al*, 1998). Prostate-targeted *Pten* knockout mice develop prostate hyperplasia, intraepithelial neoplasia, and ultimately invasive cancer (Wang *et al*, 2003; Ma *et al*, 2005).

Activating mutations in *AKT1* or *PI3K* are other mechanisms that lead to stimulation of the PI3K-AKT pathway. PI3K is a heterodimer composed of a regulatory subunit (p85α), encoded by *PIK3R1*, and a catalytic subunit (p110 α), encoded by *PIK3CA*. *PIK3CA* has frequently been reported as being mutated in various human cancers (Samuels *et al*, 2004; Levine *et al*, 2005). *PIK3R1* mutations are less common, although recently it was shown that *PIK3R1* was mutated in up to 10% of glioblastomas (Cancer Genome Atlas Research Network, 2008; Parsons *et al*, 2009). Mutations in either gene lead to a disruption of the interaction between the regulatory and catalytic subunits, which enhances enzymatic activity (Huang *et al*, 2007), thereby activating the PI3K-AKT pathway. In prostate cancer, however, activating mutations in *PIK3CA* or *PIK3R1* have not been reported thus far (Majumder and Sellers, 2005; Ligresti *et al*, 2009).

Recently, a unique mutation in the pleckstrin homology domain of *AKT1* was identified in breast, ovarian and colorectal cancer (Carpten *et al*, 2007). This G > A mutation results in a lysine substitution for glutamate at position 17 (E17K) and leads to a PI3K-independent activation of AKT1. The mutation was mutually exclusive with respect to mutations in *PI3K* and loss of PTEN protein expression. Others confirmed the mutation in breast and colorectal tumours (Bleeker *et al*, 2008) and incidental cases were identified in bladder, endometrial, lung, and skin cancer (Davies *et al*, 2008; Do *et al*, 2008; Malanga *et al*, 2008; Zilberman *et al*, 2009; Shoji *et al*, 2009). An analogous E17K substitution in AKT3

has also been found in melanomas (Davies *et al*, 2008). Previously, we reported on the E17K substitution in AKT1 in a ductal adenocarcinoma of the prostate in a patient who had a very long cancer-specific survival (Boormans *et al*, 2008).
In this study, we analysed the prevalence of AKT1(E17K) in a larger cohort of prostate cancer patients. We investigated whether the E17K substitution in AKT1 was associated with a specific growth pattern of prostate cancer and whether it corresponded with clinical outcome.

MATERIAL AND METHODS

AKT1

Genomic DNA was available from 184 freshly frozen clinical prostate cancer samples. A total of 85 samples were primary prostate tumours obtained by radical prostatectomy, 88 samples were locally advanced or recurrent tumours obtained by transurethral resection of the prostate, and 11 samples were hormone-naïve prostate cancer lymph node metastases obtained by pelvic lymph node dissection. An additional 30 formalin-fixed, paraffin-embedded primary prostate tumours were selected, including 18 ductal adenocarcinomas of the prostate and 12 primary prostate tumours with the following characteristics: pathological T stage ≥ pT3a, prostate specific antigen-level ≥ 4.0 ng/µl, any Gleason score, and a cancer-specific survival of > 13 years. From the cancerous regions of the paraffin-embedded samples 1-mm core biopsies were taken (Beecher Instruments, Silver Spring, MD, USA). In mutated samples, a core biopsy was taken from adjacent benign prostatic tissue to test whether the mutation was truly somatic. Genomic DNA was isolated using the Puregene DNA isolation kit (BIOzym, Landgraaf, the Netherlands), according to the manufacturer's instructions.
PCR analysis was carried out to yield a 198-bp genomic fragment of *AKT1* (see Supplementary Table 1 for primer sequences). PCR products were identified by 2% agarose gel electrophoresis and ethidium bromide staining. After ExoSapI treatment (USB, Staufen, Germany), the PCR fragments were sequenced with the reverse *AKT1* primer. Sequence reaction products were analysed on the ABI 3700 automated DNA sequencer (Applied Biosystems, Carslbad, CA, USA). The freshly frozen samples containing the E17K substitution in AKT1 were also analysed for *PTEN* mutations and for mutations in the mutational hotspots of exons 9 and 20 of *PIK3CA* (see Supplementary Table 1 for primer sequences of *PIK3CA* and *PTEN*).

Table 1a - Clinical and histopathological characteristics of three prostate cancer patients harbouring the E17K substitution in AKT1

Patient	Age at diagnosis (years)	Initial PSA (ng/ml)	Primary treatment	Secondary treatment	Tertiary treatment	cT-stage	pT-stage	Gleason score
I	74	8.8	WaWa	TURP	TURP + ET	cT2b	NA	4+4=8
II	72	13.0	RP and PLND	none	none	cT2b	pT4a	3+3=6
III	62	5.6	RP and PLND	none	none	cT3x	pT3a	3+3=6

Patient	Surgical margins	N+	Tissue	Histotype	Death	OS (years)	PCa death	CSS (years)
I	NA	NA	TURP freshly frozen	Ductal adenocarcinoma	Yes	18.4	No	≥ 18.4
II	Positive	Yes	RP freshly frozen	Acinar adenocarcinoma	No	≥ 17.0	No	≥ 17.0
III	Positive	No	RP FFPE	Acinar adenocarcinoma	Yes	16.8	No	≥ 16.8

Abbreviations: CSS = cancer-specific survival; cT-stage = clinical T stage; ET = endocrine therapy; NA = not applicable; OS = overall survival; PCa death = prostate cancer death; FFPE = formalin-fixed, paraffin-embedded; PLND = pelvic lymph node dissection; PSA = prostate-specific antigen; pT-stage = pathological T-stage; RP = radical prostatectomy; TURP = transurethral resection of the prostate; WaWa = watchful waiting.

Table 1b - Genetic characteristics of three prostate cancer patients harbouring the E17K substitution in AKT1

Patient	Sequence of *PTEN* exons	Sequence of *PIK3CA* exons 9 and 20	Sequence of *PIK3R1* exons 14 and 15
I	Wild type	Wild type	Wild type
II	Wild type	Wild type	Unknown
III	Unknown	Unknown	Unknown

Abbreviation: *PTEN* = phosphatase and tensin homologue.

PIK3CA and *PIK3R1*

Mutational analysis of *PIK3CA* and *PIK3R1* was also performed on a subset of 63 freshly frozen tissue samples: 61 transurethral resection of the prostate samples and two lymph node metastases. The exons known to contain mutational hotspots were sequenced, that is, exons 9 and 20 for *PIK3CA* and exons 14 and 15 for *PIK3R1* (see Supplementary Table 1 for primer sequences).

RESULTS AND DISCUSSION

The PI3K-AKT signalling pathway is a central factor in various cellular processes that are involved in carcinogenesis. The E17K substitution in AKT1 is an important mechanism that leads to a PI3K-independent activation of AKT1 (Carpten *et al*, 2007). Rare E17K substitution in AKT3 has also been described (Davies *et al*, 2008). In this study, we focussed on the more common AKT1(E17K) substitution in prostate cancer. Previously, we identified AKT1(E17K) in a pure ductal adenocarcinoma of the prostate in a patient who had a long survival (Boormans *et al*, 2008). Here, we analysed an additional 18 ductal prostate cancer samples; however, none of these samples contained the E17K substitution in AKT1. Therefore, we concluded that an association between AKT1(E17K) and a specific ductal growth pattern of prostate cancer is unlikely. This is in contrast to AKT1(E17K) in breast and lung cancer. In breast cancer, the mutation was unique for lobular and ductal histotypes (Bleeker *et al*, 2008), whereas in lung tumours AKT1(E17K) was only seen in squamous cell carcinomas (Bleeker *et al*, 2008; Do *et al*, 2008; Malanga *et al*, 2008).

Next, we randomly extended our search for AKT1(E17K) in 184 freshly frozen clinical prostate cancer samples. One extra patient harbouring the mutation was identified. The sample was a primary prostate tumour obtained by radical prostatectomy. The tumour was a moderately differentiated adenocarcinoma (Gleason score 3+3 = 6) with bladder neck involvement (pathological T stage: pT4a) and positive surgical margins. Moreover, occult pelvic lymph node metastases were present at the time of radical prostatectomy. Despite these prognostic unfavourable characteristics, the patient is still alive at the end of follow-up (survival ~ 17 years). These findings were in agreement with the clinical course of the patient we described in our previous report (Boormans *et al*, 2008). That patient was diagnosed with a poorly differentiated adenocarcinoma (Gleason score 4+4 = 8), but he also had a long survival (> 18 years) and he did not die from prostate cancer (see Table 1a for the clinical and histopathological characteristics of the patients).

To investigate whether AKT1(E17K) in prostate cancer was associated with a favourable clinical outcome, as suggested by the first two patients harbouring the *AKT1* mutation, we selected an additional 12 paraffin-embedded primary prostate tumours. All patients had unfavourable clinicopathological characteristics; nevertheless, they had a survival of > 13 years (see Materials and Methods section for selection criteria). In these additional 12 prostate tumours, we identified one extra patient having the E17K substitution in AKT1. Pathology showed a moderately differentiated adenocarcinoma (Gleason score 3+3 = 6) with extracapsular extension (pathological T stage: pT3a) and positive surgical margins. Almost 18 years after the radical prostatectomy, the patient died from causes other than prostate cancer.

In a recent series from our institution on patients with clinical T3 prostate tumours who were treated by radical prostatectomy and pelvic lymphadenectomy, cancer-specific and

overall survival after 15 years was 66% and 37%, respectively (Hsu *et al,* 2009). In a recent review, it was hypothesised that the presence of AKT1(E17K) is associated with a less-aggressive form of cancer (Brugge *et al,* 2007). The findings of our present series could be in agreement with such a hypothesis: all three patients harbouring the E17K substitution in AKT1 had a very long survival despite aggressive clinicopathological characteristics. Obviously, the findings of this study do not prove an association with better outcome because of the low prevalence of AKT1(E17K) in prostate cancer.

The mutational profiling of the mutational hotspots of *PIK3CA* and *PIK3R1* in a subset of 63 prostate cancer patients did not reveal any mutated sample, which is consistent with earlier findings (Ligresti *et al*, 2009).

In conclusion, we have identified three patients harbouring the E17K substitution in AKT1 in prostate cancer, rendering the reported frequency for AKT1(E17K) in prostate cancer at 1.4% (3 out of 214 patients examined). The mutation showed to be truly somatic, as the sequence of *AKT1* derived from benign prostatic tissue of patients carrying the mutation was of wild type (data not shown). AKT1(E17K) seemed to be associated with a favourable clinical course, but no correlation with a ductal growth pattern of prostate cancer was found. Furthermore, the E17K substitution in AKT1 was mutually exclusive with respect to mutations in *PTEN* and *PIK3CA*, as in the freshly frozen samples mutational profiling of all nine *PTEN* exons and of the mutational hotspots of *PIK3CA* (exons 9 and 20) showed no mutations (Table 1b). The possibility of loss of PTEN function by loss of expression was not assessed in this study.

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Supplementary Table S1 - Primer sequences of primers used for PCR and sequence analysis

Target	Forward 5'->3'	Reverse 5'->3'
AKT1	*tagagtgtgcgtggctctca*	*ctgaatcccgagaggccaa*
AKT1 Seq		*ctgaatcccgagaggccaa*
PI3KCA exon 9	*gattggttctttcctgtctctg*	*ccacaaatatcaatttacaaccattg*
PI3KCA exon 9 Seq	*ttgctttttctgtaaatcatctgtg*	
PI3KCA exon 20	*tggggtaaagggaatcaaaag*	*cctatgcaatcggtctttgc*
PI3KCA exon 20 Seq	*tgacatttgagcaaagacctg*	
PI3KR1 exon 14 and 15	*acttgaagaagcaggcagct*	*cattgcccaaccactcgtt*
PI3KR1 exon 14 and 15 Seq		*cattgcccaaccactcgtt*
PTEN exon 1	*agtcgctgcaaccatcc*	*ctaagagagtgacagaaaggta*
PTEN exon 1 Seq	*agtcgctgcaaccatcc*	
PTEN exon 1 Seq	*agtcgctgcaaccatcc*	
PTEN exon 2	*ttagtttgattgctgcatatttc*	*acatcaatatttgaaatagaaaatca*
PTEN exon 2 Seq	*ttagtttgattgctgcatatttc*	
PTEN exon 3	*tgttaatggtggctttttg*	*gcaagcatacaaataagaaaac*
PTEN exon 3 Seq	*tgttaatggtggctttttg*	
PTEN exon 4	*ttcctaagtgcaaaagataac*	*tacagtctatcgggttaagt*
PTEN exon 4 Seq	*ttcctaagtgcaaaagataac*	
PTEN exon 5	*gcaacatttctaaagttaccta*	*ctgttttccaataaattctca*
PTEN exon 5 Seq		*ctgttttccaataaattctca*
PTEN exon 6	*gaaataactataatggaaca*	*atggaaggatgagaatttcaagc*
PTEN exon 6 Seq	*gaaataactataatggaaca*	
PTEN exon 7	*atcgtttttgacagtttg*	*tcccaatgaaagtaaagtaca*
PTEN exon 7 Seq	*atcgtttttgacagtttg*	
PTEN exon 8	*tgcaaatgtttaacataggtga*	*cagctgtactcctagaatta*
PTEN exon 8 Seq		*cagctgtactcctagaatta*
PTEN exon 9	*gttcatctgcaaaatgga*	*ggtaatctgacacaatgtccta*
PTEN exon 9 Seq		*ggtaatctgacacaatgtccta*

Abbreviations: Seq = primer used for sequence analysis.

Chapter VIII

General Discussion

The detection of prostate cancer has rapidly increased over the past two decades as a result of PSA-based screening, whereas mortality rates have only slightly decreased (1). A negative down-side of PSA-based screening is the identification of insignificant cancers, so-called overdiagnosis (2). Overdiagnosis results in overtreatment and this faces treating physicians with a dilemma: which tumour should receive curative treatment because it will lead to disease-related symptoms in the future and which tumour will almost certainly follow an indolent course? A proper risk stratification of prostate cancer according to the characteristics of both the tumour and the patient seems, therefore, imminent. Histopathological features of the tumour are well-known prognosticators, although they all have certain limitations. With improvement of molecular techniques over the past decades, the genetic profile of a tumour has become more and more available and it has been shown that tumour-specific genetic alterations also carry prognostic significance. The aim of the present thesis was to investigate the prognostic value of certain important molecular alterations in prostate cancer. The main focus of this discussion is on ETS gene fusions and on aberrations in the PI3K-AKT signaling pathway and their correspondence with clinical outcome.

Prognosis of prostate cancer

Prostate cancer is a heterogeneous disease that can range from an indolent disease to a very aggressive and lethal illness. Indolent prostate tumours are defined according to criteria set by Epstein and colleagues (3). The criteria were shown to predict the presence of an insignificant tumour defined as a lesion that is confined to the prostate without primary or secondary Gleason pattern 4 or 5 and a tumour volume of ≤ 0.5 cm^3. Recently, active surveillance programs have been developed with the objective to safely follow prostate cancer patients without exposing them to treatment-related side-effects. Patients who are suitable for active surveillance have tumours with a Gleason score < 7, a clinical T stage ≤ 2, do not have more than two positive prostate biopsies, have a PSA density < 0.2 µg/ml per ml and a PSA-level < 10 µg/ml. In a retrospective analysis, these patients showed an excellent cancer-specific survival (4).
Locally advanced prostate tumours have a less favourable course. The involvement of regional lymph nodes in prostate cancer is regarded as a poor prognostic factor and it is a reason why most urologists are reluctant to advocate treatment of the primary tumour (prostatectomy or radiotherapy). Long-term survival is nevertheless possible for patients with node-positive disease (5). In prostatectomy series, cancer-specific survival at 10 years was reported to be 80% (6). The role of radiotherapy on the prostate in a node-positive setting is less clear, because PLND is not always carried out prior to radiotherapy. In a series by Bolla *et al.*, randomizing locally advanced tumours to radiation or radiation combined with three years of endocrine therapy, both arms were reported to

include only 4% node-positive patients, although here too a PLND was not always done (7). A risk stratification of node-positive prostate cancer could be helpful in deciding whether to synchronously treat the primary tumour. The 2002 TNM system for prostate cancer, however, does not contain different subclasses for node-positive disease (N0 vs. N1). In Chapter 3, we carried out a retrospective analysis of a large cohort of node-positive prostate cancer patients in order to identify histopathological characteristics of the lymph node metastases that were of prognostic significance. In the vast majority of the 142 patients analysed, the primary tumour was not treated. The nodal Gleason score and the diameter of the largest metastasis were independent predictors of cancer-specific survival in this study. It must be noticed that the Gleason score was originally developed to grade primary prostate cancer and not prostate cancer metastases (8), but previously it has already been shown that lymph node metastases can be graded using the Gleason score (9). Our results and that of others warrant a subclassification for node-positive prostate cancer. It might very well be that patients with favourable characteristics of their nodal metastases will benefit from local therapy to the prostate. Another hypothesis is that patients with adverse nodal parameters are candidates for immediate endocrine therapy, whereas those with favourable characteristics can be followed expectantly and they will not longer than necessary be exposed to the side-effects of castration. Preferably, these questions should be addressed in a prospective trial including a PLND as a standard diagnostic procedure.

Prognostic significance of ETS gene fusions in prostate cancer

Frequent chromosomal translocations involving gene fusions used to be the exclusive domain of haematological malignancies and sarcomas (10) but in 2005, Tomlins *et al.* identified recurrent gene fusions in prostate cancer (11). Gene fusions were shown to be the most frequent molecular alteration in prostate cancer as more than half of all clinical prostate cancers harboured a rearrangement (12, 13). Fusion of the androgen-regulated and prostate-specific gene *TMPRSS2* (14) to the ETS transcription factor *ERG* was the most common fusion product but multiple other fusions between ETS genes and a variety of partners have been described.

The role of *TMPRSS2-ERG* gene fusion in the prognosis of prostate cancer remains largely elusive. Conflicting results have been reported regarding the correlation between *TMPRRS2-ERG* and clinical outcome (12, 13, 15). This is probably due to the differences in: 1) the populations examined (number of patients, available clinical data, and length of follow-up; 2) the techniques used to detect gene fusions (RT-PCR, quantitative PCR or FISH); 3) the kind of tissue examined (freshly-frozen versus paraffin-embedded materials) 4) the origin of the samples tested (prostate needle biopsies, radical prostatectomy specimens, transurethral resections of the prostate or metastases); 5) the treatment of

the tumour (conservative management, surgery, radiation therapy or endocrine therapy). Furthermore, the mechanism of gene fusion itself can also differ as it can be a result of either a true chromosomal translocation or an interstitial deletion of the genomic region between *TMPRSS2* and *ERG* (16, 17). Fusion by deletion and not by translocation was claimed to be correlated with poor outcome (17, 18). The hypothesis for this finding was that the genomic fragment between *TMPRSS2* and *ERG* might contain tumour suppressor genes that influence the oncogenic properties of the gene fusion.

Another aspect that must be taken into account when addressing the association between *TMPRSS2-ERG* gene fusion and prognosis is the fact that many different fusion transcripts have been described (19). Multiple fusion transcripts can even be detected within a single tumour sample (20) and although translation leads in the vast majority to the synthesis of same N-truncated ERG protein, the functionality of gene fusions is merely unknown. It is, however, suggestive that different fusion products lead to different metabolic arrangements. A correlation with poor outcome for tumours expressing transcripts containing exon 2 of *TMPRSS2* and exon 4 of *ERG* was proposed (21). Translation of this transcript starts at the initation codon in exon 2 of *TMPRSS2*, resulting in a slightly truncated fusion protein. *In vitro*, this fusion transcript enhanced proliferation, invasion, and motility (22). The authors suggested that the coding sequences in this fusion transcript changed protein-protein interaction resulting in a more potent transcriptional activity towards target genes. In Chapter 4, we found that patients expressing *TMPRSS2(exon 0)-ERG* fusion transcripts had a longer BRFS after radical prostatectomy than patients who did not express this fusion transcript, but the expression of *TMPRSS2-ERG* itself had no correlation with biochemical recurrence (23). As an explanation for its correlation with favourable outcome, we speculated that *TMPRSS2(exon 0)-ERG* expression might occur in a more differentiated phenotype of prostate cancer. Although the population examined was rather small, the strength of our study was the availability of clinical data, the length of follow-up (median > 10 years), and the homogeneity of the population examined (patients that were known to harbour other gene fusions or whose primary treatment was not a radical prostatectomy were excluded).

In conclusion, the conflicting results in the literature on the prognostic value of *TMPRSS2-ERG* gene fusion and the heterogeneity of *TMPRSS2-ERG* expression in prostate cancer warrant further investigation. For an accurate establishment of its prognostic significance well-defined, homogeneous patient cohorts with a long follow-up are needed. In addition, the tissue samples to be examined must contain high quality RNA, and patients known to harbour other ETS gene fusion should be excluded from the analysis. As these cohorts and tissue samples are relatively scarce, collaboration between research groups is imminent. Recently, we have set up an initiative to validate *TMPRSS2(exon 0)-ERG* expression as an independent predictor of good outcome in a large cohort (n = 111) of primary prostate tumours from a Scandinavian institution. Remarkably, our

findings were confirmed by the group from Finland; patients expressing *TMPRSS2(exon 0)-ERG* had longer BRFS after radical prostatectomy than patients who did not express the fusion transcript (data not shown). This confirmation argues in favour of a prospective analysis of *TMPRSS2(exon 0)-ERG* expression in prostate cancer patients as patients expressing this fusion transcript might be candidates for a more conservative approach. Lastly, a recently developed specific anti-ERG antibody (24) raises the opportunity to correlate expression of fusion transcript with the results of IHC for ERG. Currently, we are testing whether ERG expression corresponds with the expression of *TMPRSS2(exon 0)-ERG* fusion transcripts as this might have important implications for prostate cancer prognosis.

ETS gene fusions and androgen responsiveness of prostate cancer

As most 5' ETS fusion partners are androgen-responsive, the presence of androgens typically stimulates the transcription of ETS gene fusions. The expression of the *TMPRSS2-ERG* fusion gene was shown to be tightly controlled by androgens (11). This androgen dependence might explain the response of prostate cancer to androgen deprivation. Unfortunately, the effect of androgen deprivation is only temporarily as eventually almost all prostate tumours will progress to a castration-resistant stage. Generally, CRPC patients have an increasing PSA indicating that prostate cancer cells can adapt in such a way that they still can depend on a functional AR-axis. In late-stage CRPC, downregulation or inactivation of the AR is also possible, which leads to an AR-independent growth of the tumour.

The relevance of androgen-responsive ETS gene fusions in the progression of prostate cancer to castration resistance remains controversial. Mostaghel *et al.* found in healthy men expression of *TMPRSS2* to be decreased after short-term castration but this was not found for the androgen-responsive genes *AR* and *PSA* (25). In the prostate cancer cell line VCaP, expression of *TMPRSS2* and *ERG* rapidly declined after treatment with an anti-androgen but *ERG* expression, like *PSA* and *AR*, returned to precastration levels when the tumour had relapsed (26). In therapy-naïve and castration-resistant tumour biopsies from a small group of patients participating in a trial on the administration of an inhibitor of androgen synthesis, Attard *et al.* assessed by Q-RT-PCR that the level of *ERG* expression in therapy-naïve and castration-resistant samples was comparable (27). Furthermore, in the study by Attard *et al.* it was shown by FISH that *ERG* gene status of circulating tumour cells, CRPC tumour biopsies, and matched archival diagnostic, therapy-naïve tumour tissue was invariably the same. This phenomenon was also observed in CRPC patients from a rapid autopsy program; the same gene fusion molecular subtype was found across multiple metastatic sites from an individual case (28). It must be noted, that both reports analyzed *ERG* rearrangement at the DNA level and nothing was men-

tioned on the expression of *TMPRSS2-ERG*. This is of importance as we know from an earlier series from our institution that in late-stage, androgen-independent xenografts, which were known to harbour *TMPRSS2-ERG* gene fusion, no expression of the fusion transcript could be detected (16).

The results from the aforementioned series suggested that *TMPRSS2-ERG* gene fusion remains dependent on androgen signalling, even in case of progression to a castration-resistant stage of the disease and that only in very late-stage prostate cancer, *TMPRSS2-ERG* gene fusion is not that relevant any more. Therefore, we hypothesized that the expression of *TMPRSS2-ERG* in lymph node metastases of hormone-naïve prostate cancer patients would correspond with the duration of response to endocrine therapy. However, we did not detect such a correlation nor did we observe a difference in time to PSA nadir after start of endocrine therapy between gene fusion-positive and gene fusion-negative patients (29). Recently, our findings were concurred by a larger series with comparable median follow-up, in which prostate biopsies from hormone-naïve patients were analysed for *ERG* rearrangement by FISH (30). Although the study by Leinonen *et al.* and our series differed in the techniques used to detect gene fusion (FISH vs. quantitative RT-PCR), the tissue samples examined (prostate biopsies vs. lymph node metastases), the percentage of fusion-positive cases (34% vs. 59%), and the percentage of cancer-specific deaths (7% vs. 63%), *TMPRSS2-ERG* gene fusion also did not correspond with PSA progression after start of endocrine therapy in the Finnish cohort.

Taken together, our data and that of others show that *TMPRSS2-ERG* acts as a novel androgen-responsive oncogene in prostate cancer. Therapeutic strategies that target androgen signalling may function through inhibition of ETS gene fusions. Questions remain, however, why some gene fusion-positive tumours are less responsive than others and why some *TMPRSS2-ERG*-negative tumours respond better to the endocrine therapy than others. In Chapter 3, we proposed several explanations for these observations. First, to retain a functional androgen-AR axis and not the presence or absence of the fusion gene itself might determine the sensitivity of a prostate tumour to androgen deprivation. Furthermore, the presence of other tumour-growth-promoting pathways may contribute to tumour growth to different extents, making the relevance of *TMPRSS2-ERG* gene fusion less important. Finally, it remains possible that other, as yet unidentified androgen-regulated mechanisms determine the response to endocrine therapy in the fusion gene-negative group.

To explore further the role of *TMPRSS-ERG* expression in relation to androgen deprivation therapy it would be worthwhile to test it prospectively by using freshly-frozen, hormone-naïve tissue samples. Gene fusion status (*TMPRSS2-ERG*, *TMPRSS2(exon 0)-ERG,* and other fusion partners) should be assessed by FISH, quantitative RT-PCR, and IHC and the status of the AR should also be tested for. Patients must be routinely followed-up every three months after start of endocrine therapy to monitor biochemical or clinical

progression. Endocrine therapy should consist of one regimen in all patients. Because the obtainment of freshly-frozen tissue samples requires invasive procedures a different approach would be to collect blood or urine from hormone-naïve patients that are planned to start endocrine therapy. Gene fusion can be tested by FISH on circulating tumour cells or by quantitative RT-PCR on prostate cells in urine after DRE. *TMPRSS2-ERG* fusion transcripts can be detected in the urine of prostate cancer patients (31, 32). One must take into account, however, that assays using urine samples have certain limitations and that a quantitative assessment of *TMPRSS2-ERG* gene fusion is technically challenging. Moreover, it might be that that the tumour load in the prostate of fusion-positive cancers leads to higher levels of *TMPRSS2-ERG* expression in the urine, although this can be corrected for using PSA expression as a control.

Functional role of *TMPRSS2-ERG* gene fusion in prostate cancer

The significance of fusion genes involving ETS family members in the development and the progression of prostate cancer is still largely unknown. It is thought that gene fusions play a role in tumour initiation in prostate cancer, as the precancerous lesion high-grade PIN can harbour *TMPRSS2-ERG* gene fusion (33). In transgenic mice, PIN was also detected (34, 35) but invasive cancer was only seen in the context of concomitant loss of the tumour-suppressor gene, *Pten* (36, 37). The effect of *ERG* overexpression in the mouse prostate was only minor but overexpression of *ERG* in immortalized, non-tumourigenic prostate cells suggested that it induces the expression of genes involved in invasion (34). Knockdown of *ERG* in VCaP inhibited the invasive capacity of the cells and it induced the expression of genes expressed in differentiated luminal epithelial cells. *ERG* may therefore function by keeping prostate cancer cells in a dedifferentiated state. These data show that overexpression of *ERG* seems insufficient for prostate cancer development; additional molecular events are necessary for neoplastic transformation. Activation of the PI3K-AKT pathway, i.e. by loss of PTEN expression, is another very common molecular aberration in (prostate) cancer and it might very well be that both molecular hits are necessary for cancer initiation. One must bear in mind, however, that due to the fact that both alterations are very common in prostate cancer, a possible correlation between *ERG* rearrangement and PI3K-AKT activation in the development of prostate cancer might also be a coincidence.

Analyses of gene expression profiles have implicated different molecular pathways associated with *ERG*-rearranged prostate cancer. Distinct expression signatures involving the plasminogen activation pathway (34), the WNT pathway (38), estrogen signalling (39), and chromosome 6q21 alterations (40) have all been described. In addition, several target genes of *ERG* have been proposed that might be of interest for therapeutic intervention like *HDAC1* and *c-MYC* in *ERG*-rearranged tumours (38, 41). Analysis of 194

prostate tumours for copy number alterations associated with *TMPRSS2-ERG* gene fusion revealed thee regions of copy-number loss: the tumour suppressor genes *PTEN* and Tumour protein p53 (*TP53*), and a prostate-specific region at chromosome 3p14 (42). This region contained *FOXP1*, which previously was shown to be involved in a gene fusion with *ETV1* in a primary prostate tumour (43). Recently, we identified *TDRD1*, a gene known to be involved in normal spermatogenesis (44), to be the strongest correlated gene with *ERG* overexpression in primary prostate tumours based on RNA expression profiles. We validated our findings by quantitative RT-PCR in an independent second cohort of primary prostate tumours. During mouse development, *Tdrd1* showed similar kinetics as *Erg*. In late-stage prostate cancer, *TDRD1* was also co-expressed with *ERG* overexpression, although in a proportion of *ERG*-negative tumour samples *TDRD1* was also expressed. Furthermore, *TDRD1* expression showed to be upregulated in late-stage tumours compared with primary tumours, whereas *ERG* expression was not.
Previous gene expression profiling studies did not identify *TDRD1* to be overexpressed in *ERG*-rearranged in prostate cancer (45-48). An explanation for this might be the fact that *TDRD1* is expressed at a low level and that not all expression arrays contained a *TDRD1* probe. Moreover, if the cohorts studied by others contained many late-stage tumours, the correlation between *ERG* and *TDRD1* expression becomes less clear. There is one earlier report by Jhavar *et al.* that was in agreement with our observations indicating that high *TDRD1* expression is correlated with *ERG* overexpression in primary prostate tumours (49), however this observation was not further analyzed in their report. In our study, *TDRD1* expression did not have prognostic significance. In *ETV1*-rearranged prostate tumours, *TDRD1* was hardly expressed and even seemed to be downregulated. Although *ERG* and *ETV1* both encode ETS transcription factors, they are both fusion partners of *TMPRSS2*, and they both seem to have similar functions in prostate oncogenesis, our findings and those of others (50) indicate that different pathways are involved in *ERG*- and *ETV1*-rearranged prostate cancer. Therefore, when addressing the prognostic significance of ETS gene fusions in prostate cancer, one should consider *ERG*- and *ETV1*-rearranged cancers as separate groups. At present, we extend our descriptive findings with functional studies focussed on manipulation of *TDRD1* expression in prostate cancer cell lines. Furthermore, it would also be interesting to increase our knowledge on the difference between ETV1 and ERG function in prostate cancer. As a first step we are enlarging our cohort of *ETV1*-positive primary tumours in order to obtain a robust gene expression signature of *ETV1*-rearranged prostate cancer.

PI3K-AKT signalling pathway in prostate cancer

AKT kinases regulate diverse cellular processes controlling cell size and growth, proliferation, motility, survival, and angiogenesis (51). Many oncoproteins and tumour sup-

pressors are connected to the AKT pathway and under benign conditions, activating and inactivating proteins are in a steady state. In cancer, this equilibrium is disrupted and the pathway becomes activated, which can occur by different mechanisms. In prostate cancer, the percentage of AKT activation is ~50% (52) and the most common perturbation in the pathway is loss of the tumour suppressor gene *PTEN*. We carried out immunohistochemistry (IHC) to detect PTEN inactivation in primary prostate tumours and prostate cancer lymph node metastases. The hypothesis was that loss of PTEN had prognostic value and was correlated with time to biochemical progression after radical prostatectomy or survival. We did not find such a correlation (unpublished results) but the results of the IHC seemed not very reliable. This might have been be due to the quality of the paraffin-embedded materials used, the quality of the antigen used for IHC, and the fact that loss of expression is difficult to quantify. Not surprisingly, the literature is also scarce on the correlation between loss of PTEN expression and clinical outcome in prostate cancer (53-55).

Another important mechanism of upregulation of the AKT signalling pathway is the presence of activating mutations in the AKT family members AKT1, AKT2, and AKT3 or in the catalytic and regulatory subunits of the upstream enzyme PI3K (encoded by the genes *PIK3CA* and *PIK3R1*). For prostate cancer, however, these mechanisms seem not very relevant. We and others were not able to detect activating mutations in *PIK3CA* or *PIK3R1* (56, 57). Only very recently in two out of 218 tumours analyzed, a mutation was found in *PIK3CA* (42). The recently identified E17K substitution in AKT1 (58) was shown to have a frequency of < 2% in prostate cancer (56). Although relatively rare, the AKT1(E17K) substitution is of importance as it might correspond with a good prognosis of cancer. In 2004, Hutchinson *et al.* already reported that AKT1 activation showed opposing effects on tumour growth and metastatic progression in mammary tumorigenesis (59). Furthermore, knock in of AKT1(E17K) in human breast epithelial cells was shown to have only minimal phenotypic consequences and it did not increase sensitivity to the mTOR inhibitor rapamycin (60). Emerging data confirm that AKT1 mutant cancers may have a more favourable prognosis (56, 61, 62) and it is hypothesized that it requires additional genetic events in other pathways to realize its oncogenic potential. In Chapters 6 and 7, we identified the expression of *TMPRSS2-ERG* gene fusion as a second genetic hit in the freshly-frozen prostate tumours harbouring the AKT1(E17K) substitution. The sequences of *PTEN*, *PIK3CA*, and *PIK3R1* were wild-type in these tumours, which is in agreement with results from the literature that *PTEN*, *PI3K*, and *AKT1* mutations occur in a mutually exclusive way (58, 63). Remarkably, the AKT1(E17K) patients from our series had a cancer-specific survival of >15 years, despite aggressive histopathological characteristics of their tumours.

In conclusion, AKT activation is frequent in prostate cancer and it is therefore an interesting target of therapeutic intervention. In prostate cancer, oncogenic mutations in *AKT1*

or *PI3K* are (extremely) rare but the E17K substitution in AKT1 can be of importance because of its presumed correlation with good outcome. To further investigate this possible association *in vitro* and mouse model experiments have to be carried out to test for the invasive properties of prostate cancer cells infected with mutant AKT1 and to see whether the prostates of mice overexpressing mutant *AKT1* show phenotypic changes or neoplastic transformation. In addition, as a second molecular event might be needed for the oncogenic potential of the mutation, crossing AKT1(E17K) mice with transgenic mice with targeted, prostate-specific overexpressing of *Erg* or conditional Pten knockout mice would be of interest. Given the high incidence of prostate cancer (> 8000 cases / year in The Netherlands), a prevalence of ~1.5% of AKT(E17K) in prostate cancer would still be relevant as it might assist in selecting patients that will be suitable for conservative management.

As histopathological and molecular characteristics of prostate cancer carry prognostic significance, they can be very helpful in the risk stratification of prostate cancer patients. The assessment of the histopathology of the tumour is nowadays standard of care but a further subclassification can be of additional value. The incorporation of specific molecular markers for the prognosis of prostate cancer remains under continuous investigation. The challenge with the development of molecular markers is that high-quality DNA or RNA is needed and that the assays to test for specific molecular alterations are labour-intensive and expensive. IHC, which is less labour-intensive, has important limitations especially in case of testing for loss of expression of a certain (tumour suppressor) gene. Furthermore, as one molecular hit is insufficient for prostate cancer development or progression, multiple molecular changes must be taken into account when addressing prostate cancer prognosis. Finally, as specific genetic alterations can be more or less relevant during the various stages of prostate cancer, different molecular tests should be developed for a specific stage of prostate cancer, i.e. hormone-naïve, hormone-responsive, or castration-resistant disease.

To summarize, the work presented in this thesis argues for a subclassification of node-positive prostate cancer based on histopathological characteristics of the nodal metastases. Secondly, we showed that expression of the androgen-regulated *TMPRSS2-ERG* fusion gene did not correspond with the duration of response to castration in hormone-naïve, node-positive disease. In contrast, in primary prostate cancer, the expression of a specific *TMPRSS2-ERG* fusion transcript containing an alternative first exon of *TMPRSS2* did carry prognostic significance as it was an independent predictor of favourable outcome. Furthermore, we assessed *TDRD1* as a candidate target gene of *ERG* but not of *ETV1* in primary prostate cancer. Finally, we identified for the first time the E17K substitution in AKT1 in prostate cancer and we found arguments that it corresponded with a relatively mild clinical course, which is in agreement with the current literature on this topic.

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Part IV

Appendices

Summary

Prostate cancer can vary from an indolent disease that almost never will lead to disease-related symptoms to an aggressive and rapidly fatal illness. An accurate risk stratification of prostate cancer patients, therefore, is of importance for optimization of treatment. Molecular and histopathological parameters of the tumour can be helpful in distinguishing indolent from clinically relevant cancers. In the present thesis, histopathological characteristics and major genetic alterations of prostate cancer were analysed to see whether they carried prognostic significance and if they were correlated with clinical outcome.

Chapter 1 is a general introduction on the different aspects of prostate cancer diagnosis and treatment. The most important genetic alterations that are linked to prostate cancer are discussed. In Chapter 2, a large cohort of prostate cancer lymph-node metastases was analysed. Histopathological characterisation of the lymph node metastases showed the nodal Gleason score and the diameter of the largest lymph node metastasis to be independent predictors of cancer-specific survival.

The presence of recurrent gene fusions in prostate cancer was only recently discovered. Approximately half of all primary prostate tumours harboured overexpression of the oncogene *ERG* as a result of a genetic rearrangement with the androgen-regulated and prostate-specific gene *TMPRSS2*. In Chapter 3, we hypothesized that the expression of this androgen-regulated fusion gene would correspond with the duration of response to endocrine therapy in hormone-naïve prostate cancer lymph node metastases. By RT-PCR it was shown that *TMPRSS2-ERG* was expressed in 59% of the 71 patients analysed. However, no significant difference in the duration of response to endocrine therapy was found between the patients that expressed *TMPRSS2-ERG* and those who did not. There were also no significant differences between the two groups for the time to PSA nadir after start of endocrine treatment, nor for the cancer-specific and overall survival. The results suggested that expression of *TMPRSS2-ERG* is not a candidate marker to select for metastatic patients who will benefit more from endocrine treatment.

Transcription of the gene *TMPRSS2* can not only start from its first exon, exon 1, but it can also start from an alternative first exon, here denoted exon 0. In Chapter 4, we characterized by quantitative RT-PCR the expression of both *TMPRSS2* transcripts and of *TMPRSS2(exon 0)-ERG* and *TMPRSS2(exon 1)-ERG* fusion transcripts. *TMPRSS2(exon 0)* and *TMPRSS2(exon 1)* transcripts were similarly androgen regulated in prostate cancer cell lines but the expression levels of *TMPRSS2(exon 1)* transcripts were much higher. Analysis of expression of both transcripts in various normal tissues showed *TMPRSS2(exon 0)* expression to be much more prostate specific than *TMPRSS2(exon 1)*. In AR-positive prostate cancer xenografts, *TMPRSS2(exon 1)* transcripts were expressed at similar levels, whereas *TMPRSS2(exon 0)* transcripts were expressed at very variable levels. The same phenomenon was observed for *TMPRSS2-ERG* fusion transcripts. In clinical prostate cancer, the expression of *TMPRSS2(exon 0)-ERG* was even more variable,

whereas *TMPRSS2(exon 1)-ERG*, again, was expressed at similar levels. The expression of *TMPRSS2(exon 0)-ERG* transcripts was detected in 55% of the gene fusion-positive primary tumours but only in 15% of the gene fusion-positive recurrences and at much lower levels. Furthermore, in primary tumours, expression of *TMPRSS2(exon 0)-ERG* transcripts was a predictor of longer biochemical progression-free survival after radical prostatectomy, independent of stage, grade and surgical margin status of the tumour. Recently, we have been able to confirm this observation in a larger cohort from a Scandinavian institution.

In Chapter 5, RNA expression profiles were used to detect genes that were co-expressed with *ERG* in prostate cancer cell lines, xenografts, primary tumours, lymph node metastases and late-stage tumours. The different stages of prostate cancer (primary tumours versus late-stage and metastases) were compared for differences in gene expression. Genes that were shown to be strongly correlated with *ERG* overexpression were confirmed by quantitative RT-PCR. *TDRD1* was identified as the strongest correlated gene with *ERG* overexpression in primary prostate tumours. During mouse development, *Tdrd1* showed similar kinetics as *Erg*. In late-stage prostate cancer, *TDRD1* was also co-expressed with *ERG* overexpression, however, not only *ERG*-positive tumour samples but also a proportion of *ERG*-negative late-stage tumours showed expression of *TDRD1*. Furthermore, *TDRD1* expression showed to be upregulated in late-stage tumours compared with primary tumours, whereas *ERG* expression was not. *TDRD1* expression did not have prognostic significance in the cohorts examined. In *ETV1*-rearranged prostate tumours, *TDRD1* was, like *ERG*, hardly expressed and even seemed to be downregulated.

In Chapter 6 and 7, the prognostic significance of certain alterations in the PI3K-AKT signalling pathway in prostate cancer was assessed. This pathway is frequently activated in various cancers, among which prostate cancer. The first identification of an activating mutation in *AKT1* in prostate cancer is stated in Chapter 6. In Chapter 7, we showed that the exact prevalence the E17K substitution in AKT1 in prostate cancer is 1.4%. An association of AKT1(E17K) with a specific growth pattern of prostate cancer was not found but it did seem to be associated with a relatively favourable clinical course. Furthermore, no activating mutations were found in the mutational hotspots of the catalytic and regulatory subunits of *PI3K*.

The results of this thesis described in Chapters 2-7 are discussed in Chapter 8 and suggestions for further research are mentioned. To summarize, this thesis argues for a subclassification of node-positive prostate cancer based on histopathological characteristics of the nodal metastases. Future perspectives will be to test whether patients with favourable characteristics of their nodal metastases will benefit from local therapy to the prostate or that patients with adverse nodal parameters might be candidates for immediate endocrine therapy instead of expectant management.

Secondly, we found evidence that the androgen-regulated *TMPRSS2-ERG* fusion gene cannot be used as a prognostic marker to select patients that will benefit from endocrine therapy. In contrast, in primary prostate cancer, the expression of a specific *TMPRSS2-ERG* fusion transcript, *TMPRSS2(exon 0)-ERG*, did carry prognostic significance as it was a predictor of favourable outcome in two independent patient cohorts. A prospective analysis to further explore the role of *TMPRSS2(exon 0)-ERG* expression as a predictor of favourable outcome in prostate cancer would be worthwhile. As *TMPRSS2-ERG* expression can be measured in urine of prostate cancer patients, the incorporation of such a test to detect *TMPRSS2(exon 0)-ERG* transcripts in the urine from patients participating in an active surveillance program would be very interesting. It can be hypothesized that patients who express *TMPRSS2(exon 0)-ERG* fusion transcripts will not show progression and therefore will be candidates to continue active surveillance.

Thirdly, we identified *TDRD1* as a candidate target gene of *ERG*, but not of *ETV1* in primary prostate cancer. This observation should be further analyzed *in vitro* with functional studies focussed on manipulation of *TDRD1* expression in prostate cancer cell lines. Knock-down of *ERG* in an *ERG*-positive prostate cancer cell line should lead to a decrease in expression of *TDRD1*. Furthermore, as more evidence is provided that different pathways are involved in *ERG*- and *ETV1*-rearranged prostate cancer, it would be interesting to enlarge our cohort of *ETV1*-positive primary tumours in order to obtain a gene expression signature of *ETV1*-rearranged prostate cancer.

Finally, we identified the E17K substitution in AKT1 in prostate cancer, which was shown to correspond with a relatively mild clinical course. Given the high incidence of prostate cancer, its low prevalence of ~1.5% would still be relevant as it can assist in selecting patients that will be suitable for conservative management. In the future, *in vitro* and mouse model experiments have to be carried out to test for the invasive properties of prostate cancer cells infected with mutant *AKT1* and to see whether the prostates of mice overexpressing mutant *AKT1* show phenotypic changes or neoplastic transformation.

Samenvatting (Dutch)

Prostaatkanker is een veelvoorkomende kanker bij mannen met een Westers leefpatroon. Prostaatkanker kan een mild beloop hebben waarbij de patiënt vrijwel nooit ziektegerelateerde symptomen ontwikkelt, maar het kan ook een zeer agressieve kanker zijn met een grote kans om te overlijden aan de ziekte. Een adequate risicostratificatie van prostaatkankerpatiënten is daarom belangrijk om tot de juiste keuze van behandeling te komen. Niet alleen histopathologische karakteristieken, maar ook genetische veranderingen van de tumor zijn van belang om een goed onderscheid te kunnen maken tussen indolente en klinisch relevante tumoren. In het huidige proefschrift zijn histopathologische kenmerken en belangrijke genetische veranderingen van prostaattumoren geanalyseerd met als doel de prognostische waarde van deze parameters te bepalen door ze te correleren met het klinisch beloop.

Hoofdstuk 1 is een algemene introductie waarin de verschillende aspecten van de diagnostiek en de behandeling van prostaatkanker worden besproken. Belangrijke genetische veranderingen in prostaatkanker die in de literatuur zijn beschreven passeren de revue. In hoofdstuk 2 is een groot cohort lymfkliermetastasen van onbehandelde prostaatkankerpatiënten geanalyseerd. Histopathologische kenmerken van de lymfkliermetastasen werden verzameld en gecorreleerd met overleving. De Gleason score van de lymfklieren en de diameter van de grootste lymfkliermetastase waren onafhankelijke voorspellers van prostaatkankersterfte.

Recentelijk zijn specifieke chromosomale veranderingen in prostaatkanker geïdentificeerd die frequent voorkomen. Ongeveer de helft van alle prostaattumoren bleek het oncogen *ERG* tot overexpressie te brengen als gevolg van een fusie van *ERG* met het androgeen gereguleerde en prostaatspecifieke gen *TMPRSS2*. In hoofdstuk 3 is de hypothese getest dat de expressie van dit androgeen gereguleerde fusiegen in hormonaal onbehandelde lymfkliermetastasen correspondeerde met de duur van respons op endocriene therapie. Tweeënveertig van de 71 geanalyseerde patiënten (59%) brachten het *TMPRSS2-ERG* fusiegen tot expressie. Er werd echter geen verschil gevonden in de duur van respons op endocriene therapie tussen de patiënten met en zonder expressie van *TMPRSS2-ERG*. Bovendien waren de tijd tot de PSA-nadir en de (kankerspecifieke) overleving tussen beide groepen niet verschillend. De resultaten suggereerden dat de expressie van *TMPRSS2-ERG* geen goede marker is om gemetastaseerde patiënten te selecteren die meer baat zouden hebben bij endocriene therapie.

Transcriptie van het gen *TMPRSS2* kan starten vanaf het eerste exon, maar ook vanaf een alternatief eerste exon, hierna exon 0 genoemd. In hoofdstuk 4 werd door middel van kwantitatieve RT-PCR de expressie van *TMPRSS2(exon 0)*, *TMPRSS2(exon 1)*, als ook de expressie van beide fusie transcripten (*TMPRSS2(exon 0)-ERG* en *TMPRSS2(exon 1)-ERG*) bepaald. In prostaatkankercellijnen bleken *TMPRSS2(exon 0)* en *TMPRSS2(exon 1)* beide in gelijke mate te worden gereguleerd door androgenen, maar de expressie van *TMPRSS2(exon 1)* was veel hoger. Een analyse van de expressie van *TMPRSS2(exon 0)* en

TMPRSS2(exon 1) in verschillende goedaardige humane weefsels toonde dat de expressie van *TMPRSS2(exon 0)* veel prostaatspecifieker was. In prostaatkanker xenografts die de androgenreceptor tot expressie brengen, bleek de expressie van *TMPRSS2(exon 1)* transcripten min of meer vergelijkbaar, terwijl de expressie van *TMPRSS2(exon 0)* transcripten sterk variabel was. Dit zelfde fenomeen werd ook waargenomen voor beide fusie transcripten. In klinische prostaatkanker samples bleek de expressie van *TMPRSS2 (exon 0)-ERG* nog veel variabeler dan in de xenografts het geval was, terwijl het niveau expressie van *TMPRSS2(exon 1)-ERG* ook hier vergelijkbaar was. In de primaire prostaattumoren werden in 55% van de fusiegen-positieve tumoren *TMPRSS2(exon 0)-ERG* transcripten gedetecteerd. In de recurrences bleek slechts 15% *TMPRSS2(exon 0)-ERG* tot expressie te brengen en bovendien was het niveau veel lager dan in de primaire tumoren. Tot slot was de expressie van *TMPRSS2(exon 0)-ERG* een gunstige voorspeller voor de tijd tot het optreden van een biochemisch recidief na radicale prostatectomie, onafhankelijk van het stadium, de gradering, of de sneevlakken van de tumor.
In hoofdstuk 5 zijn met behulp van RNA expressie technieken genen geïdentificeerd die tot overexpressie komen in prostaattumoren met *ERG* overexpressie. De expressie van genen werd voor de verschillende stadia van prostaatkanker (primaire versus laatstadium tumoren en metastasen) vergeleken. Genen die sterk correleerden met overexpressie van *ERG* werden bevestigd met kwantitatieve RT-PCR. In primaire tumoren bleek het gen *TDRD1* het sterkst gecorreleerd met *ERG* overexpressie. Deze bevinding werd gevalideerd in een tweede, onafhankelijk cohort van primaire prostaattumoren. Gedurende de ontwikkeling van de prostaat in de muis vertoonde *Tdrd1* een expressieprofiel overeenkomstig met dat van *Erg*. In laatstadium prostaatkanker bleek *TDRD1* expressie nog steeds gecorreleerd met *ERG* overexpressie, hoewel meerdere *ERG*-negatieve tumoren ook *TDRD1* expressie toonden. In prostaattumoren met *ETV1* overexpressie kwam *TDRD1*, net als *ERG*, nauwelijks tot expressie en leek er zelfs een omgekeerde correlatie te bestaan.
In de hoofdstukken 6 en 7 is de prognostische waarde van bepaalde veranderingen in het PI3K-AKT pathway in prostaatkanker bepaald. Dit pathway is vaak geactiveerd in verschillende vormen van kanker waaronder prostaatkanker. Zo is de eerste casus van een activerende mutatie in het gen *AKT1* in prostaatkanker beschreven in hoofdstuk 6. In hoofdstuk 7 is aangetoond dat deze mutatie, AKT1(E17K), een prevalentie heeft van slechts 1.4% in prostaatkanker Een verband tussen het voorkomen van de mutatie en een specifiek groeipatroon van prostaattumoren werd niet gevonden, maar de mutatie leek wel geassocieerd met een mild klinisch beloop. Tot slot konden activerende mutaties in de mutatie hotspots van het gen *PI3K* niet worden geïdentificeerd.

De resultaten uit de hoofdstukken 2 t/m 7 worden besproken in hoofdstuk 8 waarbij eveneens suggesties voor verder onderzoek in de toekomst worden gegeven. Samen-

vattend pleiten de bevindingen in dit proefschrift voor een subclassificatie van lymfogeen gemetastaseerd prostaatkanker op basis van histopathologische kenmerken van de lymfkliermetastasen. In de toekomst zou moeten onderzocht of patiënten met gunstige histopathologische kenmerken baat hebben bij de lokale behandeling van de primaire tumor (bijvoorbeeld middels radiotherapie). Een andere hypothese is dat bij patiënten met ongunstige kenmerken niet afgewacht kan worden, maar dat er direct een hormonale behandeling zou moeten worden gestart.

De expressie van het androgeen-gereguleerde *TMPRSS2-ERG* fusiegen kan niet als prognostische moleculaire marker worden gebruikt om prostaatkankerpatiënten te selecteren die baat hebben bij hormonale behandeling. Daarentegen, bleek de expressie van een specifiek *TMPRSS2-ERG* fusie transcript in primaire prostaattumoren wel prognostisch van belang, daar de expressie van *TMPRSS2(exon 0)-ERG* een voorspeller was in twee onafhankelijke patiënten cohorten. Een prospectieve analyse om de waarde van *TMPRSS2(exon 0)-ERG* expressie als een gunstige voorspeller van prostaatkanker te bekijken is de moeite waard. Aangezien eerder reeds is aangetoond dat *TMPRSS2-ERG* fusietranscripten kunnen worden gevonden in de urine van prostaatkanker patiënten, is het interessant de detectie van de expressie van *TMPRSS2(exon 0)-ERG* toe te voegen aan active surveillance programma's. De hypothese zou zijn dat prostaatkankerpatiënten waarbij expressie van *TMPRSS2(exon 0)-ERG* kan worden gedetecteerd, geen progressie ontwikkelen. Deze patiënten zouden daarom kandidaat zijn het active surveillance beleid te continueren.

Ten derde, identificeerden wij het gen *TDRD1* als een mogelijk targetgen van *ERG* in primaire prostaattumoren. Deze observatie dient in de toekomst verder *in vitro* gevalideerd te worden met behulp van functionele studies die gericht zijn op de manipulatie van TDRD1 in prostaatkankercellijnen. Downregulering van de expressie van *ERG* in *ERG*-positieve prostaatkankercellijnen door middel van 'short interfering RNA' zou moeten leiden tot een verminderde expressie van *TDRD1*, hetgeen een directe relatie tussen *ERG* en *TDRD1* impliceert.

Tot slot detecteerden wij de E17K vervanging in AKT1 in prostaatkanker die bleek te corresponderen met een gunstig klinisch beloop. Gezien de hoge incidentie van prostaatkanker is deze zeldzame mutatie (prevalentie +/- 1.5%) toch relevant, aangezien de aanwezigheid van de mutatie patiënten kan selecteren die kandidaat zijn voor een afwachtend beleid. In de toekomst dienen *in vitro* experimenten gedaan waarbij de invasieve eigenschappen van prostaatkankercellijnen die geïnfecteerd zijn met mutant AKT1 dienen te worden bepaald. Daarnaast zouden in muismodellen moeten worden bekeken of er neoplastische veranderingen optreden in het fenotype van prostaten van muizen die mutant AKT1 tot overexpressie brengen.

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Curriculum Vitae

Joost Boormans was born on the 13th of January 1976 in the city of Tilburg, the Netherlands. He spent his entire youth in 'The most beautiful city of the country'. In 1994, he finished high school (Atheneum, cum laude, Theresialyceum, Tilburg) and he continued with Medical School at Utrecht University. In December 2001 he graduated from Medical School and in that same year he passed Step II of the United States Medical Licensing Examination.

In January 2002, he started as a resident in urology at the Department of Children's Urology, Sophia's Children's Hospital in Rotterdam (head: Prof. dr. J.M. Nijman). In July 2002, he moved to the adults at the Department of Urology, Erasmus MC, Rotterdam (head: Prof. dr. C.H. Bangma). His surgical training started in September 2003 at the Department of Surgery, Albert Schweitzer Hospital, Dordrecht (heads: dr. K.G. Tan and dr. R.J. Oostenbroek). After finishing his training in general surgery, he participated in the research on genetic alterations in prostate cancer under supervision of dr. P.C.M.S. Verhagen, which eventually have lead to the current thesis. From January 2006 till December 2008 he followed his academic training in urology at the Department of Urology, Erasmus MC, Rotterdam (head: Prof. dr. C.H. Bangma / mentor dr. G.R. Dohle). During this 3-year period, he spent one year full-time in the research laboratory of Prof. dr J. Trapman at the Josephine Nefkens Instituut of the Erasmus University. His final year of training was conducted at the Department of Urology, Amphia Hospital, Breda (head: drs. D.K.E. van der Schoot).

Since January 1st 2010, Joost Boormans is working at the Department of Urology, Erasmus MC – Daniel den Hoed Cancer Centre, as a fellow in oncological urology. He shares his life with Janneke Thomassen. Together they have a son, named Michiel.

PhD Portfolio

SUMMARY OF PHD TRAINING AND TEACHING

Name PhD student: Joost Laurens Boormans	PhD period: 2005-2010
Erasmus MC Department: Pathology and Urology	Promotors:
Research School: -	Prof. dr. C.H. Bangma, Prof. dr. J. Trapman
	Supervisor:
	Dr. P.C.M.S. Verhagen

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
- Biomedical English Writing and Communication	2007	5
- Workshop ´How to write a successful grant proposal´	2010	0.4
Specific courses (e.g. Research school, Medical Training)		
- Obligatory courses for residents in training 'Nederlandse Vereniging voor Urologie'	2006-2009	8
- 'Basisopleiding stralingsbescherming deskundigheidsniveau 4A/M'	2006	1
- Courses 'Discipline Overstijgend Onderwijs'	2008	1
Erasmus MC	2009	2
- European Urology Residents Education Program	2009	2
- Fellow of the European Board of Urology	2010	0.5
Seminars and workshops		
- Department's Journal Club	2005-2008	1
- Department's Campbell Club	2005-2008	1
- Department's 'refereeravond'	2006-2009	1
- Department's 'promovendiavond'	2006-2010	1
Presentations		
- Multiple presentations at different conventions and meetings of the Department of Urology and the Department of Experimental Pathology	2005-2010	5
(Inter)national conferences		
- Multiple poster and oral presentations at (semi)-annual meetings of: American Urological Association (Chicago) European Association of Urology (Paris, Berlin, and Milan) Nederlandse Vereniging voor Urologie (5x)	2005-2010	9
Other		
-		

2. Teaching

	Year	Workload (Hours/ECTS)
Lecturing		
- Physiotherapists Erasmus MC 'Oncological Urology'	2008	0.4
- Nurses Department of Urology Erasmus MC	2010	0.4