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The detection of *TMPRSS2:ERG* fusion gene in blood of prostate cancer patients

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ABBREVIATIONS

AR	Androgen receptor
ARE	Androgen receptor element
BPH	Benign prostate hyperplasia
CDK	Cyclin-dependent kinase
CGH	Comparative genomic hybridization
CRPC	Castration resistant PC
CTC	Circulating tumor cell
DHT	5α-dihydrotestosterone
EMT	Epithelial-to-mesenchymal transition
ERG	V-ets erythroblastosis virus E26 oncogene homolog (avian)
ETS	Avian erythroblastosis virus E26 homolog
EZH2	Enhancer of zeste homolog 2
GS	Gleason score
GnRH	Gonadotropin releasing-hormone
HDAC	Histone deacetylase
HGPIN	High-grade prostatic intraepithelial neoplasia
HPC	Hereditary prostate cancer
LOH	Loss of heterozygosity
mRNA	Messenger ribonucleic acid
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
NKX3.1	NK3 homeobox 1
PC	Prostate cancer
PC-3	Human PC cell line 3
P53	Tumor protein 53
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3-kinase
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
QRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
TMPRSS2	Transmembrane protease, serine 2
TNM	TNM classification of malignant tumors
TURP	Transurethral resection of prostate
UTR	Untranslated region
VCaP	Vertebrae PC cell line

ABSTRACT

Prostate cancer (PC) is a heterogeneous disease caused by multiple complex genetic, epigenetic and chromosomal abnormalities within cells. The discovery of total prostate specific antigen (total PSA) testing in the blood of PC patients has led to an increased number of PC diagnoses in western populations. However, PSA is not only PC specific, since other conditions which disrupt the normal prostatic architechture can also cause PSA to appear in blood, therefore more reliable biomarkers are needed to improve PC detection. Currently, the best predictor of PC survival is provided by the combination of PSA levels, clinical and pathological tumor stage, and Gleason score. In order to better define the type of cells undergoing malignant transformation, analysis of specific tumor phenotypes involved in PC recurrence and finally predict patient outcome in the future, more tumor- and tissue-specific (as well as blood based) biomarkers are needed.

Translocations of the androgen-induced 5´ *TMPRSS2* (transmembrane protease, serine 2) chromosomal region to the oncogenic transcription factor coding family member 3´ *ERG* (v-ets erythroblastosis virus E26 homolog avian) have been shown to be present in approximately 50% of PC cases. It has been reported that the translocation between *TMPRSS2* and *ERG* is an early lesion in PC progression and the frequency of *TMPRSS2:ERG* fusions increases from moderately to poorly differentiated tumors. There have been several studies utilizing different molecular techniques to investigate the role of *TMPRSS2:ERG* fusion in PC progression. However, the real biological role of the fusion in PC tumorigenesis remains to be elucidated.

The main aim of the present study was to validate the sensitivity of TaqMan, SYBR Green and iScript One RT-PCR assays in the detection of the *TMPRSS2:ERG* transcript in the fusion positive VCaP prostate cancer cell line. Further, *TMPRSS2:ERG* status in 163 clinical PC patients' blood samples was studied and the frequency of the fusion was determined with a TaqMan assay, which was considered to be the most sensitive assay for the detection of the fusion. The association between *TMPRSS2:ERG* fusion and clinicopathological data was evaluated using Fisher's exact test. The Kaplan-Meier progression-free survival analysis and the Mantel-Cox hazard model were used to define the correlation between the fusion status and patients' clinical outcome.

In the present study, the fusion status was not shown to be significantly associated either with clinicopathological data or patient outcome, due to low frequency and high variability of the *TMPRSS2:ERG* fusion transcript detected in the blood of respective PC patients. According to the results obtained from clinical samples, the TaqMan assay was not confirmed to be a sufficiently stable and reproducible method for the detection of *TMPRSS2:ERG* fusion transcripts in the blood of PC patients. Thus, this TaqMan assay cannot be used as a clinical diagnostic method for PC. In the future, it would be interesting to further evaluate whether or not the sensitivity of the TaqMan assay and the detection of *TMPRSS2:ERG* fusion positive PC cases could be improved by using different combinations of blood-based biomarkers.

1. INTRODUCTION

1.1 CANCER

Cancer is a heterogeneous disease caused by multiple sets of genetic alterations. During cancer development the normal process of the mammalian cell cycle is disrupted leading to impaired replication of DNA. Defects in the double-strand break DNA repair process can lead to inhibition of homologous recombination between strands, increasing chromosomal instability, and finally accumulation over time can lead to the tumor formation (Kumma and Jackson 2001). Moreover, according to Kumma and Jackson (2001), chromosomal translocations and inversions usually arise during the error prone non-homologous end joining (NHEJ) process where the strands which are not repaired are aberrantly joined together initiating tumorigenesis. Thus, mutations, chromosomal rearrangements and epigenetic changes occur during constant and rapid cell cycle divisions. Due to the fast mutation and proliferation rate of tumor cells, adaptation to the constantly changing microenvironment is a requirement for tumor cell survival. This leads to the formation of heterogeneous and complex tumor cell tissue (Hanahan and Weinberg 2000).

1.1.1 Oncogenes

Oncogenes are mutated cancer-promoting genes that originate from proto-oncogenes. They are dominant genes which require only one mutation for malignant transformation. Proto-oncogenes encode essential proteins such as transcription factors (e.g. MYC), signal transducers (e.g. kinases), apoptosis regulators (e.g. BCL-1 and BCL-2), and growth factors, as well as growth factor receptors that regulate cell growth and differentiation (Croce 2008). When the proto-onco genes become structurally altered due to gain-of-function mutations such as gene amplification, chromosomal rearrangements, or point mutations they become oncogenes and start to directly encode oncoproteins (Croce 2008). Oncogenes can alter the normal function of growth by inducing the receptor and cytoplasmic tyrosine kinase phosphorylation, leading to ligand-independent activation of receptors. The *BCR-ABL gene fusion* is one example of an oncogene; found on the Philadelphia chromosome in chronic myelogenous leukemia patients. *BCR-ABL* encodes receptor tyrosine kinase, which is constitutively active, accelerating cell proliferation during cancer tumorigenesis. Oncogenes can permanently activate epidermal growth factor receptors (EGFR) and vascular endothelial growth factor receptor (VEGFR), inducing epidermal growth and angiogenesis in cancer, respectively. (Croce 2008).

1.1.2 Tumor suppressor genes

Tumor suppressor genes (TSGs) are recessive genes with cancer-preventive effects, such as the regulation of DNA damage detection and repair, tumor vascularization, and responses at cell cycle checkpoints (Sherr 2004). Mutations in TSGs lead to the inhibition of cancer-suppressive functions, increasing the risk of malignant growth. Due to the recessive characteristic of TSGs, at least two mutational events are required in both alleles in order to silence the gene (Knudson 1971, Berger et *al*. 2011). The first allelic mutation in TSGs usually occurs at the germline level, whereas inactivation of the other allele occurs at the somatic level. The second mutational event is called loss of heterozygocity (LOH), which occurs due to interstitial deletions of the chromosomal regions close to the respective gene, and by mitotic recombination or gene conversion (Berger et *al*. 2011, Sherr 2004). Haploinsufficient genes are TSGs which need only one allelic loss for tumor growth, especially when combined with other mutations which alter the function of oncogenes and TSGs (Cook and McCaw 2000).

1.2 PROSTATE CANCER

1.2.1 The anatomy and physiology of the prostate

The prostate gland is located in anterior to the rectum, and inferior to the bladder within the lower pelvis, and is connected to the seminal vesicles, forming the accessory glands of reproduction. The main function of prostate gland is to secrete a milky, slightly acidic fluid which is essential for ejaculation. One crucial component of this fluid is serum prostate specific antigen (PSA); used during the screening of PC patients. The prostate is a glandular and muscular organ supported by a stroma and extracellular matrix (ECM) components including neurovascular tissue, fibroblasts, smooth muscle cells, and lymphocytes. There are four different cell types in the prostatic epithelium: secretory, basal, transient amplifying (intermediate between secretory and basal cells), and neuroendocrine cells. (Isaacs and Coffey 1989)

Cellular compartment of the prostate

Secretory cells which line the prostate glands and ducts are involved in the secretion of an alkaline fluid that is crucial for the motility and nourishment of sperm. Basal cells are attached to the basement membrane and are adjacent to the secretory cells; they have a high proliferative capacity and long life span. Neuroendocrine cells are spread throughout the prostate gland, their main function being the regulation of cell growth and prostatic secretory activity (Bonkhoff et *al*. 1994, De Marzo et *al*. 1998, Foster et *al*. 2002). The prostate gland is divided into four anatomical zones: the peripheral, central, transition, and fibromuscular zones. The peripheral zone is the largest, comprising approximately 75% of the prostate gland, from which about 70-80% of PCs originate. Precursor lesions to PC, such as high-grade prostatic intraepithelial neoplasia (HGPIN), prostatic intraepithelial neoplasia (PIN), and primary prostate carcinoma develop in the peripheral zone. However, tumors may also develop in the transition zone. Benign prostatic hyperplasia (BPH), which is not considered to be a precursor lesion to PC, usually develops in the transition zone (Appelwhite et *al*. 2001, Ayala and Ro 2007, Kristal et *al*. 2010). Most PCs are multifocal and grow in the multiple zones of the prostate.

1.2.2 Incidence and risk factors

Incidence

In recent years, PC incidence has increased throughout the developed world. This can largely be attributed to increased PSA testing, as well as increased positive biopsy rate in aging populations. However, mortality has decreased or remained at the same level (Jemal et *al*. 2010). PC incidence in the aging population of developed countries is approximately 16% (Jemal et *al*. 2010, Siegel et *al*. 2012), suggesting that on average, one in every six men in these countries will be diagnosed with PC. According to Siegel et *al*. (2012), PC is the most commonly diagnosed malignancy in men in the United States and the second (after lung cancer) most common cause of all cancer deaths in the USA. In the developed world, about 650 000 men are diagnosed with PC, while approximately 136 000 men die from PC annually (Jemal et *al*. 2011).

Risk factors

Little is known about the risk factors of PC, though several studies have shown that the most common risk factors significantly affecting PC progression are: advanced age, ethnicity, and family history (Damber and Aus 2008, Haas et *al*. 1997). According to different epidemiological studies, PC risk increases by two to three fold if any first degree relatives (i.e. father or brother) have PC (Cerhan et *al*. 1999, Zeegers 2003). Hereditary PC (HPC) – a type of familial PC – accounts for 5-10% of PC cases, and is more common in families with significantly earlier onset of PC (Cerhan et *al*. 1999, Grönberg et *al*. 1997). High penetrance genes increase the PC risk among HPC cases. However, only a few HPC cases have been found to interact with high penetrance genes, including *breast cancer type 2 susceptibility protein* (*BRCA2)*, *elaC homolog 2 (Escherichia coli)* (*ELAC*), *macrophage scavenger receptor 1* (*MSR1*), and *ribonuclease L* (*RNASEL*) (Carpten et *al*. 2002, Edwards et *al*. 2003). Low penetrance genes are more common in HPC. In addition, several chromosomal loci including 8q24, 17q21, Xp11 and 10q21 have been found to be associated with PC risk (Al Olama et *al*. 2009, Witte 2009).

1.2.3 Early signs and symptoms

Morphological changes

The morphology of prostate cells changes during the aging process; known as benign prostatic hyperplasia (BPH), which is not a precursor to PC as mentioned earlier. In BPH the number of epithelial cells increases but the nuclear appearance of the cells remains normal. BPH is a very common lesion among elderly men; affecting approximately 90% of 80-year-old men (Bushman 2009, Kristal et *al*. 2010). However, constantly proliferating prostate epithelial cells can acquire changes in their nuclear histology and columnar appearance. When the nuclear compartment is enlarged and the nucleoli are prominent the lesion is considered to be a precursor lesion to PC. Such pre-malignant lesions are either called prostatic intraepithelial neoplasia (PIN) (leading to nuclear enlargement and accumulation of luminal epithelial cells) or high-grade prostatic intraepithelial neoplasia (HGPIN) (leading to dysplasia of prostatic epithelial cells). Multifocal lesions, which may be characterized by a variety of differentiation phenotypes at histological level, arise in the constantly changing micro-environment and cell compartments during PC progression (Ayala and Ro 2007).

Genetic and epigenetic changes

Several genetic and epigenetic aberrations have been shown to be involved in the development of malignant PC. The most frequently found alterations are *TMPRSS2:ETS* translocations, androgen receptor (AR) gene amplification, *MYC* oncogene overexpression, loss of *NKX3.1*, loss of *PTEN*, gain-of-function mutations in *EZH2*, and inactivation of *TP53* (Asatiani et *al*. 2005, Bowen et *al*. 2000). Loss of *NKX3.1* has been found to be associated with the early stages of HGPIN. In addition, *TMPRSS2:ETS* translocations and gain-of-function mutations of *MYC* have been reported to be associated with localized PC. Furthermore, amplification of the *AR* gene, loss of *PTEN*, and mutations in *EZH2* and *TP53* have been found more often in advanced stage of PC (Asatiani et *al*. 2005, Bowen et *al*. 2000).

Epigenetic alterations, such as *GSTP1* (glutathione S-transferase pi 1) promoter hypermethylation (Maruyama et *al*. 2002) and *APC* (adenomatous polyposis coli) hypermethylation (Kang et *al*. 2004) have been found to be involved in early HGPIN lesions as well as in primary tumors. *GSTP1* is a caretaker gene that is involved in the detoxification and inactivation of carcinogens by conjugating

them with glutathione. Moreover, *GSTP1* promoter hypermethylation has been observed in 70% of PIN and 90% of primary tumors (Meiers et *al*. 2002). Further, the well-known tumor suppressor gene *APC* in colon cancer has been shown to be involved in the regulation of downstream targets such as β-catenin, *MYC*, and Wnt signaling (Markowitz and Bertagnolli 2009). It has been reported that *APC* is hypermethylated in 30% of PIN cases, and around 55-85% of prostate carcinomas (Kang et *al*. 2004). Disruption of *APC* activity leads to altered cellular division, growth and migration of the prostate cells.

1.2.4 Diagnosis

PSA testing

Prostate specific antigen, PSA, is a serine protease which is a member of the human kallikrein family genes. PSA is produced by both normal and malignant prostate epithelial cells, and has been reported to be involved in the regulation of semen liquefaction. PSA can be detected in the blood and PSA testing is currently used for early PC detection and as part of the clinical application for measuring PC progression after prostatectomy, therapeutic response, and staging (Lilja et *al*. 2008). The common threshold level of PSA for the detection of PC is 4 ng/ml, and men who have PSA levels > 4 ng/ml are recommended to undergo prostate biopsy. However, PSA is not a PC-specific marker; elevated serum PSA levels can reflect other disorders affecting the integrity of the prostate architechture, such as BPH and proliferative inflammatory atrophy (PIA) (Lilja et *al*. 2008, Thompson et *al*. 2004). So in order to avoid overdiagnosis, PSA testing must be carefully interpreted and combined with results obtained from digital rectal examination (DRE), and transrectal ultrasound (TRUS)-guided prostate needle biopsy, which is analyzed according to the Gleason grading system and pathological tumor, node, metastasis staging system.

Gleason grading system

One of the most important and widespread indicators of PC prognosis is the Gleason grading system, based on examination of prostate biopsy samples by light microscopy (Epstein 2010). Due to the heterogeneic nature of PC, multiple biopsies of the prostate tumor have to be obtained. The urologist commonly takes 12 core-biopsies of the prostate (the majority from the peripheral zone), and prepares hematoxylin & eosin (H&E) stained slides for histological examination (Heidenreich et *al*. 2011) using a light microscope. The pathologist will examine the architectural pattern and histology of tumor samples and determine the relative degree of tumor differentiation according to the two most prevalent tumor patterns within the biopsy (Humphrey 2004). Thus, PC aggressiveness is determined according to five basic Gleason grade patterns, numbers ranging from 1 to 5: 1 being very well differentiated (looks most like normal prostate tissue and least aggressive), 5 being poorly differentiated (most abnormal and most aggressive prostate tissue glandular structure) (Humphrey 2004) (Figure 1).

The total Gleason score (GS) is determined by adding the two most predominant morphological grade patterns together. For example a GS of 5+2 = 7 means that final GS is 7 (PC is at an intermediate stage), with the most prevalent glandular structure being poorly differentiated, while the second most abundant glandular structure was quite well differentiated. However, if the GS was 2+5 = 7, the most prevalent structure would be well differentiated (GS = 2) meaning that this type of prostate tumor has less aggressive features compared to the previous tumor. Overall, high-grade prostate tumors (GS > 8) are more likely to spread and grow quickly compared to low-grade prostate tumors (GS < 6). (Humphrey 2004).

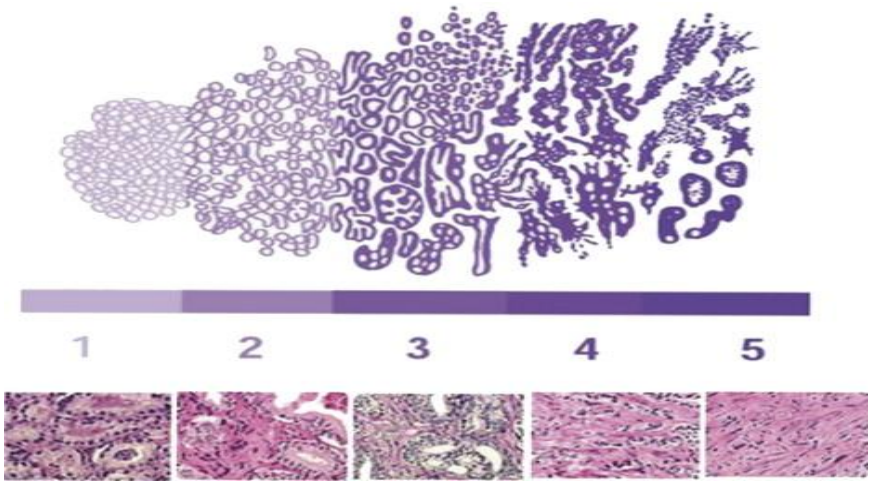

Figure 1. **Gleason grading system**. Prostate cancer aggressiveness is defined according to five Gleason patterns found on a hematoxylin & eosin stained prostate tissue biopsy slide. Final Gleason score (GS) is determined by adding the two most predominant patterns together. Gleason grades vary between 1 (well differentiated, almost normal tissue) and 5 (poorly differentiated, most abnormal and aggressive prostate tissue structure). Higher final GS implicates more aggressive prostate cancer.

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Tumor, Node, Metastasis (TNM) staging systems

There are two different TNM staging systems: clinical and pathological. Clinical staging (cT) is based on the information obtained before prostate surgery (such as PSA levels, DRE findings, imaging etc.). Pathological staging (pT) is based on the information obtained after prostate surgery, i.e. it is based on the histological findings of the prostate tissue sample examination. The PC diagnosis is always determined according to histological examination, while the type of treatment is determined according to PSA levels, T stage and pathological findings. (Heidenreich et *al.* 2011)

TNM-staging describes how much the tumor tissue has spread within the prostate and to the surrounding structures (Figure 2). Stages are as follows: pT I, tumor is not palpable or visible by rectal examination; pT II, tumor is confined within the prostate; pT IIa, tumor involves ≤ 50% of one prostate lobe; pT IIb, tumor involves more than 50 % of the lobe; (pT IIc, tumor involves both lobes, here shown as IIb); pT III, tumor extends through the prostatic capsule into seminal vesicles; pT IV, tumor has spread to lymph nodes and other organs. Tumor lymph node spread and distant metastasis are described as (N) and (M), respectively. (Heidenreich et *al.* 2011)

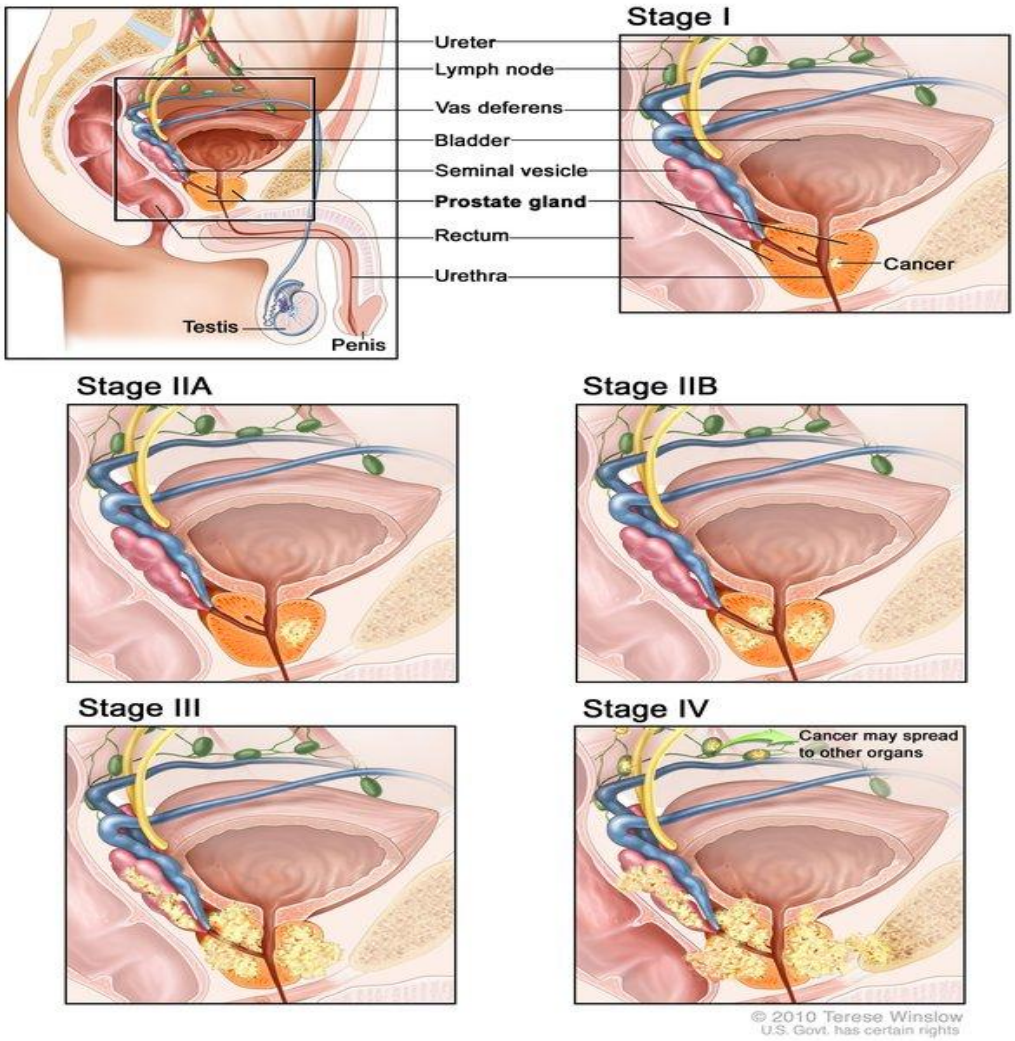

Figure 2. **Tumor, Node, Metastasis (TNM)-staging system**. Stage I, prostate tumor is not visible or palpable; Stage IIA, prostate tumor involves ≤ 50% of the lobe; Stage IIB, prostate tumor involves ≥ 50% of the lobe; IIc (here shown as IIb), prostate tumor involves both lobes, but is confined within prostate; Stage III, prostate tumor has spread into seminal vesicles; Stage IV, prostate tumor has spread to lymph nodes and other nearby tissues.

PC patients are commonly placed to different risk groups according to PSA levels, GS, cT and TNM-staging (Table 1) (Heidenreich et *al.* 2011).

Table 1. Prostate cancer risk groups

Risk Group	PSA[a] (ng/ml)	Gleason Score[b] (GS)	Clinical stage[c] (cT)
Low	< 10	≤ 6	T1a-c, T2a
Intermediate	≥ 10 - ≤ 20	7	T2b
High	> 20	≥ 8	T2c, T3a-c

[a] PSA, prostate specific antigen; [b] is based on the pathological findings on the prostate tumor biopsy; [c] clinical stages; T1a-c, tumor is not palpable or visible by digital rectal examination or imaging; T2, tumor is palpable and confined within prostate; T2a, tumor involves ≤ 50% of one prostate lobe; T2b, tumor involves > 50% of one lobe; T2c, tumor involves both prostate lobes; T3a-c, tumor extends through the prostatic capsule and the growth is no longer limited within the prostate lobes.

1.2.5 The role of the androgen receptor in prostate cancer

Androgen receptor signaling

Androgens such as testosterone and dihydrotestosterone (DHT) are steroid hormones which bind to androgen responsive elements (AREs) on the androgen receptor (AR). They both control and stimulate the development of male sex organs. The production of testosterone is dependent on the regulation of luteinizing hormone (LH) by the anterior pituitary. Secretion of gonadotropin releasing hormone (GnRH) in the hypothalamus is essential for LH regulation by the anterior pituitary; LH inducement by the anterior pituitary leads to testosterone production by Leydig cells in the testicles. Indeed, the prostate needs androgens for its development and normal physiological function. ARs are responsible for the specification of the lineage-specific differentiation of prostate cells through the activation of prostate-specific gene expression. Moreover, in the normal prostate AR maintains the differentiated phenotype of the prostate epithelial cells (Heinlein & Chang 2004).

AR, also called *NR3C4*, is a member of the nuclear DNA-binding transcription factors that regulate the action of androgens. The AR protein has four main domains, including the regulatory N-terminal domain, a hinge region that connects the DNA binding domain (DBD) with the ligand binding domain (LBD), DBD and LBD (Heinlain & Chang 2004). In association with heat shock proteins (HSPs), chaperones, and cytoskeletal proteins the tertiary structure of AR is stabilized and AR is held inactive in the cytoplasm. Further, when testosterone enters the cell it is converted to DHT in the presence of a resident enzyme: 5α-reductase. After DHT conversion, inhibitory HSPs are released and the AR undergoes a conformational change. The AR is then phosphorylated and further translocated to the nucleus where it binds to AREs in the promoter regions and to DNA binding sites of *AR* target genes. Furthermore, transcriptional initiation of target genes is facilitated in the presence of coregulators including histone acetylases and chromatin remodeling complexes, which are recruited after nuclear localization of AR (Heinlein & Chang 2004, Hodgson et *al*. 2011).

Androgen receptor signaling in prostate cancer

PC tumorigenesis is highly dependent on the stimulation of AR signaling by androgens during all stages of the disease progression. Thus, AR signaling is one of the key factors in the promotion of PC cell survival and proliferation (Chen et *al*. 2004, Linja and Visakorpi 2004). There are different hypotheses concerning AR functioning during PC development, including AR reprogramming by other transcription factors such as FOXA1 (forkhead box A) and ETS transcription factors. Both of these transcription factors have specific binding sites located on AR binding sites. It has been reported that FOXA1 can both suppress and induce AR signaling (Sahu et *al*. 2011). According to Sahu et *al*. (2011), altered expression of the FOXA1 transcription factor may induce AR target gene expression (hormonal signaling and cell proliferation), thus mediating AR signaling in PC. Moreover, Sahu et *al*. (2011) observed that in the presence of FOXA1 a large percentage of AR binding sites were masked, suggesting that FOXA1 is involved in the regulation of AR-mediated signaling. Additionally, ETS can regulate AR binding to DNA. According to Massie et *al*. (2007) and Yu et *al*. (2010), AR recruitment to AR DNA binding sites also enhanced *ERG* and ETS binding to AR binding sites. Overall, it can be suggested that overexpression of ETS and FOXA1 transcription modulators may lead to the AR reprogramming and increased expression of oncogenes, modulating the normal differentiation of the prostate epithelia (Sahu et *al*. 2011 Yu et *al* 2010).

The mechanism of *AR* activation is usually changed during PC tumorigenesis, especially in castration resistant PC (CRPC). Androgen ablation (anti-androgen) therapy and total androgen blockade (surgical or chemical castration combined with anti-androgen treatment) are widely used treatments for CRPC patients. Due to the withdrawal of androgens, prostate cells have to adapt to very low levels of androgens, which they do via different mechanisms. In advanced PCs, mainly in CRPC cases, AR signaling activation occurs by several different mechanisms, including *AR* amplification, which has been shown to occur in around one-third of CRPC patients (Visakorpi et *al*. 1995a). Moreover, AR activation has been shown to occur by other steroid hormones or anti-androgens such as progesterone and estrogens due to mutations in the *AR* gene (Taplin 2007). In addition, intratumoral steroidogenesis (e.g. DHT is produced from cholesterol or dehydroepiandrosterone (DHEA) has been reported to activate AR (Cai and Balk 2011). AR has also been shown to be activated through alternative splicing of the *AR* gene (Sun et *al*. 2010). Overall, altered expression levels of *AR* co-activators and co-repressors can lead to AR activation (Gregory et *al*. 2001).

AR activity increases in correlation with an increase in the number of gain-of-function mutations. However, *AR* mutations are rare in untreated tumors and it has been observed that 10-30% of anti-androgen treated patients have *AR* mutations (Linja and Visakorpi 2004). Moreover, an increasing amount of evidence suggests that truncated isoforms of *AR* splice variants (which lack LBD) lead to ligand-independent activation of AR which further leads to formation of constitutively active AR in CRPC patients. In addition, due to the altered structure of *AR* splice variants there is a high possibility that the interaction with coregulators and binding to AREs may be altered (Sun et *al*. 2010). *AR* coregulators such as NCOA1, 2, and 3 regulate histone methylation as well as acetylation leading to transcriptional initiation and *AR* activation (Heinlein and Chang 2004). *AR* corepressors including NCOR1 and NCOR2 promote chromatin condensation, resulting in inhibition of the transcriptional activation of *AR* (Taylor et *al*. 2010). Thus, loss of *AR* corepressors and gain of *AR* coregulators may stimulate the development of CRPC tumor progression in low-hormone microenvironments.

1.2.6 Localized prostate cancer

PC growing within the prostate gland which has not extended through the prostate capsule is known as localized PC. The clinical stages that best define localized PC are T1-T2 (Table 1). Thus, the patients that belong to low or intermediate risk groups most commonly have localized PC. Nowadays, an increasing number of localized, slow growing, well differentiated PCs are diagnosed due to a widespread increase in the use of PSA testing (Klotz 2010). The clinical challenge is now to avoid overtreatment among the patients who have been diagnosed with localized, slow growing PC. Often the most suitable treatment for patients diagnosed with localized slow growing PC is active surveillance (close monitoring of PSA levels and prostate tissue morphology). Furthermore, the risk of prostate tumor progression is low (approximately 15-20 years) among nearly 50% of the patients who have been diagnosed with localized slow growing PC (Heidenreich et *al*. 2011, Klotz 2010).

1.2.7 Metastatic prostate cancer

PC can either metastasize locally or to distant organs. PC is referred to as "locally advanced" when the prostate tumor has broken the prostate capsule (clinical stage T3a) and spread just outside of the prostate gland, to the seminal vesicles for example (clinical stage T3b). Moreover, after invading the seminal vesicles, the prostate tumor may spread into nearby structures including bladder, rectum, muscles, and pelvic cavity. Clinical stages T3 and T4 commonly refer to locally advanced PC. Contrastingly, in advanced stage of PC (clinical stage T4) distant metastases are found most commonly in the bones, liver, or lungs. According to Bubendorf *et al*. (2000), over 90% of advanced PC tumors metastasize to bone, around 40-50% metastasize to the lungs, and 25-30% metastasize to the liver. PC bone metastases can be also called PC osteoblasts due to their ability to form bone tissue (Ibrahim et *al*. 2010). Still, the molecular mechanisms of the crosstalk between metastasized PC cells and the cells involved in the bone formation (osteoblasts, osteoclasts, and immune cells) remain to be investigated.

1.2.8 Castration-resistant prostate cancer

The growth of PC is a highly androgen dependent process. Indeed, the key treatment for metastatic PC is androgen deprivation therapy by way of chemical or physical castration. It has been shown that approximately 80-90% of metastatic PC patients have favorable initial biochemical responses after castration therapy, including decreased levels of PSA and serum markers in the blood. However, cancer cells are able to adapt to low levels of androgens by utilizing alternative signal transduction mechanisms including steroidogenesis, AR reactivation (*AR* amplification, LBD mutations in *AR*), as well as bypass pathways (overexpression of anti-apoptotic proteins such as *BCL-2*) (Attard et *al*. 2006). Moreover, castration-resistant PC (CRPC) commonly develops within 18-24 months of initial castration therapy (Damber and Aus 2008) and, according to Halabi et *al*. (2009), survival after CRPC progression is only around 20 months. Overall, castration therapy is not curative and one of the major clinical challenges is to develop better therapies against CRPC which are curative.

1.2.9 Traditional treatments

Active surveillance

Active surveillance has been shown to be a safe and promising treatment option for low risk PC patients who harbor small, slow-growing, well differentiated prostate tumors with good prognosis (Table 1), as well as for older patients with limited life expectancy (Klotz 2010) whose cause of death is likely to be unrelated to their PC. During active surveillance PC progression is followed by periodic PSA assessments (every 3-6 months). Prostate tissue biopsies are obtained and analyzed every 3-5 years (Heidenreich et *al*. 2011, Klotz 2010). Indeed, the purpose of active surveillance is to avoid overtreatment among low risk patients as long as possible, until the PC shows signs of progression; defined by a PSA level increase to over 10 ng/ml and GI progression to ≥ 7.

Radical prostatectomy

Radical prostatectomy (RP) is the surgical excision of all or part of the prostate gland and seminal vesicles. After prostatectomy, PSA should be totally absent from the blood, therefore PSA levels are regularly followed after RP treatment (PC is deemed to have recurred if PSA levels increase above 0.5 ng/ml after prostatectomy). RP is commonly used as a curative treatment for low or intermediate risk (Table 1) PC patients diagnosed with clinically localized organ-confined cancer (not broken through the prostatic capsule) with more than a 10 year life expectancy. In contrast, potential benefits decrease after radical prostatectomy among high-risk PC patients who have locally advanced or advanced prostate cancer (tumor has already spread through the prostatic capsule) (Heidenreich et *al*. 2011, Kumar et *al*. 2006). It has been shown that the effects (PSA level decrement) are good after RP based upon long-term cancer follow-up and high cancer-specific survival rates. Progression-free survival has been shown to be improved significantly among high-risk PC patients who have been treated with adjuvant radiation therapy immediately after RP. It has not yet been concluded if pre- or postoperative neoadjuvant endocrine therapy would significantly improve progression free survival after RP (Heidenreich et *al*. 2011, Kumar et *al*. 2006).

Radiation therapy

Radiation therapy is used as a treatment for localized PC as well as advanced PC. Radiation therapy can be either external (external beam radiation therapy (XRT)) or internal (brachytherapy). During XRT, a curative dose of targeted radiation is delivered into the prostate in order to kill the cancer cells without causing harm to the surrounding tissues. In low-dose-rate brachytherapy radioactive seeds are implanted permanently to prostatic tissue under transrectal ultrasound guidance, whereas in high-dose-rate brachytherapy the implantation of seeds is temporary and it is usually combined with XRT. (Heidenreich et *al*. 2011, Haas and Sakr 1997, Peeters et *al*. 2006).

Endocrine therapy (GnRH, antiandrogens, MAB)

PC patients who are treated with endocrine therapy most commonly have either locally advanced or metastasized disease. Already in 1941, Huggins and Hodges observed that PC progression is strongly related to testosterone action, while at the same time making the first observations about the clinical and therapeutic effects of surgical castration and endocrine therapy for advanced PC (Huggins and Hodges, 1941). Nowadays, endocrine therapy is a common treatment for advanced and metastatic PC, or for PC that has recurred after primary treatment (prostatectomy or radiation therapy) (Mottet et *al*. 2011). Since testosterone is strongly associated with PC tumorigenesis, the main purpose of endocrine therapy is to silence and eliminate its mechanism of action. Today, several different

endocrine therapies for advanced or recurred PC have been used, such as GnRH agonist therapy (inhibits testosterone synthesis from testes due to reduced amount of luteinizing hormone (LH) after inhibition of GnRH receptor function), antiandrogen therapy (inhibits AR activation by preventing the binding of testosterone and DHT to AR), and maximum androgen blockage (MAB) (combined inhibition of testosterone synthesis from testes and AR activation by testosterone and DHT using both GnRH and anti-androgens). Overall, endocrine therapy is initially highly effective; however endocrine therapies are never curative due to the resistance development to AR-signaling targeting agents (Fitzpatrick et *al*. 2008).

1.3 *TMPRSS2:ETS* TRANSLOCATIONS

1.3.1 The discovery of *TMPRSS2:ETS* translocations in prostate cancer

As mentioned earlier, gene expression alterations, e.g. loss of function mutations in TSGs and gain-of-function mutations in oncogenes are the most common hallmarks for malignant transformation of the normal cellular tissue. Most often, such mutations result from chromosomal translocations or deletions leading to fusion formation of two different gene transcripts and further altered and modified gene expression. The most common recurrent disease specific chromosomal rearrangements and gene fusions have been mainly found to be associated with hematologic malignancies (lymphomas and leukemias) and sarcomas.

Cancer outlier profile analysis (COPA)

Over seven years ago, in 2005, Tomlins and colleagues reported an interesting discovery of gene fusions in PC that is one of the most prevalent epithelial malignancies in the world. By analyzing DNA microarray data with a specific unconventional bioinformatics approach called the cancer outlier profile analysis (COPA) algorithm (described in Tomlins et *al*. 2005), they discovered recurrent *TMPRSS2:ETS* (E-twenty six) translocations in PC. They performed COPA analysis in Oncomine 3.0 with 132 different gene expression data sets (10,486 microarray experiments). Instead of using cancer samples and analyzing them with common analytical methods to search for activation of genes (e.g. t test) they used COPA for the search of genes that have marked overexpression profiles (outlier profiles) in specific cancer types. Furthermore, Tomlins and co-workers found that *ERG* (21q22.3) and *ETV1* (7p21.2), which encode ETS oncogenic transcription factors, had the strongest outlier profiles in several data sets observed in PC and the respective genes were among the top 10 outlier genes in six different PC profiling studies.

Moreover, Tomlins et *al*. (2005) found that *ETV1* and *ERG* were overexpressed in 40%-70% of PC cases. RNA was obtained from PC samples and analyzed in exon-walking quantitative RT-PCR. They observed that 3´ ends of *ETV1* and *ERG* were constantly overexpressed while 5´ regions of respective genes were missing. Furthermore, by utilizing different molecular analyses Tomlins et *al*. discovered that the 5´ regions of *ETV1* and *ERG* were consistently replaced with 5´ UTR of transmembrane protease serine 2, *TMPRSS2* (a prostate-specific androgen regulated gene). The prevalence of recurrent gene fusion at the chromosomal level was investigated by high-throughput fluorescent *in situ* hybridization (FISH) assays, and was confirmed to occur at the same frequencies. Thus, it was shown that the increased expression of *ETV1* and *ERG* genes found in PC occur due to the structural rearrangements of transmembrane serine 2, 5´ *TMPRSS2* to 3´ *ETV1* or 3´ *ERG* (Tomlins et *al*. 2005).

Other novel 5´ and 3´ fusion gene partners

Since the first discovery of *TMPRSS2:ERG* and *ETV1* gene fusions by Tomlins et *al*. in 2005, various different novel 5´ and 3´ gene partners have been found to be associated in PC. *TMPRSS2:ERG* and *TMPRSS2:ETV1* are the most common gene fusions and translocations associated in PC, and have been found to occur in approximately 40-70% and 2-30% of PC cases, respectively. Furthermore, fusions of 5´ *TMPRSS2* to 3´ *ETV4* and 3´ *ETV5* have been found in 2-5% and < 1% of PC cases, respectively (Shah and Chinnaiyan 2009). Helgeson et *al*. (2008) reported that other fusions such as *HERV-K* (11q22): *ETV1* (7p21), *SLC45A3* (1q32): *ETV1* (7p21), *SLC45A3* (1q32): *ETV5* (3q28), *Cl5ORF21* (1q25): *ETV1* (7p21) and *HNRPA2B1* (7p15): *ETV1* (7p21) are involved in the molecular network of PC at significantly lower levels (<1%). Moreover, in the same year Hermans and colleagues observed that additional *KLK2* (19q13): *ETV4* (17q21) and *CANT1* (17q25): *ETV4* gene fusions occurred at similar frequencies (<1%) in PC (Hermans et *al*. 2008). However, the molecular function of respective fusions remains to be investigated.

1.3.2 *TMPRSS2* (*Transmembrane protease, serine 2*)

Transmembrane protease serine 2 is a type II transmembrane protein encoded by the *TMPRSS2* gene and located on 21q22.2 in humans. TMPRSS2 protein contains five types of domains, a protease domain, a scavenger receptor cysteine-rich domain, a receptor class A lipoprotein domain, type II transmembrane domain, and a cytoplasmic domain (Paoloni-Giacobino et *al*. 1997). *TMPRSS2* belongs to a serine protease gene family, the members of which have crucial roles in different physiological and pathological processes such as in digestion, blood coagulation, remodeling of tissues, invasion of tumor cells, inflammatory responses, and apoptosis. Furthermore, *TMPRSS2* has been shown to be expressed at low levels in liver, kidney, pancreas, lung, and colon (Vaarala et *al*. 2001). *TMPRSS2* is highly expressed in both normal prostate and PC cells and contains androgen responsive elements (AREs) in its promoter region. It has been reported (Afar et *al*. 2001) that the expression of *TMPRSS2* is increased by androgens in PC cells and decreased in androgen independent PC (Afar et *al*. 2001, Lin et *al*. 1999).

1.3.3 *ERG* (*v-ets erythroblastosis virus E26 oncogene homolog (avian)*)

ERG (*v-ets erythroblastosis virus E26 oncogene homolog (avian)*) is an oncogene and is located on 22q22.3. *ERG* belongs to the family of *ETS* (E-twenty six) coding transcription factors. *ETS* family encodes for transcription factors regulating the expression of genes that are involved in cellular proliferation and differentiation, inflammation, embryonic development, angiogenesis (platelet adhesion, stem cell maintenance and hematopoiesis) and apoptosis (Seth and Watson 2005, Wang et *al*. 2011). ERG contains conserved ETS DNA-binding domain and protein-protein interaction domain (PNT). Moreover, ERG has been shown to regulate the expression of genes involved in PC progression pathways such as DNA damage, cell invasion and proliferation, epithelial to mesenchymal transformation (EMT) as well as cellular differentiation and epigenetic control (Sreenath et *al*. 2011). In addition, *ERG* gene has been shown to be associated with chromosomal translocations leading to the formation of fusion gene product with different genes. The most common *ERG* related gene fusions have been observed in PC (*TMPRSS2:ERG*), Ewing's sarcoma (*EWS:ERG*) and acute myeloid leukemia (*FUS:ERG*).

1.3.4 *TMPRSS2:ERG* fusion

The frequency of *TMPRSS2:ERG* fusions

As mentioned earlier, *TMPRSS2:ERG* is the most dominant fusion and according to different FISH, array comparative genomic hybridization (aCGH) and RT-PCR assays it is detected in approximately 40-70% of clinical PCs (Tomlins et *al*. 2005, Perner et *al*. 2006, Mosquera et *al*. 2008). In addition, *TMPRSS2:ERG* fusions have been found in approximately 10-20% of high HGPIN precursor lesions and in 29%-69% of advanced or hormone refractory PCs, but not in benign epithelial cells or stromal cells. This suggests that *TMPRSS2:ERG* fusion is an early event in PC progression.

TMPRSS2:ERG fusion is formed either by chromosomal translocation (found commonly in early stages of PC) or interstitial deletion (found commonly in aggressive forms of PC) on a single copy of chromosome 21 (Perner et *al*. 2006, Mosquera et *al*. 2008). Both of the *TMPRSS2* and *ERG* genes are located on chromosome 21q and the distance between AR regulated *TMPRSS2* and *ERG* oncogene is approximately 3 mega bases (MB). The relatively short distance between these genes on the same chromosome could explain the higher frequency of *TMPRSS2:ERG* fusions in PC compared to other observed fusions of different 5´ and 3´ *ETS* partners.

TMPRSS2:ERG transcript variants

Different PC-related *TMPRSS2:ERG* fusion transcripts have been characterized by Rapid Amplification of cDNA ends (RACE) assays and RT-PCR assays (Tu et *al*. 2007). Most commonly, a transcript variant of *TMPRSS2* exon 1 or 2 is fused to *ERG* exon 2, 3, 4 or 5. The fusion of *TMPRSS2* exon 4 or 5 to *ERG* exon 4 or 5 is less frequent. Overall, nineteen different alternatively spliced transcript variants of *TMPRSS2:ERG* have been found. The exons of 5´ *TMPRSS2* involved in the formation of the fusion transcript are usually non-coding and are rarely translated into protein products. Furthermore, an N-terminal truncated form of the ERG protein is the protein which is commonly translated by all of the fusion transcripts (Tu et *al*. 2007). However, there are also fusion protein transcript variants such as *TMPRSS2* exon 2:*ERG* exon 4 expressing true fusion protein product (both the 5´ *TMPRSS2* exons and 3´ *ERG* exons are translated). It may be possible that most of the protein products have similar functions as wild-type ERG. However, the biological association of N-terminal truncated ERG proteins and true fusion products encoded by *TMPRSS2:ERG* transcript variants in PC progression remains to be further determined (Tu et *al*. 2007).

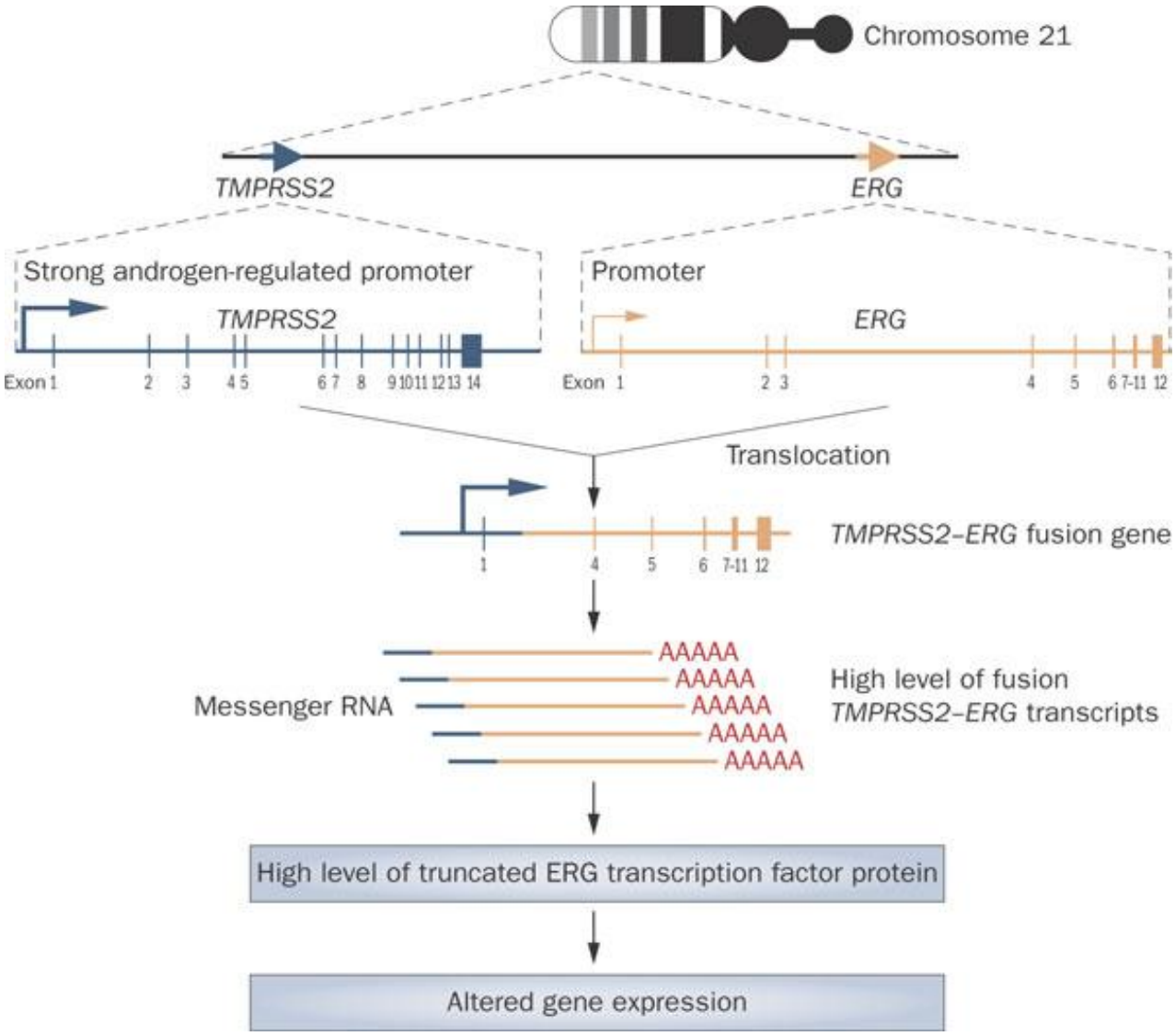

Figure 3. **The formation of *TMPRSS2:ERG* gene fusion by translocation**. The most common *TMPRSS2:ERG* gene fusion found in prostate cancer is the deletion between 5′ UTR end of *TMPRSS2* exon1 and 5′ end of *ERG* exon 4. The prostate specific androgen regulated *transmembrane protease serine 2* (*TMPRSS2*) is located on 21q22.2. *V-ets erythroblastosis virus E26 oncogene homolog (avian)* (*ERG*) is an oncogene and is located on 21q22.3. After translocation the level of *TMPRSS2:ERG* mRNA transcripts increases which leads to the translation of N-terminal truncated ERG oncogenic transcription factor proteins and altered gene expression.

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1.3.5 The formation of *TMPRSS2:ERG* fusion

The genomic events leading to the formation of gene fusions are of great interest to researchers in the hopes of find new possible molecular drug targets for the treatment of cancer. The molecular mechanisms leading to the formation of *TMPRSS2:ERG* gene fusions are under investigation. Several studies have been done and different trigger points that may be associated in the fusion event have been found. Liu et *al*. (2006) found consensus sequences homologous to the human restriction endonucleases (Alu-Sq and Alu-Sp subfamily) after fine-mapping the intronic deletion breakpoint regions of *ERG* and *TMPRSS2*. Moreover, Liu and colleagues concluded that there is a correlation between the presence of *TMPRSS2:ERG* gene fusions and the respective restriction endonuclease sequences, which may be one of the possible factors leading to fusion formation by deletion at 21q22.2 - 21q.22.3. In 2009 Luedeke and colleagues performed genome-wide linkage studies of familial PC and found a significant correlation between the polymorphism in DNA repair genes and the presence of the *TMPRSS2:ERG* fusion gene in the familial form of PC. Moreover, they discovered that the defects in the variants of *POLI* that protects chromosomal stability, and *ESCO1* which is needed for sister chromatid pairing (by preventing chromosomal damage during S-phase) may play a role in *TMPRSS2:ERG* fusion gene translocation event in familial PC (Luedeke et *al*. 2009).

Furthermore, Mani et *al*. (2009) studied the fusion formation using the fusion-negative LNCaP cell line. By irradiating LNCaP cells, Mani and colleagues introduced double strand breaks into the cells before further stimulating them with DHT. According to the results of FISH assays, Mani et *al*. (2009) observed that after irradiation and stimulation of LNCaP cells with DHT, the *TMPRSS2:ERG* fusion transcripts could be detected in the previously fusion-negative LNCaP cells. Haffner et *al*. (2009) reported that androgen signaling stimulates the recruitment of *AR* and topoisomerase II beta (*TOP2B*) (involved in the breaking and rejoining of two strands of duplex DNA) to the androgen responsive genes, and *TMPRSS2* and *ERG* regions. Moreover, they discovered that the recruitment of *AR* and *TOP2B* leads to the formation of DNA double-strand breakpoints and *TMPRSS2:ERG* fusion formation (Haffner et *al*. (2009).

In addition, chromosomal alterations and rearrangements occurring at an elevated rate (called chromosomal instability) could also aid in explaining the formation of the *TMPRSS2:ERG* fusion gene in PC. A well known TSG called phosphatase and tensin homologue, *PTEN*, is commonly deleted in PC. This genomic aberration leads to increased chromosomal instability through activation of the cell cycle stimulating protein kinase, AKT. The deletion of *PTEN* further leads to phosphorylation and inactivation of Chk1, which is known to prevent cell cycle progression after DNA damage. Thus, it may be argued that *PTEN* deficiency could delay the DNA damage repair process, and thus lead to the formation of *TMPRSS2:ERG* fusions.

1.3.6 Prostate cancer and *TMPRSS2:ERG* fusion

Several studies using *in vivo* models have reported that the overexpression of *ERG* due to *TMPRSS2:ERG* fusion formation leads to prostate cell migration and invasion (Klezovitch et *al*. 2008, Tomlins et *al*. 2008). In the study by Tomlins et *al*. (2008), transgenic mice were generated to overexpress a truncated version of *ERG* (*ERG* expression was activated by an androgen-inducible probasin promoter) to further study the effects of ERG. In total, 3 of 8 respective transgenic mice developed PIN by 12-14 weeks of age. This study was supported by Klezovitch et *al*. (2008), who showed that high levels of *ERG* (under the control of probasin promoter) lead to the formation of murine PIN (mPIN) by five to six months of age. Furthermore, they observed that transgenic mice

overexpressing *ERG* at lower levels (but still at significantly higher levels than normal *ERG* expression) developed mPIN by 10-12 months of age. Furthermore, the basal cell layer of the prostate gland was observed to be disrupted in the *ERG*-overexpressing mice, which could be a potential early sign of PC development. However, none of the studies reported malignant tumors of the prostate, indicating that *TMPRSS2:ERG* fusion alone may only induce the transformation of the prostate epithelium.

Different *in vivo* studies have shown that there is a co-operation between phosphatase and tensin homologue (*Pten*) inactivation and *TMPRSS2:ERG* expression during the PC tumorigenesis. It has been shown that transgenic mouse expressing only *TMPRSS2:ERG* fusion did not develop HGPIN lesions or prostate tumors. However, it has been reported that when the respective fusion transgenic mouse was crossed with either a *Pten*-deficient mouse or a mouse that had high Akt (serine/threonine specific protein kinase) activity, the offspring developed HGPIN preneoplasia but PC progression was not observed (King et *al*. 2009). Similar results with haploinsufficient *Pten* or *Pten* knockdown mice overexpressing *ERG* under the control of an androgen-inducible probasin promoter have been observed (Carver et *al*. 2009). However, Carver et *al*. (2009) observed PC progression in the offspring. In addition, they demonstrated that *Pten* haploinsufficiency or increased activity of Akt leads to increased cell proliferation in PC cell lines, whereas cell migration is increased due to *ERG* expression (Carver et *al*. 2009). Thus, it can be assumed that development of HGPIN in prostate cells is stimulated by the co-operation between *PTEN* loss, constitutively activated AKT, and *TMPRSS2:ERG* fusion (Figure 4). In addition to *in vivo* animal models, several PC cell culture models have been designed to study the biological role of *ERG* overexpression *in vitro*. The invasiveness of VCaP cells, which are known to express *AR* and are *TMPRSS2:ERG* fusion positive, was significantly inhibited after knockdown of *ERG* by small interfering RNA (siRNA) (Tomlins et *al*. 2008). Moreover, Tomlins et *al*. (2008) reported that cellular invasiveness of PC cells was inhibited after blockage of urokinase plasminogen activator (uPa) and plasminogen activator pathways which are known to be associated with *ERG* overexpression.

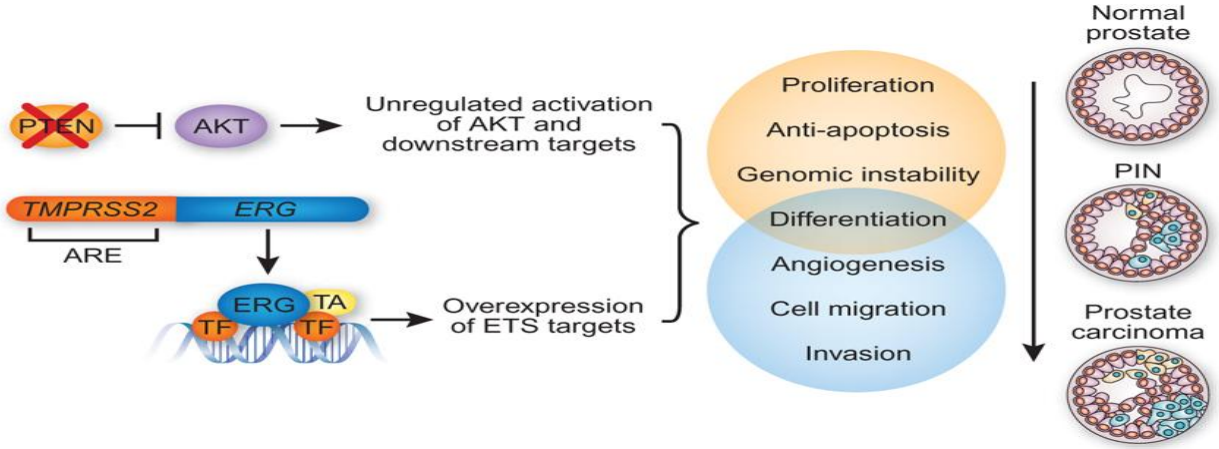

Figure 4. **The role of PTEN loss, TMPRSS2:ERG fusion formation and concominant activation of AKT in PC progression**. Loss of tumor suppressor gene *PTEN* may act as an initiating lesion in PC progression leading to constant activation of AKT and increased cell survival. Furthermore, *TMPRSS2:ERG* may act as a driver lesion and stimulate cellular transformation from PIN to prostate carcinoma in situ (HGPIN) due to increased overexpression of *ERG* oncogenes. *PTEN*, phosphatase and tensin homologue; *AKT*, serine/threonine specific protein kinase; *TMPRSS2*, transmembrane protease serine 2; *ERG*, v-ets erythroblastosis virus E26 oncogene homolog (avian); ARE, androgen responsive elements; TA, transcriptional activator; TF, transcription factor; *ETS*, E-twenty six family of transcription factors; PIN, prostatic intraepithelial neoplasia.

1.4 OTHER GENES INVOLVED IN PROSTATE CANCER PROGRESSION

1.4.1 *MYC*

MYC (*c-MYC*) is an oncogene located at the 8q24 chromosomal region. *MYC* is one of the commonly amplified loci in PC (Jenkins et *al*. 1997, Visakorpi et *al*. 1995b); it encodes a transcription factor which is crucial for the regulation of cell proliferation, cell growth, apoptosis, and stem cell renewal (Albihn et *al*. 2010). *MYC* has been shown to be overexpressed in different types of tumors such as in lymphomas and leukemias, hematopoietic tumors, breast, prostate, and colon cancers, as well as in hepatocellular carcinoma and neuroblastoma (Toyoshima et *al*. 2012).

The MYC protein has been reported to be overexpressed in different stages of PC tumorigenesis including HGPIN, localized primary tumors, and in advanced metastatic tumors (Jenkins et *al*. 1997). Moreover, MYC has been reported to have the ability to downregulate *NKX3.1* TSG expression and increase the expression of *EZH2* transcriptional repressor gene, suggesting its role in different stages of PC progression (Iwata et *al*. 2010, Koh et *al*. 2011). It has been shown that up to 70% of CRPC patients have the gain of 8q and one third of these patients have *MYC* amplification (Nupponen et *al*. 1998a, Saramäki et *al*. 2001). However, amplification has not been confirmed to be the primary mechanism for elevated *MYC* expression levels (Gurel et *al*. 2008). The gain of 8q24 is rarely observed in localized PC but *MYC* is commonly overexpressed in such cases. The reasons for *MYC* overexpression remain unknown but it has been suggested that inactivation of *APC* and the downregulation of *FOXP3* could be associated with *MYC* upregulation (Kang et *al*. 2004, Wang et *al*. 2009a).

1.4.2 *NKX3.1*

One of the most frequently observed deletions in PC is the loss of the *NKX3.1* locus at 8p21 region. *NKX3.1* is a TSG that encodes homeobox-containing transcription factor (Voeller et *al*. 1997). Moreover, *NKX3.1* inhibits the proliferation and differentiation of epithelial cells in the prostate and it can be expected that cell proliferation and cell differentiation increase in the prostatic epithelia after the deletion of the *NKX3.1* locus. Further, according to Asatiani et *al*. (2005) *NKX3.1* might be epigenetically regulated through promoter methylation. However, mutations are rare in the 8p21 region and no additional mutations have been observed in the remaining *NKX3.1* allele (Ornstein et *al*. 2001, Voeller et *al*. 1997). In addition, Bowen et *al*. (2000) found that the expression of *NKX3.1* is lost in advanced tumor stage as well as in hormone refractory disease. Furthermore, Lei et *al*. (2006) observed that due to the deletion of *PTEN*, the expression level of *NKX3.1* is decreased leading to increased activity of AR and further to tumor progression.

Taken together, the facts that LOH has been observed in up to 85% of prostate adenocarcinomas and in 60% of PIN at the 8p21 chromosomal region, and no additional mutations have been found at the aforementioned region, it may be assumed that *NKX3.1* is a haploinsufficient TSG. This assumption can be supported by the additional studies of Abdulkadir et *al*. (2002) and Bhatia-Gaur et *al*. (1999), in which the researchers observed that both heterozygous and homozygous knockout mice develop hyperplasias and PIN lesions in their prostates.

1.4.3 PI3K pathway and *PTEN*

PTEN is a TSG located on the chromosome 10; it is involved in the regulation of cell signaling pathways by targeting the proteins that regulate cell growth and survival. According to Zhang and Yu (2010), *PTEN* is among the most commonly inactivated TSGs in many human cancers. Kwabi-Addo et *al*. (2001) and Trotman et *al*. (2003) showed that functional loss of just one *PTEN* allele in mice confers to advantage for PC growth.

Moreover, *PTEN* mutations have been observed in around 40% of localized PC cases (Bismar et *al*. 2011, Taylor et *al*. 2010), whereas in advanced PCs the incidence of *PTEN* deletions is much higher being around 70-80% (Liu et *al*. 2009, Taylor et *al*. 2010). Recently, Krohn et *al*. (2012) analyzed several hormone-naïve and hormone-refractory tissue microarray (TMA) samples by FISH and observed that *PTEN* deletions are significantly associated with high Gleason grade, advanced tumor stage (pT), presence of *ERG* gene fusion, lymph node metastasis, CRPC disease, and early PSA recurrence.

PI3K is a phosphoinositide 3-kinase that regulates the conversion of phosphatidylinositol (4,5) bis-phosphate ($PI(4,5)P_2$) to $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ is involved in the activation of serine/threonine specific protein kinase (AKT) whose activity facilitates cell cycle progression, protein synthesis, cell growth and cell survival (Cantley 2002, Sulis and Parsons 2003). The activity of PI3K is negatively regulated by PTEN lipid phosphatase. Thus, inactivation of *PTEN* in somatic cells during PC by LOH or complete loss of *PTEN* increases the levels of $PI(3,4,5)P_3$, leading to enhanced PC cell growth as well as enhanced PC cell survival (Uzoh et *al*. 2009).

Moreover, according to Carver et *al*. (2011) and Mulholland et *al*. (2011) there is a linkage between Akt, PI3K and AR signaling. Indeed, they observed that AR inhibition leads to the activation of Akt signaling and in the similar manner PI3K inhibition activates AR signaling. Thus, the inhibition of one of the signaling pathways activates the other pathway leading to tumor cell survival. Further, Carver and colleagues found cancer regression in $Pten^{-/-}$ PC mice after inactivation of PI3K/Akt and AR signaling pathways.

1.4.4 *EZH2*

EZH2 is a transcriptional repressor gene that belongs to polycomb group family. *EZH2* is located at 7q36 chromosomal region and it has been shown to regulate cell proliferation *in vivo* – especially in metastatic prostate carcinomas (Varambally et *al*. 2002). According to Yu at *al*. (2007), gain-of-function mutation of *EZH2* could be one of the reasons for epigenetic silencing of TSGs during PC development leading to poor prognosis of the patients. In a recent study, Yu et *al*. (2010) observed that *TMPRSS2:ERG* fusion is associated with the regulation of EZH2 signaling. They found that the epigenetic silencing mediated by *EZH2* is increased due to increased *EZH2* gene expression and decreased *EZH2* target gene expression by *ERG*.

1.4.5 *TP53*

One of the most commonly inactivated genes in human cancers is a tumor suppressor *TP53*. *TP53* encodes tumor protein p53: a homotetrameric transcription factor. Tumor protein p53 is a crucial regulator of the cell cycle; involved controlling the transition from G1 phase to S phase. Further, *TP53* is activated under conditions conducive to DNA damage, such as during hypoxia, irradiation, and oncogene activation or during genotoxic damage leading to cell cycle arrest (DNA repair) or induction of apoptosis (Prives 1998, Morris 2002). Mutated p53 protein can be detected by

immunohistochemistry due to nuclear accumulation caused by prolonged half-life. According to Visakorpi et *al*. (1992), Bookstein et *al*. (1993) and Navone et *al*. (1993), early localized PC cases with *TP53* gene mutations are rare. In contrast, mutations in *TP53* have been observed in about 20-40% of hormone-refractory or metastatic prostate carcinoma cases. In addition, nuclear accumulation of mutated *TP53* has been reported to be associated with poor prognosis (Visakorpi et *al*. 1992).

1.5 CIRCULATING TUMOR CELLS (CTCs) IN PROSTATE CANCER

The molecular nature of PC metastasis remains to be elucidated. The aberrant migration of PC cells to the blood and to distant organs in the body is accelerated due to the induction of epithelial to mesenchymal transition (EMT), increasing the development of invasive epithelial cells. During EMT, prostate tumor cells acquire mesenchymal cell morphology and decrease their epithelial characteristics, which lead to phenotypic variability of tumor cells. Overall, decreased expression of integrins (key regulators of the signal transduction exchange between extracellular matrix (ECM) and cells), repression of E-cadherin (a cell adhesion molecule) expression, as well as elevated expression of N-cadherin and increased degradation of the surrounding stroma by proteases are involved in the PC metastatic progression and CTC invasion. (Gravdal et *al*. 2007, Jin et *al*. 2011).

Most often, circulating prostate tumor cells metastasize into bone marrow (BM). Moreover, when CTCs extravasate into BM and form micrometastases in bones they are called as disseminated tumor cells (DTCs). However, not all disseminated tumor cells from primitive tumor site are capable of forming metastases, suggesting that metastasis can be a relatively inefficient process (Chambers et *al*. 2002). Moreover, it has been reported that the immune system, e.g. natural killer cells, can detect tumor cell antigens and destroy tumor cells in the circulation and in the bone marrow (Zamai et *al*. 2007). Several different techniques have been developed for the capture, isolation and characterization of CTCs according to their phenotypic properties.

1.5.1 The definition of CTC

CTCs can be found either in peripheral blood (PB) or bone marrow (BM). They are relatively rare events, occurring at levels as low as one CTC per 10^6 or 10^7 leukocytes in blood. Thus, a high degree of assay sensitivity is required in order to detect these rare events in PB. Moreover, the assay has to be highly specific in order to detect as many true positive events as possible and the fewest false positive and false negative events. Commonly used threshold values for CTC count in blood of PC patients have been shown to be controversial; standard definitions of unfavorable and favorable CTC counts have been reported to be $\geq$5 CTCs/7.5ml and <5 CTCs/7.5ml, respectively. (de Bono et *al*. 2008).

The DNA, RNA or protein surface markers of CTCs are commonly used to detect these rare CTCs in PB and BM. Multiple PCR-based methods and immunohistochemical methods can be used to identify captured CTCs. After identification, CTCs can be potentially used as biomarkers to predict patient outcome and to predict the response to a specific therapy (e.g. pre- and postoperative CTC count). Additionally, CTCs can be used as response-indicators to confirm if the specific treatment has led to a pharmacologic or physiologic response after therapy, such as a decline in PSA level in PB after PC therapy, or if the therapy has resulted in treatment resistance such as treatment failure or PC progression. (Danila et *al*. 2011)

1.5.2 Current methods for the detection of CTCs

An increasing number of molecular biological assays has been developed to enumerate, isolate, and characterize CTCs from blood. However, the development of the most analytically valid assay for clinics has shown to be difficult. As mentioned earlier, during EMT, tumor cells acquire mesenchymal characteristics and lose their epithelial characteristics. Thus, the essential step of CTC detection from blood is to use specific cell surface markers for phenotypic differentiation between epithelial and hematopoietic tumor cells. Before CTCs can be identified by nucleic acid-based methods, e.g. RT-PCR, CTCs have to be captured and enriched from blood, which can be done via different methods. Initially, CTCs are separated from blood by immune affinity methods (e.g. magnetic beads and immunoseparation) based on their specific cell surface markers, for example, epithelial cell adhesion molecule, EpCAM. Alternatively, CTCs can be enriched from blood by cell adhesiveness or size filtration methods. After enrichment, CTCs are labeled with fluorescent antibodies specific for epithelial cells (CK 8, 18, and 19), leukocytes (CD45) and the nuclei of tumor cells (DAPI). (Jones et *al*. 2013).

1.5.2.1 Immunology based methods

Immunological techniques are used to enrich and detect CTCs based on the expression of epithelial markers such as epithelial cell adhesion molecules (EpCAM) or cytokeratins (CK) on the surface of CTCs. The strength of immunological assays is that mRNA from dead cells as well as free mRNA can be detected and thus differentiated from living mRNA. However, compared to RT-PCR methods, immunological methods have lower sensitivity and specificity.

CellSearch

The CellSearch (Veridex LLC) system is currently the only CTC detection assay that is approved by USA Food and Drug Administration (FDA) for the detection of metastatic prostate cancer, breast cancer, and colorectal cancer and for the prediction of patients' prognosis. The enrichment step and immunostaining of CTCs is combined in the CellSearch system. CellSearch is based on the isolation (by magnetic separation) of EpCAM expressing tumor cells (Figure 5) (Danila et *al*. 2007).

In the beginning of the assay, a patient's blood sample is mixed with anti-EpCAM coated magnetic beads in order to positively select EpCAM expressing cells. To further identify specific tumor cells of interest, the sample is incubated with anti-cytokeratin antibodies such as CK 8, 18 or 19 and anti-CD45 antibodies to eliminate the presence of leukocytes. Furthermore, the nuclei of enriched tumor cells are labeled with fluorescent nucleic acid dye (4, 2-diamidino-2-phenylindole dihydrochloride, DAPI) in order to visualize the cell population of interest on the automated fluorescent microscopy. Thus, after enrichment and capture of CTCs they are identified as $EpCAM^{+}$-$CD45^{-}$-CK^{+}-DAPI+. The CellSearch system provides accurate information of the quantity of CTCs present in the blood sample according to the specificity of EpCAM, CK and DAPI antibodies. However, as mentioned before, the epithelial characteristics of tumor cells decrease during EMT, which may lower the detection of metastatic CTCs when using the CellSearch assay (Danila et *al*. 2007, Jones et *al*. 2013).

Circulating Tumor Cell (CTC)-Chip

CTC-Chip is a microfluidic platform containing an array of 78 000 microposts coated with anti-EpCAM antibodies. The blood sample is processed through the CTC-chip in order to obtain EpCAM-expressing cells on the microfluidic platform. Thus, EpCAM expressing CTCs bind to microposts, while anuclear red blood cells and nucleated leukocytes are washed away. Following

that, the CTC-chip is washed in order to remove leukocytes. Captured CTCs are then fixed and immunostained with specific antibodies – such as CK and DAPI – for better identification of epithelial cells. Finally, the cells are analyzed with fluorescence microscope (Figure 5). One of the major advantages of CTC-Chip assay is that several different gene expressions can be studied simultaneously (array technique). (Stott et *al*. 2010).

MagSweeper

MagSweeper system is also based on the immunomagnetic separation of EpCAM positive circulating epithelial cells (CepC) from blood. A blood sample is incubated with magnetic beads which are coated with anti-EpCAM antibodies in specific wells. Further, the robotically guided magnetic rod covered with plastic sheath is swept through the capture well that contains the EpCAM positive CePCs. After that EpCAM-coated antibody CepCs are captured to plastic sheath and unbound cells are washed away. Finally magnetic rod is disengaged from the nonadherent plastic covers in new well containing buffer solution and the release of EpCAM labeled CepCs is stimulated with external magnetic beads which are located under the wells. One of the advantages of MagSweeper assay is that after incubating the blood sample with anti EpCAM antibodies, no other handling of the blood sample is required, leading to higher purity of the CTCs of interest and reliable results. Moreover, in the MagSweeper assay individual CTCs can be extracted based on their physical properties (Talasaz et *al*. 2008). The MagSweeper assay has been mainly used for the detection of CepCs in breast cancer (Figure 5).

Functional assays

Epithelial ImmunoSPOT (EPISPOT)

Epithelial immunospot is based on the detection of viable cells after negative selection of CD45 cells. A blood sample is mixed with antibody complexes which recognize unwanted cells, such as CD45 and Glyco A on red blood cells. After negative selection of CD45 and red blood cells, the blood sample is centrifuged by the Ficoll density gradient method in order to obtain the target tumor cells. CTCs or DTCs are cultured on an antibody-coated membrane that captures the secreted proteins. Secondary fluorochrome-labelled antibodies are used to detect viable DTCs or CTCs after 48 h of *ex vivo* cultures based on the secretion of proteins such as PSA and FGF2 (a stem cell fibroblast growth factor 2). (Alix-Panabières et *al*. 2012) (Figure 5).

Collagen Adhesion Matrix (CAM)

During CTC metastasis, tumor cells are commonly able to invade collagenous matrices. A collagen adhesion matrix assay is used for the separation of CTCs based on their invasive phenotype *in vitro*. In the beginning of the reaction, CTCs are separated from peripheral blood by mixing blood cells with fluorescently labeled collagen adhesion matrix fragments (CAM+). The unbound cells are thenwashed away. Those CTCs which are capable of ingesting CAM+ are confirmed to be tumor cells after specific immunostaining with CK marker Epi+. Thus, CTCs are separated from leukocytes, since leukocytes do not express Epi+ cytokeratin markers. CTCs are identified as CAM^{+}-Epi^{+}-CK^{+}-$CD45^{-}$. In order to obtain mononucleated cells (MNC) from the blood, CAM+ expressing CTCs are centrifuged by Ficoll density gradient centrifugation. Subsequently, MNCs are seeded to a fluorescently-labeled collagen matrix, and non-adherent cells are washed away. Thus, more viable aggressive types of CTCs can be detected and separated by CAM assay (Paris et *al*. 2009) (Figure 5). However, CTC detection is based solely on their functional properties, meaning limited numbers of CTCs (mainly metastasized CTCs) are able to be detected.

1.5.2.2 RT-PCR methods

Standard RT-PCR methods are used for differential gene expression and genetic profiling studies (e.g. targeting specific genes expressed in tumor cells). RT-PCR assay is based on the reverse transcription of isolated mRNA (e.g. from blood) to cDNA followed by target cDNA amplification in standard PCR. The assay can be considered as semiquantitative method (Bustin S. A. 2000). Since CTCs are rare events in the blood, many markers (target gene expression) have to be studied simultaneously in order to increase reliability. Thus, due to the fact that a single PCR reaction can detect only one gene in the target CTC, the cost is a major limitation (many markers have to be studied separately).

Different RT-PCR methods have been widely used to detect and measure specific gene expression in CTCs extracted from PC patients. PC-specific antigen mRNAs detected by RT-PCR methods include *PSA*, prostate-specific membrane antigen (*PSMA*), prostate stem cell antigen (*PSCA*), as well as cytokeratin 19 (*CK 19*) antigens. All of the respective antigens can be detected in blood of PC patients (Panteleakou et *al*. 2009). PSA is a serine protease regulated by androgens and secreted by the prostate, as well as by benign and malignant tumors (Lilja et *al*. 2008). PSMA is a membrane bound glycoprotein that has been shown to be absent in benign tumors and highly expressed in CRPC and metastatic lesions (Olson et *al*. 2007). PSCA is also a membrane surface glycoprotein that has been shown to be highly expressed in PC and may be a good prognostic and diagnostic biomarker for PC due to its low expression in normal tissues (Raff et *al*. 2009). CK 19 belongs to the keratin family and is a type of intermediate filament protein; it has been shown to be expressed in the PB epithelial cells of PC patients (Machado et *al*. 2005).

Nested RT-PCR

The sensitivity of an RT-PCR assay can be increased by using two step nested or multiplex RT-PCR. In nested RT-PCR, a small aliquot (1%) of target gene product from the primary RT-PCR reaction is used in a second round of PCR amplification. During the second round of PCR, the target sequence from the first RT-PCR reaction is used as a template and new specific primer pairs (called as nested primers) are used to detect a sequence in target gene amplicon. Moreover, by using nested primers, non-specific amplification is decreased and sensitivity in amplifying the specific sequence of interest is increased (Goode et *al*. 2002, Panteleakou et *al*. 2009).

Multiplex RT-PCR

Multiplex RT-PCR can be used for the detection of mutations in the genes and for genotyping assays. Multiple primers are used to detect genetic aberrations, such as deletions and duplications in the genome. In multiplex RT-PCR, different sizes of amplicon are generated in single PCR reaction. Thus, a different target sequences can be analyzed during each PCR run. In addition, the internal control (housekeeping gene) can be co-amplified in order to eliminate gene expression variability and to increase the reliability of the results. However, major challenges in multiplex RT-PCR include the optimization of reaction conditions, multiple primer designs, and difficulties in determining the number of CTCs needed in specific sample volume (Panteleakou et *al*. 2009).

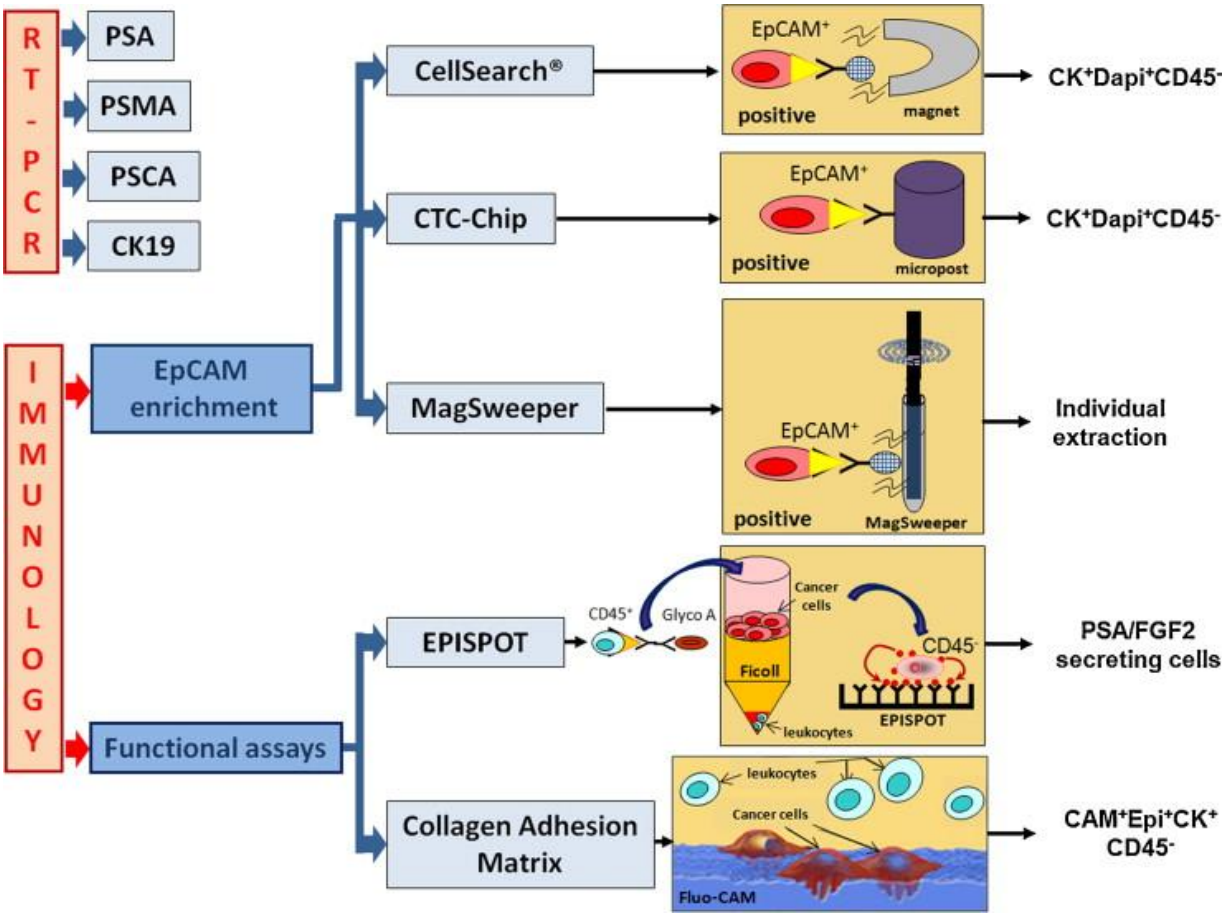

Figure 5. **Current molecular biology- and immunology -based methods for enrichment and detection of circulating tumor cells (CTCs) in blood of prostate cancer patients**. RT-PCR assays: are used to detect and measure specific gene expression (such as *PSA*, *PSMA*, *PSCA* and *CK 19*) in CTCs of PC patients. Immunological methods: The CTCs are enriched from blood based on the epithelial expression of EpCAM and CK. **CellSearch®**: blood is mixed with EpCAM coated magnetic beads and incubated with anti-CK antibodies and anti-CD45 antibodies to eliminate the presence of leukocytes. The nuclei of enriched tumor cells are labeled with fluorescent nucleic acid Dapi in order to visualize the tumor cells with fluorescent microscopy. **CTC-Chip**: blood is processed through the EpCAM coated micropost. EpCAM expressing CTCs bind to microposts. CTCs are further stained with CK and Dapi. **MagSweeper**: blood is incubated with anti EpCAM coated magnetic beads. EpCAM positive cells are captured by magnetic rod covered with plastic sheath. Functional assays: **EPISPOT**: blood sample is mixed with antibody complexes which recognize CD45 and Glyco A on red blood cells and centrifuged by Ficoll density gradient method. DTCs/CTCs are identified after 48 h of *ex vivo* cultures based on the secretion of proteins such as PSA and FGF2. **Collagen Adhesion Matrix**: blood is mixed with CAM+ fluorescent fragments. CTCs that are capable to ingest CAM+ are confirmed to be tumor cells after CK, Epi+ immunostaining. After Ficoll density gradient centrifugation mononuclear cells are seeded to fluorescently labeled collagen matrix and non adherent cells are washed away. CTCs are characterized based on their functional properties. **PSA**, prostate specific antigen; **PSMA**, prostate specific membrane antigen; **PSCA**, prostate stem cell antigen; **CK**, cytokeratin; **EpCAM**, epithelial adhesion molecule; **Dapi**, 4,2-diamidino-2-phenylindole dihydrochloride; **CTC-Chip**, circulating tumor cell-microfluidic platform; **EPISPOT**, epithelial immunospot; **Glyco A**, glycophorin A, a 131 amino acids protein present at the extra-cellular surface of the human red blood cells; **CD45**, leukocyte specific marker; **FGF-2**, a stem cell fibroblast growth factor; **CAM**, collagen adhesion matrix.

Reproduced with permission from the copyright holder: Doyen J., Alix-Panabières C., Hofman P., Parks S. K., Chamorey E., Naman H., Hannoun-Lévi J. M. (2012) **Circulating tumor cells in prostate cancer: A potential surrogate marker of survival**. *Critical Reviews in Oncology/Hematology* **81**, 241-256

1.6 QUANTITATIVE REAL-TIME PCR

In quantitative real-time PCR (qRT-PCR) the amplified PCR product is quantitatively measured after each PCR cycle. Different fluorescent dyes that bind to double-stranded (ds) DNA can be used for the detection of amplified cDNA. During the reaction, fluorescence signals are monitored by a computer-based program in real-time and the intensity of fluorescence signals is directly correlated with the quantity of the target PCR product present after each cycle (Kubista et *al*. 2006). Moreover, due to the low intensity of the fluorescence signals they cannot be distinguished from the background in the beginning of the qRT-PCR reaction.

With each PCR cycle the concentration of target cDNA sequence increases and fluorescence signals become detectable above background, leading to an exponential increment of fluorescence levels. The cycle numbers required to cross a set threshold fluorescence signal level is represented with Ct (cycle threshold) value. The background value is determined according to the relatively stable fluorescence signal levels during the first cycles. Furthermore, threshold value is defined in order to detect the fluorescence above background (Kubista et *al*. 2006).

In order to obtain Ct values, the fluorescence from the amplified cDNA has to cross the threshold. Moreover, the relative concentration of target cDNA during the exponential phase of the reaction is obtained by plotting fluorescence signals against cycle number on a logarithmic scale. In addition, known concentrations of serially diluted PCR products are used for the generation of a standard curve in order to generate a linear relationship between Ct values and starting quantities of the template. Furthermore, based on the Ct values of unknown samples the concentrations can directly be determined from the standard curve. Housekeeping genes are used as control genes to correct the variability in gene expression (Kubista et *al*. 2006).

TaqMan assay

A TaqMan assay reaction is based on the binding of specific TaqMan probe to target DNA sequence and the 5´exonuclease activity of Taq DNA polymerase to hydrolyze the TaqMan probe from the target region. 5´end of TaqMan probe is usually labeled with reporter (R) fluorescent dye and 3´ end of the probe is labeled with quenching (Q) fluorescent dye. During denaturation cDNA is separated into two single strands. The following annealing step allows the two target template specific primers (5´ forward and 3´ reverse) to define the endpoints of the sequence by binding to respective cDNA sequence. After the respective primers have been annealed to the sequence the target specificity is increased further when the dual-fluorescent labeled TaqMan probe hybridizes specifically to the target sequence of interest between the two PCR primers during the annealing step. (Bustin et *al*. 2000)

When the probe is bound there is an energy transfer between R and Q and little or no fluorescence is detected due to reporter fluorescence suppression by quencher. During polymerization, 5´ Taq DNA polymerase starts to synthesize the target sequence. TaqMan probe remains bound to the sequence until Taq polymerase reaches the reporter end and cleaves the probe by utilizing its 5´exonuclease activity. Thus, the reporter is released and is no longer suppressed by the quencher, allowing the fluorescence to be detected. The fluorescence intensity is increased if the target sequence is complementary to the TaqMan probe. (Bustin S. A. 2000)

SYBR Green assay

The SYBR Green reaction is based on the binding of SYBR Green fluorescent dye to dsDNA during DNA polymerization. During denaturation, the background fluorescent signal is very weak and SYBR Green is unbound. After the denaturation process, specific primers bind to target sequence (annealing) and some SYBR Green molecules bind to annealed sequence. The fluorescence signal is weak and only light emitted upon excitation is detected. During polymerization, new DNA is constantly synthesized leading to increased binding of SYBR Green molecules to dsDNA sequences. After each PCR cycle, fluorescence levels are exponentially increased and directly monitored in real-time. (Bustin S. A. 2000)

iScript One-Step RT-PCR assay

The iScript One-Step RT-PCR assay is also based on the SYBR Green dye binding to ds DNA. What is different compared to TaqMan assay and SYBR Green assay is that mRNA reverse transcription into cDNA and qPCR (SYBR Green) occurs in the same reaction. Thus, mRNA is used as a template in iScript One-Step RT-PCR assay, in contrast to the TaqMan and SYBR Green assays. Initially, mRNA is transcribed to produce cDNA by the specific iScript RT enzyme. After cDNA synthesis the reaction continues according to the standard RT-PCR protocol.

2. AIMS OF THE STUDY

The main aim of this study was to detect the *TMPRSS2:ERG* fusion gene in clinical prostate cancer patient samples. The specific aims were as follows:

1. to validate the sensitivity and specificity of TaqMan, SYBR Green and iScript One-Step RT-PCR assays for detecting *TMPRSS2:ERG* fusion gene in fusion positive VCaP prostate cancer cell line.

2. to find the most suitable assay for detecting *TMPRSS2:ERG* fusion gene in prostate cancer patient material to study the occurrence of the fusion gene in blood samples.

3. to examine how the *TMPRSS2:ERG* fusion gene occurrence affects patient outcome.

3. MATERIALS AND METHODS

3. 1 Cell lines and clinical samples

PC-3 and VCaP cells were cultured as a monolayer in 75cm^2 flasks. PC-3 cells were maintained in HAM´S F-12 medium (Lonza, Verviers, Belgium) supplemented with L-glutamine and 10% fetal bovine serum. PC-3 cells were passaged twice weekly (after PC-3 cells achieved 75-80% confluency) prior to use. VCaP cells were grown in Dulbecco's Modified Eagle´s medium (DMEM, Lonza, Verviers, Belgium) supplemented with L-glutamine and 10% Hyclone (HC) fetal bovine serum (Lonza, Verviers, Belgium). VCaP cells were examined under the microscope and passaged once weekly (after VCaP cells achieved 85-90% confluency) prior to use. VCaP and PC-3 cells were cultured in humidified conditions at 37°C, 5% CO_2.

Blood samples from 163 PC patients (Table 2) and 16 control samples (not shown) were utilized to study the incidence of the *TMPRSS2:ERG* fusion gene in a clinical setting. Blood samples were collected between 1993 and 2001 at the Tampere University Hospital in Finland. All of the blood specimens were initially processed; the RNA was extracted with Trizol (extraction method described in 3.4.5) from the blood samples and stored at -80°C for further use at the Institute of Biomedical Technology in Tampere, Finland in 2010. The follow-up data for these patients is available from 1993 to 2001 and the full clinical data (e.g. treatment, diagnostic PSA value, TNM stage, GS) is shown in Appendix 1.

Table 2. Clinical data of the prostate cancer patients (n=163)

Median age, years	(range)	**63.9**	(47.8 - 82.5)
Median PSA, ng/ml	(range)	**28.4**	(0.5 - 2480)
Median follow-up, months	(range)	**97.09**	(9.47 - 163.20)
Pathological tumor stage (pT)			
pT: 2	(%)	**85**	(52.2)
pT: 3	(%)	**62**	(38)
pT: ND[d]	(%)	**16**	(9.8)
Gleason score (GS)			
GS < 7[e]	(%)	**69**	(42.3)
GS = 7[f]	(%)	**64**	(39.3)
GS > 7[g]	(%)	**29**	(17.8)
GS = ND[h]	(%)	**1**	(0.6)
WHO[i]			
1	(%)	**32**	(19.6)
2	(%)	**113**	(69.3)
3	(%)	**18**	(11.1)
Primary treatment			
Hormonal therapy	(%)	**13**	(8.0)
Surgical castration	(%)	**8**	(4.9)
Chemical castration	(%)	**5**	(3.1)
Prostatectomy	(%)	**147**	(90.2)
Radiotherapy	(%)	**3**	(2.0)

[d] ND**,** not determined due to biopsy atypia / transurethral resection of the prostate gland (TURP); [e] low grade; [f] intermediate; [g] high grade; [h] GS is unknown; [i] PC stages according to World Health Organization (1, 2, 3: low grade, intermediate, high grade); PSA, prostate specific antigen

In the cohort of 147 prostatectomy-treated patients, a total of 42.9% (63 of 147) of the patients had PC recurrence after prostatectomy (Table 3). Biochemical failure was considered when the postoperative PSA serum level increased above 0.5ng/ml after prostatectomy.

Table 3. PSA progression in the prostatectomy treated patients (n=63)

Median age, years	(range)	**63.1**	(52.1 – 71.9)
Median PSA, ng/ml	(range)	**11.7**	(2.8 – 48.1)
Median PSA progres. free months[j]	(range)	**54.2**	(9.5 – 138)
Pathological tumor stage (pT)			
pT: 2	(%)	**26**	(41.3)
pT: 3	(%)	**37**	(58.7)
Gleason score (GS)			
GS < 7[k]	(%)	**14**	(22.2)
GS = 7[l]	(%)	**33**	(52.4)
GS > 7[m]	(%)	**16**	(25.4)
WHO[n]			
1	(%)	**6**	(9.5)
2	(%)	**47**	(74.6)
3	(%)	**10**	(15.9)

[j] median prostate specific antigen (PSA) progression free months after prostatectomy;
[k] low grade; [l] intermediate; [m] high grade; [n] PC stages according to World Health Organization (1, 2, 3: low grade, intermediate, high grade); PSA, prostate specific antigen

3. 2 RNA isolation from PC-3 & VCaP cells and first strand cDNA synthesis

Total PC-3 and VCaP RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA pellet was dissolved in RNase-free H_2O and the concentration and purity of isolated PC-3 and VCaP RNA was determined by measurement of the optical density at 260 and 280 nm using the NanoDrop. For cDNA synthesis, 1 µg of total PC-3 RNA and 1 µg of total VCaP RNA were mixed and converted into first strand cDNA using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). The cDNA synthesis was done in a 20 µl reaction volume which contained 10 µl of total RNA (PC-3 and VCaP RNA), 1 µl random hexamer primers, 1 µl dNTP mix, 4 µl 5x First-Strand Buffer, 2 µl DTT, 1 µl RNase inhibitor and 1 µl (200 units) of SuperScript ™ III reverse transcriptase. Furthermore, for cDNA synthesis 1 µg of total VCaP RNA was converted into first strand cDNA as described above and five-fold serial dilutions of VCaP cDNA were prepared.

3. 3 Validation of real-time quantitative RT-PCR assays

Three different qRT-PCR assays: TaqMan Gene Expression Assay (Applied Biosystems), quantitative Real-Time PCR with SYBR Green (Fermentas), and iScript™ One-Step Real Time-PCR with SYBR® Green (Bio-Rad) were tested for the validation of the specificity and sensitivity of the TMPRSS2:ERG cDNA amplification. Real-Time PCR primer pairs were designed according to the complete cDNA sequences deposited in GenBank (Table 4). The starting amounts of VCaP and PC-3 cDNA and mRNA template mixtures used in respective qRT-PCR assays are shown in Table 5.

TMPRSS2 exon 1:ERG exon 4 and ERG primer sequences used in iScript One-Step RT-PCR and SYBR Green assays were obtained from Tomlins et *al*. (2005) (Table 4). *TMPRSS2:ERG* gene fusion specific TaqMan gene Expression Assay (Applied Biosystems) was designed based on the fusion sequences used in Tomlins et *al*. (2005). TBP primer sequences were used as internal controls and designed based on the cDNA sequences deposited in GenBank (Table 4).

Table 4. Primer sequences used in TaqMan, SYBR Green and iScript One-Step RT-PCR.

Amplified region	**Primers (5´-3´)**	**PCR (bp)**	**Ta (°C)**	**Accession no. (GenBank)**
ERG	**F**: TCTTGGACCAACAAGTAGCC **R**: GTCGGGATCCGTCATCTTG	151	60	NM_182918
TBP	**F**: GAATATAATCCCAAGCGGTTTG **R**: ACTTCACATCACAGCTCCCC	226	59	NM_00319
***TMPRSS2*E1**: ***ERG* E4**	**F**: TAGGCGCGAGCTAAGCAGGAG **R**: GTAGGCACACTCAAACAACGACTGG	126	68	NM_005656 NM_004449
	TaqMan gene Expression Assay (Applied Biosystems)			
TMPRSS2: ***ERG***	**Assay ID** Hs03063375_ft	106	60	DQ204772.1

The different amounts of *TMPRSS2:ERG* fusion positive VCaP cDNA/mRNA and *TMPRSS2:ERG* fusion negative PC-3 cDNA/mRNA used as templates in qRT-PCR reactions are shown in Table 5.

Table 5.The amount of VCaP and PC-3 cDNA (TaqMan, SYBR Green) and RNA (iScript One-Step RT-PCR) templates used in the study.

	VCaP (ng)	**+**	**PC-3 (ng)**
1.	100		100
2.	20		100
3.	4		100
4.	0.8		100
5.	0.16		100
6.	$3.2x10^{-2}$		100
7.	$6.4x10^{-3}$		100
8.	$2.56x10^{-3}$		100
9.	$1.28x10^{-4}$		100
10.	$5.12x10^{-5}$		100

3.3.1 TaqMan gene expression assay

The total volume of each qRT-PCR reaction was 20 µl containing 2 µl of first strand cDNA (Table 5), TaqMan® Universal Master Mix (Applied Biosystems, Carlsbad, CA) and 0.5 µM 20xTaqMan® Gene Expression Assay (Table 4). CFX 96 Real-Time System apparatus (Bio-Rad) was used for qRT-PCR analysis. The following steps were carried out for amplification and detection at standard conditions: after 10 min polymerase activation step at 95ºC amplification was performed in a two-step cycling procedure for 45 cycles: denaturation at 95ºC for 15 s, annealing/extension at 60ºC for 15 s for TaqMan probe (FAM-labeled) and fusion primers (Table 4: TaqMan gene Expression Assay). A standard curve was established for *TMPRSS2:ERG* fusion gene using the mixture of five-fold serial dilutions of VCaP cDNA (Table 5) and the standard amount of PC-3 cDNA (Table 5).

3.3.2 SYBR Green assay

The total volume of each qRT-PCR reaction was 22µl containing 2µl of first strand cDNA (Table 5), 2xMaxima SYBR® Green/Rox qPCR Master Mix (Fermentas) and 0.625µM of TMPRSS2:ERG fusion primers (Table 4). CFX 96 Real-Time System apparatus (Bio-Rad) was used for qRT-PCR analysis. The amplification and detection were carried out as follows: after an initial 5 min polymerase activation step at 95^{o}C, amplification was performed in a three-step cycling procedure for 50 cycles: denaturation at 95^{o}C for 10s, annealing at 68°C for TMPRSS2:ERG fusion primers for 30s, and elongation at 72^{o}C for 20s (the ramp rate was 0.5^{o}C/s for all steps), and a final cooling step. Melting curve analysis was performed after the amplification to check the specificity of the qRT-PCR reaction. A standard curve was established for *TMPRSS2:ERG* fusion gene as described in 3.3.1.

3.3.3 iScript One-Step RT-PCR assay

The total volume of each qRT-PCR was 25 µl containing 2.5 µl of template RNA (Table 5), 2xSYBR® Green RT-PCR Reaction Mix (Bio-Rad, Hercules, CA) and 0.3 µM of TMPRSS2:ERG fusion primers (Table 4). CFX 96 Real-Time System apparatus (Bio-Rad) was used for qRT-PCR analysis. The following steps were carried out for cDNA synthesis, amplification, and detection: first strand cDNA synthesis for 10 min at 44°C, iScript Reverse transcriptase inactivation for 5 min at 95°C, amplification was performed in a three-step cycling procedure for 40 cycles: denaturation at 95^{o}C for 10 s, annealing at 68°C for TMPRSS2:ERG fusion primers for 30 s, and elongation at 72^{o}C for 20 s (the ramp rate was 0.5^{o}C/s for all steps), and a final cooling step. Melting curve analysis was performed after the amplification to check the specificity of the qRT-PCR reaction. A standard curve was established for *TMPRSS2:ERG* fusion gene as described in 3.3.1.

3.4 Validation of *TMPRSS2:ERG* detection in the mixture of VCaP cells and human leukocytes

3.4.1 VCaP cell line

VCaP cells were cultured as described in 3.1.

3.4.2 Human blood specimens

Blood samples from three randomly chosen individuals were collected into 9 ml x EDTA (ethylenediaminetetraacetic acid) -containing tubes at the Institute of Biomedical Technology Tampere, Finland in 2012. Blood specimens were initially processed within 1 hour of collection.

3.4.3 Isolation of human leukocytes

Leukocytes were separated from red blood cells by the dextran extraction method using 3% dextran solution in saline (282 kDa Mw, Sigma, Industrial grade, D-7265) as follows: 9-10 ml aliquots of whole EDTA blood (in 15 ml Falcon tubes) were mixed gently with 4 ml 3% dextran solution in saline and allowed to stand at ambient room temperature for 30-45 minutes to ensure that all of the red blood cells were separated from nucleated white blood cells.

After sedimentation (30-45 min), the leukocyte-containing supernatants (7 ml) remaining in the tubes were removed and transferred into fresh 15 ml centrifuge tubes (Falcon). An equal amount (7 ml) of RNase free phosphate buffered saline (1xPBS) was added to each tube and mixed gently by repeated inversions. The respective samples were centrifuged at 300 x g for 10 min at +4^{o}C. After centrifugation the supernatants were discarded and the cells (remaining in the bottom of the tube) were suspended in 2 ml of RNase free 1xPBS. These cell suspensions (2 ml) were transferred into 2 fresh 15 ml centrifuge tubes (Falcon) (1 ml of cell suspension/tube) and centrifuged at 800 x g for 10 min at +4^{o}C. The supernatants were carefully removed and the cell pellets (leukocytes) were snap frozen in liquid nitrogen and stored at -80^{o}C for further use.

3.4.4 The mixture of VCaP cells and human leukocytes

VCaP cells were washed with 1xPBS and harvested from the flask by trypsinization. The total number of VCaP cells were counted using a counting chamber (Bürker, 0. 0025 mm^2) and the final VCaP cell concentration was determined. VCaP cell suspension was diluted in 1xPBS and total of 1, 5, 10, 15, and 20 VCaP cells were mixed with the isolated human leukocytes.

3.4.5 RNA isolation from VCaP cells + leukocyte -mixture and first strand cDNA synthesis

Total RNA was extracted from each of the VCaP + leukocyte mixtures using TRIzol (Invitrogen) followed by ethanol precipitation according to the manufacturer's suggestions. All of the 1-20 VCaP RNA pellets were dissolved in RNase free H_2O and the concentration and purity of isolated 1-20 VCaP + leukocyte mixture RNAs were determined by measurement of the optical density at 260 and 280nm using a NanoDrop. For the synthesis of cDNA, 1µg of each 1-20 VCaP + leukocyte -mixture RNA were converted into first strand cDNA using a SuperScript^{TM} III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). The cDNA synthesis was performed in a 20 µl reaction volume which contained 10 µl of total 1-20 VCaP + leukocyte mix RNA, 1 µl random hexamer primers, 1µl dNTP mix, 4 µl 5x First-Strand Buffer, 2 µl DTT, 1 µl RNase inhibitor and 1 µl (200 units) of SuperScript ^{TM} III reverse transcriptase.

3.4.6 TaqMan gene expression assay for the detection of *TMPRSS2:ERG*

The total volume of each qRT-PCR was 20 µl, containing 2 µl of first strand 1-20 VCaP + leukocyte mixture cDNA, TaqMan® Universal Master Mix (Applied Biosystems, Carlsbad, CA) and 0.5µM 20xTaqMan® Gene Expression Assay (Table 4). CFX 96 Real-Time System apparatus (Bio-Rad) were used for qRT-PCR analysis. The following steps were carried out for amplification and detection at standard conditions: after an initial 10 min polymerase activation step at 95°C, amplification was performed in a two-step cycling procedure for 50 cycles: denaturation at 95^{o}C for 15 s, annealing/extension at 60°C for 15 s for TaqMan probe (FAM-labelled) and fusion primers (Table 4: TaqMan gene Expression Assay). A standard curve was established for *TMPRSS2:ERG* fusion gene using the mixture of five-fold serial dilutions of VCaP cDNA (Table 5).

3.4.7 SYBR Green assay for the detection of *ERG*

The total volume of each qRT-PCR reaction was 22 µl containing 2 µl of first strand 1-20 VCaP + leukocyte mixture cDNA, 2 x Maxima SYBR® Green/Rox qPCR Master Mix (Fermentas) and 0.625 µM of ERG primers (Table 4). CFX 96 Real-Time System apparatus (Bio-Rad) was used for qRT-PCR analysis. The amplification and detection were carried out as follows: after an initial 5 min polymerase activation step at 95^{o}C, amplification was performed in a three-step cycling procedure for 50 cycles: denaturation at 95^{o}C for 10 s, annealing at 60°C for ERG primers for 30 s, and elongation at 72^{o}C for 20 s (the ramp rate was 0.5^{o}C/s for all steps), and a final cooling step. Melting curve analysis was performed after the amplification to check the specificity of the qRT-PCR reaction. A standard curve was established for *ERG* gene as described in 3.3.1.

3.5 The detection of *TMPRSS2:ERG* fusion transcript in clinical prostate cancer blood samples

3.5.1 First strand cDNA synthesis

For cDNA synthesis 1 µg of each 163 clinical PC blood RNA sample was converted into first strand cDNA using a SuperScript ^{TM} III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). The cDNA synthesis was done in a 20 µl reaction volume which contained 10 µl of total clinical PC blood RNA, 1 µl random hexamer primers, 1 µl dNTP mix, 4 µl 5x First-Strand Buffer, 2 µl DTT, 1 µl RNase inhibitor and 1 µl (200 units) of SuperScript ^{TM} III reverse transcriptase.

3.5.2 TaqMan gene expression assay

The total volume of each qRT-PCR was 20 µl containing 2 µl of first strand clinical PC cDNA, TaqMan® Universal Master Mix (Applied Biosystems, Carlsbad, CA) and 0.5 µM 20xTaqMan® Gene Expression Assay (Table 4). CFX 96 Real-Time System apparatus (Bio-Rad) was used for qRT-PCR analysis. The following steps were carried out for amplification and detection at standard conditions: after an initial 10 min polymerase activation step at 95°C, amplification was performed in a two-step cycling procedure for 50 cycles: denaturation at 95^{o}C for 15 s, annealing/extension at 60°C for 15 s for TaqMan probe (FAM-labelled) and fusion primers (Table 4: TaqMan gene Expression Assay). A standard curve was established for *TMPRSS2:ERG* fusion gene as described in 3.4.6.

3.6 Statistical analysis

Kaplan-Meier estimator functions and Mantel-Cox proportional hazard models were used to evaluate the association between PC specific-survival and *TMPRSS2:ERG* fusion status, and to estimate hazard ratios (HR) and 95% confidence intervals (CI). Survival analysis models were examined adjusting for *TMPRSS2:ERG* status in blood at the time of PSA progression. Thus, the time to PSA recurrence (PSA levels $\geq$ 0.5 ng/ml) after prostatectomy was used as an endpoint. The Mann-Whitney test was used to examine the eventual associations between patients' clinicopathological follow-up data (PSA at diagnosis and age at diagnosis) and *TMPRSS2:ERG* fusion status in the blood. Fisher's exact test was used to assess the relationship between *TMPRSS2:ERG* fusion positive and fusion negative and pathological tumor stage (pT). The null hypothesis, $H_{0,}$ is that there are no differences in clinicopathological follow-up data or progression free survival between *TMPRSS2:ERG* fusion positive and *TMPRSS2:ERG* fusion negative patients. H_0 was kept if the statistical significance differences between *TMPRSS2:ERG* fusion positive and *TMPRSS2:ERG* fusion negative patients were $p \geq 0.05$.

4. RESULTS

4.1 TaqMan, SYBR Green and iScript One-Step RT-PCR assays

In order to validate the most sensitive and specific assay for the detection of *TMPRSS2:ERG* gene transcript in the template mixture of VCaP and PC-3 cDNA, three different quantitative RT-PCR assays TaqMan (Applied Biosystems, Carlsbad, CA), SYBR Green (Fermentas) and iScript One-Step RT-PCR (Bio-Rad) were tested. Five-fold serial dilutions of VCaP cDNA (Table 5) and standard amount of PC3 cDNA (Table 5) were used as templates in each reaction. TaqMan assay was confirmed to be the most sensitive and specific assay for the detection of *TMPRSS2:ERG* fusion gene in the mixture of VCaP and PC-3 cells (Table 6).

Table 6. The sensitivity of TaqMan, SYBR Green and iScript One-Step RT-PCR assays

VCaP cDNA[o] (ng)	**Threshold cycle[p] (C(t))**		
	TaqMan	**SYBR Green**	**iScript One-Step RT-PCR**
100	20.81	20.02	26.61
20	22.62	22.04	29
4	25.62	24.66	31.77
0.8	27.64	27.11	-
0.16	30.11	29.59	-
$3.2x10^{-2}$	33.09	32.29	-
$6.4x10^{-3}$	34.9	35.92	-
$1.28x10^{-3}$	38.39	-	-
$2.56x10^{-4}$	42.34	-	-
$5.12x10^{-5}$	43.61	-	-

[o] The amount of VCaP cDNA (TaqMan and SYBR Green) and VCaP RNA (iScript One-Step RT-PCR) as well as standard amount of PC-3 cDNA/RNA (Table 5) used as templates in each qRT-PCR reaction; **[p]** The cycle number at which the fluorescence exceeds the threshold is called the threshold cycle; -, not detectable

4.2 The detection of *TMPRSS2:ERG* and *ERG* expression in the mixture of human leukocytes and VCaP cells

Human leukocytes were isolated from blood samples of three randomly chosen individuals. Isolated leukocytes were mixed with *TMPRSS2:ERG* fusion positive VCaP cells in order to investigate the expression levels of *ERG* and fusion transcript in the resultant cell mixture. A total of 60 ml of human venous blood was collected and processed within 1 hour. The dextran extraction method was used to separate the leukocytes and red blood cells (RBC). Leukocytes were snap-frozen and stored as cell pellets at -80ºC. The following day the leukocytes were mixed with different numbers of VCaP cells (1, 5, 10, 20), each of the leukocyte + VCaP RNA -mix was extracted by TRIzol (Invitrogen) and further converted to cDNA using SuperScript™ III reverse Transcription Kit (Invitrogen). SYBR Green assay (Bio-Rad) and TaqMan assay were used to examine and validate

the expression levels of *ERG* as well as *TMPRSS2:ERG* fusion gene in the mixture of leukocyte + VCaP cDNA, respectively.

TMPRSS2:ERG fusion status in the mixture of VCaP cells and human leukocytes

For the detection of *TMPRSS2* exon1 and *ERG* exon4 (*TMPRSS2:ERG*) fusion product in different VCaP + leukocyte mixtures, TaqMan gene expression assay was used (Table 4). This study was performed total of three times. However, the expression of *TMPRSS2:ERG* fusion gene could not be detected in any of the experiments (results not shown).

The expression of *ERG* in the mixture of VCaP cells and human leukocytes

The expression levels of *ERG* in different VCaP + leukocyte mixtures were investigated by using SYBR Green assay. For the detection of *ERG* in VCaP + leukocyte mixtures, specific forward and reverse primers designed for *ERG* coding sequence (Table 4) were used. *ERG* expression was detected in the following mixtures of VCaP and leukocytes: 1 VCaP cell + leukocytes and 5 VCaP cells + leukocytes. The expression of *ERG* could not be detected in the mixtures of 10, 15, 20 VCaP cells + leukocytes (results not presented).

4.3 The frequency of *TMPRSS2:ERG* expression in clinical prostate cancer blood samples

TaqMan assay

Quantitative RT-PCR TaqMan assay (Applied Biosystems) was used to assess *TMPRSS2:ERG* fusion status in 163 clinical PC blood samples (13 of 163 patients received hormonal therapy and 3 of 163 patients received radiotherapy as their primary treatment; these samples were excluded from the data analysis). All of the blood samples were collected and the RNA was extracted at the Institute of Biomedical Technology Tampere, Finland in 2010. For the detection of *TMPRSS2* exon 1 and *ERG* exon 4 (*TMPRSS2:ERG*) fusion product in clinical PC samples, a TaqMan gene expression assay was used (Table 4).

The TaqMan assay was repeated total of three times. The fusions were detected as follows in patients who underwent prostatectomy (n=147): 1. TaqMan (n=147): 38 of 147 (25.8%). After the first TaqMan assay, the RNAs of the 38 positive samples were once again reverse transcribed into cDNA (to confirm that there are no false positive results) and used as templates in the following two TaqMan assays. The fusions were detected as follows; 2. TaqMan (n=38): 2 of 38 (5.3%), 3. TaqMan (n=38): 6 of 38 (15.8%).

TMPRSS2:ERG expressions were detected as follows in the patients who received hormonal therapy (n=13) as their primary treatment: 1. TaqMan (n=13): 4 of 13 (30.8%), 2. TaqMan (n=4): 0 of 4 (0%), 3. TaqMan (n=4): 1 of 4 (25%). Furthermore, the patients who received radiotherapy as their primary treatment (n=3), did not have *TMPRSS2:ERG* expression.

TMPRSS2:ERG fusion status and clinicopathological data of prostatectomy-treated patients are shown in Table 7 and 8 (data of the PC patients who received hormonal and radiotherapy as their primary treatment is not shown).

Table 7. *TMPRSS2:ERG* fusion status and clinicopathological data of 147 prostatectomy treated patients (1. TaqMan).

		Patients (n)	1. TaqMan *TMPRSS2:ERG* Yes (%)	No (%)
Age	< 50	2	**1 (50)**	1 (50)
	50-60	38	**6 (15.8)**	32 (84.2)
	60-70	99	**30 (30.3)**	69 (69.7)
	> 70	8	**1 (12.5)**	7 (87.5)
pT[q]	2	85	**20 (23.5)**	65 (76.5)
	3	62	**18 (29)**	44 (71)
GS[r]	≤ 6	62	**15 (24.2)**	47 (75.8)
	7	60	**14 (23.3)**	46 (76.7)
	≥ 8	24	**8 (33.3)**	16 (66.7)
	ND	1	**1 (100)**	0 (0)
PSA diag	≤ 4	16	**2 (12.5)**	14 (87.5)
(ng/ml)[s]	4-10	89	**25 (28.1)**	64 (71.9)
	> 10	42	**11 (26.2)**	31 (73.8)
WHO[t]	1	30	**9 (30)**	21 (70)
	2	101	**23 (22.8)**	78 (77.2)
	3	16	**6 (37.5)**	10 (62.5)
PSA	Yes	63	**17 (26.9)**	46 (73.1)
prog.[u]	No	84	**21 (25)**	63 (75)

[q] pathological tumor stage (pT); **[r]** Gleason score (GS); **[s]** prostate specific antigen (PSA) level (ng/ml) at diagnosis; **[t]** PC tumor stage according to World Health Organization (WHO) (1, low grade; 2, intermediate; 3, high grade); **[u]** prostate specific antigen (PSA) serum level increment after prostatectomy above 0.5 ng/ml; ND, not determined

Table 8. *TMPRSS2:ERG* fusion status and clinicopathological data of 38 prostatectomy treated patients (2. and 3. TaqMan).

		Patients (n)	2. TaqMan *TMPRSS2:ERG* Yes (%)	2. TaqMan *TMPRSS2:ERG* No (%)	3. TaqMan *TMPRSS2:ERG* Yes (%)	3. TaqMan *TMPRSS2:ERG* No (%)
Age	< 50	1	**0 (0)**	1 (100)	**1 (100)**	0 (0)
	50-60	6	**0 (0)**	6 (100)	**2 (33.3)**	4 (66.7)
	60-70	30	**2 (6.7)**	28 (93.3)	**2 (6.7)**	28 (93.3)
	> 70	1	**0 (0)**	1 (100)	**1 (100)**	0 (0)
pT[v]	2	20	**0 (0)**	20 (100)	**3 (15)**	17 (85)
	3	18	**2 (11.1)**	16 (88.9)	**3 (16.7)**	15 (83.3)
GS[w]	≤ 6	15	**1 (6.7)**	14 (93.3)	**2 (13.3)**	13 (86.7)
	7	14	**1 (7.1)**	13 (92.9)	**3 (21.4)**	11 (78.6)
	≥ 8	8	**0 (0)**	8 (100)	**1 (12.5)**	7 (87.5)
	ND	1	**0 (0)**	1 (100)	**0 (0)**	1 (100)
PSA diag.	≤ 4	2	**0 (0)**	2 (100)	**0 (0)**	2 (100)
(ng/ml)[x]	4-10	25	**2 (8)**	23 (92)	**3 (12)**	22 (88)
	> 10	11	**0 (0)**	11 (100)	**3 (27.3)**	8 (72.7)
WHO[y]	1	9	**0**	9 (100)	**1 (11.1)**	8 (88.9)
	2	23	**2 (8.7)**	21 (91.3)	**4 (17.4)**	19 (82.6)
	3	6	**0**	6 (100)	**1 (16.7)**	5 (83.3)
PSA	Yes	17	**1 (5.9)**	16 (94.1)	**2 (11.8)**	15 (88.2)
prog.[z]	No	21	**1 (4.8)**	20 (95.2)	**4 (19)**	17 (81)

[v] pathological tumor stage (pT); [w] Gleason score (GS); [x] prostate specific antigen (PSA) level (ng/ml) at diagnosis; [y] PC tumor stages according to World Health Organization (WHO) (1, low grade; 2, intermediate; 3, high grade); [z] prostate specific antigen (PSA) serum level increment after prostatectomy above 0.5 ng/ml; ND, not determined

4.4 The Mann-Whitney test

The Mann-Whitney test was used to examine how the *TMPRSS2:ERG* fusion gene occurrence affects patient outcome based on PSA at diagnosis (p= 0.7964) and age at diagnosis (p= 0.1193). The relationship between *TMPRSS2-ERG* fusion positive patients (n= 38) and fusion negative patients (n= 109) and pathological tumor stage (pT) was assessed using the Fisher exact test. Statistically significant differences were not observed between the groups (p= 0.4538). Additionally, the Chi-square test was used for the evaluation of how the GS or WHO values correlated with *TMPRSS2-ERG* fusion status. According to obtained results, there were no statistically significant correlations between GS and *TMPRSS2:ERG* fusion status (p= 0.9515) or between WHO values and *TMPRSS2-ERG* fusion status (p= 0.4745). The Mann-Whitney test was not used to examine the correlation between *TMPRSS2:ERG* fusion status (detected in second and third TaqMan assays: 2 out of 38 and 6 out of 38, respectively) and clinicopathological data due to the low number of fusion positive samples. Overall, there were no correlations between *TMPRSS2:ERG* fusion status and patient's clinicopathological follow-up data and H_0 was kept.

4.5 Kaplan-Meier progression-free survival analysis

Kaplan-Meier progression-free survival analysis (the time to eventual PSA recurrence after prostatectomy) and Mantel-Cox proportional hazard model were used to estimate hazard ratios (HRs) and 95% confidence interval (CI) and to evaluate the probability of progression-free survival based on *TMPRSS2:ERG* status in blood (Figure 6). A total of 147 patient samples (38 fusion positive, F+ (median PSA progression free months = 63.29)) and 109 fusion negative, F- (median PSA progression free months = 50.81)) were examined in the first Kaplan-Meier progression-free survival analysis. According to the results, patients' *TMPRSS2:ERG* fusion status did not correlate with the time to PSA recurrence after prostatectomy (P= 0.8919 by log-rank test; Hazard ratio (HR) = 0.9618; 95% CI (0.5485-1.687)) (Figure 6).

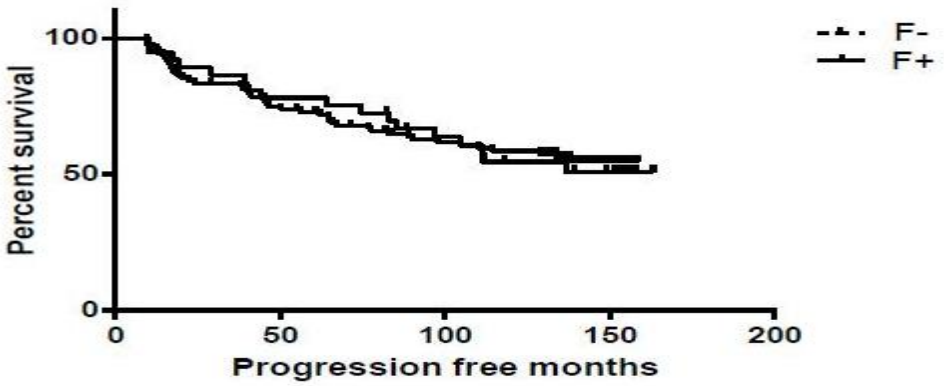

Figure 6. **Kaplan-Meier analysis of progression-free survival in *TMPRSS2:ERG* fusion negative and *TMPRSS2:ERG* fusion positive prostatectomy treated PC patients**. 1. TaqMan assay (n= 147): F-, *TMPRSS2-ERG* fusion negative (n= 109, median PSA progression free months= 50.81), F+, *TMPRSS2-ERG* fusion positive (n= 38, median progression free months= 63.29). No significant correlation between fusion status and the progression-free survival were found (P= 0.8919 by log-rank test; Hazard ratio (HR) = 0.9618; 95% CI (0.5485-1.687)).

Moreover, no significant correlations between fusion status and progression-free survival were found in second (2.) and third (3.) Kaplan-Meier analysis due to low number of fusion positive samples (data not shown).

5. DISCUSSION

Prostate cancer is a heterogeneous and complex disease with many genetic aberrations and recurring rearrangements. In order to find new molecular determinants for identification of new therapeutic targets and for better prognostication of PC patient outcome, more established, reproducible as well as accurate assays are needed. Since CellSearch (Veridex, LLC) is the only analytically validated and FDA approved method for CTC isolation and enrichment, there is an increasing interest to discover new potential non-invasive and safe methods to find predictive blood based biomarkers (CTCs) of tumor sensitivity. What makes CTC detection difficult is their rareity in PC patients' blood, accounting for approximately 1 cell in a million to a billion nucleated cells.

The main aim of this study was to detect the *TMPRSS2:ERG* fusion transcript in blood of 163 randomly selected PC patients. However, before detecting the fusion status in the real PC patients' blood samples, the sensitivity of TaqMan, SYBR Green and iScript One-Step RT-PCR assays were studied and the assay that was considered to be most sensitive was chosen for use in further analyses. Moreover, before the detection of *TMPRSS2:ERG* fusion status in the real PC blood samples, the fusion status was studied in the mixture of VCaP cells and human leukocytes.

5.1 The sensitivity of TaqMan, SYBR Green and iScript One-Step RT-PCR assays

The TaqMan assay was confirmed to be the most specific and sensitive assay to detect *TMPRSS2:ERG* fusion. The lowest quantity of VCaP cDNA needed to detect the *TMPRSS2:ERG* fusion in the mixture of VCaP and PC-3 cDNA using TaqMan assay was $5.12x10^{-2}$ pg. TaqMan assay chemistry relies on the specific binding of the probe synthesized exactly for the target gene. In order to get any fluorescent signal, specific hybridization and hydrolyzation between a precisely designed probe and target sequence is required, which also enables the direct detection of PCR products during the amplification cycles. Furthermore, non-specific binding to other DNA sequences is avoided since the TaqMan probe binds exactly to the target gene sequence of interest. Thus, the probability of non-specific amplicon formation is very low.

SYBR Green assay was confirmed to be the second most sensitive assay to detect *TMPRSS2:ERG* fusion in the mixture of VCaP and PC-3 cDNA. The lowest quantity of VCaP cDNA required to observe the *TMPRSS2:ERG* fusion gene within the cell mixture was 6.4x10 pg. SYBR Green chemistry is highly sensitive since it relies on the SYBR Green dye binding to all double-stranded (ds) DNA present in the sample including non-specific reaction products. Thus, the amplification of any dsDNA can be observed when using SYBR Green assays and the probability of getting more non-specific binding to any dsDNA is increased. In this study, primer-dimers were always detected after melting curve analysis when SYBR Green was used. Thus, the non-specific binding increased when the SYBR Green assay was used for the detection of *TMPRSS2:ERG* fusion in the mixture of VCaP and PC-3 cells.

The SYBR Green detection format is also used in the iScript One-Step RT-PCR assay. In this study the sensitivity of iScript One-Step RT-PCR assay was confirmed to be poor. Indeed, as much as 4 ng of VCaP cDNA was required to detect *TMPRSS2:ERG* fusion in the mixture of VCaP and PC-3 cDNA. The difference between iScript One-Step RT-PCR assay and the other previously described assays is that the template used in this assay is in the form of RNA and the complementary DNA synthesis as well as PCR amplification occur in the same tube. Since the reverse transcription of RNA into cDNA and the PCR amplification are carried out in the same reaction tube it is critical to add precisely the same amount of RNA in all reactions. In addition, to minimize the errors in the iScript One-Step RT-PCR assay, the extracted RNA has to be properly purified before reverse

transcription and PCR amplification in order to avoid any contaminations with additional minor illegitimate DNAs. It may be possible that the VCaP and PC-3 RNA mixture that was added to the reaction tube was not properly purified, or the quantity of RNA template mixture varied due to possible pipetting errors. It is also important to notice that when the reaction reagents were mixed together, air bubbles were constantly present in the iScript One-Step RT-PCR reaction mixture tubes. Thus, this could also be one of the reasons for the poor cDNA synthesis and PCR amplification during the RT-PCR assay.

5.2 *TMPRSS2:ERG* and *ERG* expression in the mixture of human leukocytes and VCaP cells

After validating the most sensitive assay for the detection of *TMPRSS2:ERG* gene in the mixture of PC cells (VCaP and PC-3), the *TMPRSS2:ERG* expression status was further determined in the mixture of VCaP and human leukocytes. According to the results of the present study, no expression of *TMPRSS2:ERG* could be detected in any of the VCaP and leukocyte mixtures.

ERG has been reported to be expressed in early stages of lymphocyte (early T-cell and pre B cell) development and further in mature B-cells (Rivera et *al*. 1993). The quality of VCaP and leukocyte mixtures was validated based on the *ERG* expression in the mixtures. As described previously *ERG* is expressed in leukocytes therefore it could be expected that the *ERG* expression should be detected in VCaP and leukocyte mixtures. In order to confirm that *ERG* is expressed in VCaP + leukocyte mixtures, the SYBR Green assay was used. However, *ERG* expression was only observed in the mixtures of 1 VCaP cell + leukocytes (100 ng) and 5 VCaP cells + leukocytes (100 ng). In contrast, *ERG* expression was not detected in 10, 15 and 20 VCaP cells + leukocytes (100 ng).

Due to the variable expression of *ERG* in the mixtures it may be argued that the RNA extraction or cDNA synthesis of 10, 15 and 20 VCaP cells + leukocyte mixtures were not properly performed. The extraction of VCaP + leukocyte RNA was performed with TRIzol reagent. The main advantage of the TRIzol extraction method is the high yield of total RNA. While RNA remains stable in TRIzol reagent, the isolation process is relatively long compared to other RNA extraction methods; thus, if the isolation process of RNA lasts too long at room temperature there is a possibility that RNA may be degraded.

Human leukocytes are very easily contaminated with other peripheral blood mononuclear cells during the isolation process (Nauseef W. M. 2007). In addition, if there are too many red blood cells (RBC) left in the top layer of leukocytes RBC sedimentation, and if the RBC sediment is disturbed with the pipette, there is a risk of pipetting RBCs along with leukocytes. According to Nauseef W. M. (2007), the dextran extraction method is commonly used when processing larger quantities ($\geq$ 200 ml) of blood. However, Hypague-Ficoll density gradient centrifugation is another method to isolate all of the different peripheral blood mononuclear cells from the whole blood with greater precision; it is commonly performed directly after dextran extraction of the leukocytes (Nauseef W. M. 2007). It can be argued that the risk of contamination of leukocytes may have been decreased if Hypague-Ficoll method would have been used after dextran extraction of leukocytes.

RT-PCR is one of the most frequently used methods to detect CTCs in the blood. Moreover, RT-PCR methods have been used to detect relatively low levels of circulating mRNA transcripts (propagating either from primary tumor site or from distant metastasis) in the peripheral blood, mainly in the mononuclear cell fraction of cancer patients. As mentioned earlier, qRT-PCR can even detect only one prostatic cell among million to billion peripheral mononuclear cells in blood (Moreno et *al*. 1992). However, despite the high sensitivity of qRT-PCR assays the specificity is usually relatively poor. It has been argued that illegitimate mRNA can be amplified in RT-PCR

reaction due to activation of promoters by ubiquitous transcription factors leading to high false-positive rate (Zieglschmid et *al*. 2005). Moreover, it is well known that our own immune system can detect and destroy dangerous molecules from the blood (Zamai et *al*. 2007). Thus, the mononuclear cell fraction does not necessarily include precisely the target tumor cells but instead the fraction could partly represent phagocyted tumor cells in macrophages.

5.3 The expression of *TMPRSS2:ERG* in the clinical prostate cancer blood samples

The detection frequency of *TMPRSS2:ERG* fusion transcript was confirmed to be relatively low. There were only 25.8% (38 of 147) prostatectomy treated patients who had *TMPRSS2:ERG* fusion. To confirm *TMPRSS2:ERG* fusion status (38 of 147) from the first reaction, TaqMan assay was repeated two times and the fusion positive samples were detected as follows: 5.3% (2 of 38) and 15.8% (6 of 38). It could be considered that there was a poor reproducibility and specificity in TaqMan assay due to the variable number of fusion positive samples in each reaction. Moreover, a total of 30.8% (4 of 13), 0% (0 of 4), and 25% (1 of 4) of the hormonal treated patients had the fusion. Finally, no expression of the fusion gene could be detected among radiotherapy treated patients.

The major challenges during CTC analysis are preanalytical processing of CTCs and qualitative and quantitative target CTC gene expression analysis. In order to eliminate the errors and variability of *KLK2* (kallikrein-related peptidase 2) and *KLK3* (kallikrein-related peptidase 3, i.e. PSA) expression in different steps of the assay, Helo et *al*. (2009) used target gene-specific internal standard RNA. The construction of an internal standard calibrator was described by Ylikoski et *al*. (1999). In the study by Helo et *al*. (2009) *KLK2*- and *KLK3*-specific *m3PSA* internal standard RNA was directly added into PC patients' blood samples at the beginning of RNA extraction. The cDNA synthesis was performed with sequence specific RNA primers and anchored Oligo-dT primers designed for *KLK* and internal standard RNA. An external standard curve was established with different quantities of *KLK2*, *KLK3*, and *m3PSA* mRNA, and was used for the analysis of PC patients' blood samples. In a real-time quantitative PCR assay Helo et *al*. (2009) used a target sequence-specific 5´ fluorescently labelled lanthanide chelate probe, and 5´ to 3´ exonucleolytic DNA polymerase. The difference between *m3PSA* internal standard gene expression in duplicate reactions was 0.5 Ct and the variation in *KLK2* and *KLK3* gene expression was only 1 Ct. In this study, the expression of *KLK3* or *KLK2* mRNA could be detected in the blood of 49% (37 of 76) of the CRPC patients, whereas only 8% (15 of 76) of localized PC patients had *KLK3* or *KLK2* expression, suggesting that the number of CTCs increases during the metastatic stage of PC. Therefore, it may be argued that the probability of detecting more *TMPRSS2:ERG* gene fusions in clinical PC blood samples could have possibly been increased in the present study if internal standards had been used during RNA isolation step.

In this study there were more pT2 than pT3 stage tumors. This mean there were more clinically localized PCs (i.e. tumors wheich were confined to the prostate gland) than locally advanced PCs. No metastases to other organs were reported in any of the PC patients. In addition, after prostatectomy PSA progression occurred in total of 42.9% (63 of 147) patients, whereas 57.1% (100 of 147) of prostatectomy treated patients did not have PSA progression during the follow-up period. Moreover, according to present results the expression of *TMPRSS2:ERG* fusion was more commonly found in PC recurrent cases (26.9%) compared to non-recurrent cases (25%). Since it is known that the frequency of *TMPRSS2:ERG* fusions increases from moderately to poorly differentiated tumors (Rajput et *al*. 2007), it could be argued that the possibility of detecting fusion positive CTCs may have been slightly increased had there been a larger cohort of advanced or metastatic stage of PC patients. In addition, it may be assumed that the number of CTCs and the frequency of

TMPRSS2:ERG transcript levels would have been increased if the clinical blood samples had been enriched before qualitative qRT-PCR assays.

By combining different biomarkers, Attard et *al*. (2009) characterized *ERG*, *AR*, and *PTEN* gene status in CTCs isolated from CRPC patients. Blood was collected from 89 CRPC patients who were treated with abiraterone acetate (AA), which is an androgen synthesis inhibitor. In order to isolate target CTCs, Attard and colleagues used an EpCAM-based method followed by positive selection of CK, negative selection of CD45, and DAPI staining of nucleus. In a multicolor FISH assay the expression status of the *ERG* gene was invariably the same in CRPC CTCs, metastasis, prostate tissue as well as in naïve tumors. Moreover, *ERG* gene expression persisted in CRPC and no heterogeneity of the *ERG* gene rearrangement was observed in CTCs (n=48). In contrast, heterogeneity of *AR* copy number gain and *PTEN* loss was observed, suggesting that *ERG* rearrangement may be a relatively early event in prostate tumorigenesis. Furthermore, Attard et *al*. (2009) confirmed that the isolated cells from CRPC patients were malignant CTCs (EpCAM+ CK+ CD45-). Thus demonstrating that malignant CTCs can be effectively captured from blood using immunoseparation method.

On the other hand different enrichment techniques are not necessarily the only choice for analytically valid and reproducible assays. In 2009, Helo and colleagues validated the concordance between an immunomagnetic CTC separation system (CellSearch assay) and a real-time RT-PCR assay. They found that the concordance between the real-time RT-PCR assay and the CellSearch assay was 80-85%, suggesting that the detection frequency of CTCs expressing *KLK3* and *KLK2* mRNAs is almost equal when using either the CellSearch assay or a real-time RT-PCR assay.

In 2008, Mao et *al*. studied the *TMPRSS2:ERG* fusion status in 27 prostate biopsy samples (prostatectomy-treated) and 15 CTCs (hormonal therapy) obtained from localized and advanced androgen independent PC, respectively. In this study, a total of 44% (12 out of 27) *TMPRSS2:ERG* fusion positive cases were found in prostatectomy samples using FISH. Moreover, Mao and colleagues reported that *TMPRSS2* E1:*ERG* E4 was the most common fusion isoform that was detected (9 of 12). However, despite the high frequency of fusions detected by FISH, *TMPRSS2:ERG* fusions could not be detected in 15 CTCs samples isolated from the blood of PC patients when using both standard RT-PCR and qRT-PCR assays. On the other hand, when Mao et *al*. 2008 used FISH to detect the *TMPRSS2:ERG* fusion in 10 CTCs (only 10 CTCs were available for FISH analysis) they found total of 6 fusion positive samples. Thus, it can be assumed that the detection frequency of the *TMPRSS2:ERG* fusion transcript can vary when using different techniques and biopsy samples.

Overall, one of the main reasons for the low number of *TMPRSS2:ERG* fusion positive samples detected in the blood of prostatectomy-treated patients in the present study may be explained by high variability in *TMPRSS2:ERG* amplification in TaqMan RT-PCR assays. In addition, low recovery of CTC RNA/cDNA after purification and overall purity of the RNA/cDNA obtained from the blood samples may have negatively influenced the results. Moreover, in order to eliminate the errors and variability of *TMPRSS2:ERG* gene amplification, target gene-specific internal standard RNA could have been used at the beginning of the RNA extraction. Thus, it can be assumed that immunomagnetic capture methods (EpCAM) or other specific enrichment methods could have been used to capture target CTCs. Internal controls for *TMPRSS2:ERG* target gene sequences could have been used during the preanalytic assays in order to decrease the variability of *TMPRSS2:ERG* fusion transcript expression detected by TaqMan RT-PCR assay.

5.4 Correlation between *TMPRSS2:ERG* status and clinicopathological data

One of the aims of the present study was to define whether or not there are any significant associations between PC patients' *TMPRSS2:ERG* status and clinicopathological data. Despite the fact that the molecular mechanisms of *TMPRSS2:ERG* gene fusion formation in PC tumorigenesis have been widely studied both *in vivo* and *in vitro*, the exact biological role of this fusion gene in PC remains to be fully elucidated.

As previously described, *TMPRSS2:ERG* fusion gene formation alone does not lead to the formation of prostate neoplasia. Tumorigenesis requires co-operation involving other genetic aberrations such as gain-of-function mutations in *MYC* and *EZH2* oncogenes, loss-of-function mutations in *PTEN*, *NKX3.1* and *TP53* TSGs, as well as *AR* amplification and constitutively activated serine/threonine AKT pathway are strongly involved in PC progression (Carver et *al*. 2009, King et *al*. 2009). Thus, different individual genetic alterations related to PC tumorigenesis should be considered when defining the associations between fusion status and clinicopathological data.

In the present study, the *TMPRSS2:ERG* fusion transcript was detected more frequently in patients who had pT3 tumor stage, GS either 7 or 8, and WHO either 2 or 3, which suggests that the patients who harbored the fusion had more advanced PC. PSA levels in the fusion positive patients were often found to be between 4-10 ng/ml. The results of this study are supported by the study of Rajput et *al*. (2007), in which 196 PC cases were analyzed using FISH. The *TMPRSS2:ERG* fusion was detected in a total of 101/196 PC cases. Moreover, Rajput and colleagues reported that 40.7% and 6.7% of the fusion positive cases were detected in moderately to poorly, and well differentiated tumors, respectively. The fusion positive cases were verified by RT-PCR and sequencing; the correlations identified between patients' fusion status and pT were considered to be significant.

According to the results of the present study, no statistically significant associations could be confirmed between *TMPRSS2:ERG* fusion positive patients and their clinicopathological data at diagnosis, e.g. age, pT, GS, PSA, and WHO status. This could be due to a low number of samples as well as a low and highly variable number of fusion positive patients detected overall in TaqMan assays. In addition, another supporting fact is that over 20 years, median PSA levels have only been shown to be associated with prostate size, but not with GS (Stamey et *al*. 2004). Thus, despite the long follow-up time (median follow-up 97.2 months; approximately 4 years), no significant associations were detected between clinicopathological data and fusion positive or fusion negative groups. In further studies, more samples and more sensitive analyses should be utilized to attempt to confirm the associations between *TMPRSS2:ERG* fusion status and clinicopathological data.

Prior studies have reported similar results concerning the association between fusion status and patients' clinicopathological data. Several studies (utilizing various methods) have reported that there are no significant associations between clinicopathological features (either GS or tumor stage) and *TMPRSS2:ERG* fusion positive cancers (Goapalan et *al*. 2009, Lapointe et *al*. 2007, Saramäki et *al*. 2008, Tu et *al*. 2007). For example, in the FISH study performed by Goapalan et *al*. (2009), *TMPRSS2:ERG* fusion positive tumors were found in 42% of 521 prostatectomy treated patients. Goapalan and colleagues found that 63% of the *TMPRSS2:ERG* fusion formations occurred by intergenic deletion of chromosome 21. Despite the large sample size of the cohort and long term follow-up period (medium follow-up time 95 months), *TMPRSS2:ERG* status could not be considered to be significantly associated with pT, biochemical recurrence, metastasis, or death. However, in contrast to the present study, Goapalan et *al*. (2009) found that *TMPRSS2:ERG* rearrangement alone is associated with lower tumor grade.

The strength of the present study was the long-term follow-up period (median follow-up 97.2 months), however, had there been a greater number of samples, it may have been possible to identify a better correlation between patients' clinicopathological data and their fusion status. According to these results, it can be concluded that *TMPRSS2:ERG* fusion status in the blood cannot be used as a predictive biomarker using the TaqMan assay alone. Moreover, since *TMPRSS2:ERG* fusion gene is androgen regulated, it could be argued that the combination of different biomarkers – such as different androgen regulated genes – may have been better in the prediction of patients' outcome according to clinicopathological features. Thus, in order to predict patient outcome according to individual's *TMPRSS2:ERG* status, more sensitive methods, larger sample cohorts, and eventually more combined biomarkers are needed.

In 2004, Mitsiades and colleagues reported that the combined detection of *PSMA* and *PSA* in nested RT-PCR increased the specificity of the nested RT-PCR reaction. They performed nested PCR for *PSA* and *PSMA* transcripts and found that the combined expressions of *PSA* and *PSMA* transcripts found in peripheral blood leukocytes (PBL) and in bone marrow (BM) samples indicated a significantly shorter time to PSA level increment after prostatectomy. Moreover, in 2010 Joung and colleagues reported that membrane surface glycoprotein *PSCA* mRNA detected by nested RT-PCR in the peripheral blood of high-risk PC patients is a significant factor indicative of PC recurrence after prostatectomy. These results suggest that different prostate tumor antigens detected in CTCs may have prognostic value. On the other hand, Thomas et *al*. (2002) could not confirm the clinical value of *PSA* and *PSMA* detected by combined nested RT-PCR.

5.5 Prognostic value of *TMPRSS2:ERG* status in blood of prostate cancer patients

One of the aims of the present study was to validate if *TMPRSS2:ERG* fusion status could be a possible prognostic tool to help predict PC patients' outcome and survival. In the present study *TMPRSS2:ERG* fusion was detected in 26.9% (17 of 63) of the recurrent PC cases in the first TaqMan assay. On the other hand, total of 25% (21 of 84) PC patients who did not have PC recurrence after prostatectomy possessed the *TMPRSS2:ERG* fusion. In the second TaqMan assay, there was a total of 5.9% of fusion positive cases with PC recurrence, whereas 4.8% of non-recurrent PC patients expressed the fusion. Moreover, in the third TaqMan assay, *TMPRSS2:ERG* fusions were detected more frequently (19%; 4 of 20) in non-recurrent PC cases compared to recurrent PC cases 11.8% (2 of 16). Thus, the frequency of *TMPRSS2:ERG* fusion was slightly increased in recurrent PC cases compared to non-recurrent PC cases (except in the last TaqMan assay).

Interestingly, the time to PSA recurrence was notably longer for the patients who had *TMPRSS2:ERG* fusion (63.29 months) compared to fusion negative PC patients (50.81 months). Thus, it could be argued that those patients who harbored the *TMPRSS2:ERG* fusion had more favorable prognosis compared to fusion negative patients. However, the difference between *TMPRSS2:ERG* status in recurrent and non-recurrent PC patients could not be considered to be significant in any assays. Thus, no definitive conclusions can be drawn from these results. The present results are supported by other studies, since there have been several controversial results regarding the prognostic value of *TMPRSS2:ERG* status **(**Fitzgerald et *al*. 2007, Goapalan et *al*. 2009, Hermans et *al*. 2009, Lapointe et *al*. 2007, Saramäki et *al*. 2008, Wang et *al*. 2006).

TMPRSS2:ERG fusion status has been shown to be associated both positively and negatively with the outcome of PC patients. Fitzgerald et *al*. (2007) and Wang et *al*. (2006) observed poor outcome in patients harboring the fusion. Furthermore, Wang et *al*. (2006) showed that the fusion transcript variants of *TMPRSS2* E1:*ERG* E2, *TMPRSS2* E1:*ERG* E3, and *TMPRSS2* E2:*ERG* E4 are involved in pathogenic progression of aggressive PC, seminal vesicle invasion, and early PSA recurrence. Thus,

the expression of *TMPRSS2:ERG* fusion transcript variants may negatively affect patient outcome. In contrast, Hermans et *al*. (2009) and Saramäki et *al*. (2008) found that PC patients harboring the *TMPRSS2:ERG* fusion have more favorable prognoses. Conversely, different studies have also reported that there are no significant correlations between fusion status and clinical prognosis (Goapalan et *al*. 2009, Lapointe et *al*. 2007).

In 2011, Danila and colleagues showed that *TMPRSS2:ERG* fusion status in CTCs is a predictive biomarker of sensitivity after AA treatment. They used the CellSearch system to enumerate CTCs, a TaqMan assay to confirm the fusion status in CTCs isolated from CRPC patients, and finally, FISH analysis in primary tumor samples to confirm the results. They found total of 37% (15 of 41) of fusion positive CRPC patients had a median CTC count of 17 in 7.5 ml of blood. After AA treatment a PSA decline ≥50% was found in 47% (7 of 15) of fusion positive patients and in 38% (10 of 26) of fusion negative patients. Danila et *al*. (2011) reported that patients who had five or more CTCs in their circulation after 4 weeks of AA therapy had significantly shorter overall survival. However, Danila and colleagues confirmed that there were no associations between *TMPRSS2:ERG* status and PSA decline and there were no significant differences in overall survival between fusion positive and fusion negative patients.

In the present study, *TMPRSS2:ERG* status could not be considered to be associated with patient outcome: a conclusion which is supported by several previous studies as described above. Controversial results of the prevalence and prognostic significance of *TMPRSS2:ERG* fusion status observed in different studies could be explained by the fact that researchers have been using different techniques for the detection and characterization of *TMPRSS2:ERG* gene rearrangements in blood and PC tissues. Moreover, high variability of novel potential *TMPRSS2:ERG* fusion products, in addition to individual genetic differences may have certain effects when defining patient outcome in relation to *TMPRSS2:ERG* fusion status. In order to be able to acurately predict patient outcome and discriminate between low and high risk patient groups, more combined prostate tumor-specific biomarkers, reliable assays, and larger sample cohorts are required.

6. CONCLUSIONS

According to the results of the present study, the TaqMan assay can be confirmed to be a more sensitive method for qualitative gene expression analysis than SYBR Green and iScript One-Step RT-PCR assays. However, the TaqMan assay cannot be considered to be a sufficiently sensitive and specific method in the detection of *TMPRSS2:ERG* fusion in the blood of PC patients without valid preanalytical assays. The efficiency of preanalytic target CTC isolation could be increased by using target gene-specific internal controls at the beginning of the assay, starting at the RNA isolation stage. Furthermore, different enrichment, isolation, and characterization methods could potentially increase the probability of detecting more target CTCs before validating *TMPRSS2:ERG* fusion status in the blood of PC patients by qualitative TaqMan gene expression assays. Moreover, due to the heterogeneic nature of PC and individual genetic variability, *TMPRSS2:ERG* fusion status in blood of PC patients cannot be used alone to determine patient prognosis and outcome. Therefore, in order to accurately separate tumors into distinct prognostic categories, and to better predict the outcome of the patients, more variable combinations of tumor- and tissue-specific, as well as blood based biomarkers are needed. In the future, it would be interesting to evaluate how *TMPRSS2:ERG* fusion could be used in combination with other biomarkers in order to better predict and monitor patients' prognosis.

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APPENDIX

APPENDIX 1. Clinical information of the prostate cancer patients

Nr	PSA	Age	WHO	pT	Gleason	GS	Primary treatment	PC diagnosis	PSA progression	Updated cancer registry
1	21	68,3	1	3a	3+2	5	Prostatectomy	1998-12-17	2000-10-18	2009-04-24
2	10,6	71,9	3	2a	4+5	9	Prostatectomy	1998-12-01	2000-08-04	2009-04-24
3	10,4	60,4	2	2b	3+4	7	Prostatectomy	1999-01-13	2000-02-08	2009-04-24
4	3,6	58,1	3	3a	4+4	8	Prostatectomy	1993-02-23	2000-01-14	2009-04-24
5	6,8	61,6	2	3a	3+3	6	Prostatectomy	1999-02-09	2007-10-24	2009-04-24
6	6	62,8	2	3a	3+3	6	Prostatectomy	1999-01-13	No progr.	2009-04-24
7	7,4	80,2	2	ND 1.	3+3	6	Horm. therapy 3.	1999-01-29	ND	1999-01-29
8	2,9	74,4	1	ND 2.	3+3	6	Horm. therapy 3.	1999-02-01	ND	1999-02-01
9	8,4	62,9	2	2	4+3	7	Prostatectomy	1999-01-25	2000-01-14	2009-04-24
10	43,3	71,8	2	3b	4+4	8	Prostatectomy	1999-02-12	2000-05-31	2009-04-24
11	16,7	68,4	1	2b	3+3	6	Prostatectomy	1999-02-04	No progr.	2009-04-24
12	5,21	62,8	2	3a	3+4	7	Prostatectomy	1999-03-12	No prog.	2009-04-24
13	19,5	62,3	1	2b	2+3	5	Prostatectomy	1999-02-01	No prog.	2006-06-06
14	5,2	58,8	2	2b	3+4	7	Prostatectomy	1999-03-11	2009-12-01	2009-04-24
15	0,5	71,4	2	2	3+3	6	Prostatectomy	1998-12-18	No progr.	ND
16	3,1	67,8	2	3b	3+3	6	Prostatectomy	1999-01-29	2003-04-10	2009-04-24
17	9	66,8	2	3a	4+3	7	Prostatectomy	1999-04-13	No progr.	2009-04-24
18	3,6	70,4	2	3a	3+3	6	Prostatectomy	1999-04-16	No progr.	2009-04-24
19	5	59,6	2	3a	3+3	6	Prostatectomy	1999-04-01	No progr.	2009-04-24
20	10,3	54,6	2	3a	3+3	6	Prostatectomy	1999-04-01	No progr.	2008-04-01
21	6,95	59	3	3a	4+5	9	Prostatectomy	1999-04-13	2000-02-08	2009-04-24
22	15,7	66,8	2	3a	3+3	6	Prostatectomy	1999-03-26	No progr.	2009-04-24
23	6,7	63,7	1	3a	2+3	5	Prostatectomy	1999-03-30	No progr.	2009-04-24
24	5,6	65,8	3	2b	5+4	9	Prostatectomy	1999-06-03	2002-09-17	2009-04-24
25	4,16	54,8	2	2	3+4	7	Prostatectomy	1999-03-30	No progr.	2009-04-24
26	7,3	66,6	1	2	3+3	6	Prostatectomy	1999-05-26	No progr.	2009-04-24

27	27,3	62,3	2	3b	4+3	7	Prostatectomy	1999-05-28	2000-11-02	2009-04-24
28	9,72	55	2	3a	3+4	7	Prostatectomy	1999-06-29	2008-10-21	2009-04-24
29	6,7	60,8	2	2	3+4	7	Prostatectomy	1999-05-26	2000-03-21	2009-04-24
30	4,9	52,1	2	3a	3+4	7	Prostatectomy	1999-06-07	2000-10-16	2009-04-24
31	8,7	61,7	2	2b	3+4	7	Prostatectomy	1999-06-07	2010-10-25	2009-04-24
32	29,3	55,3	2	3b	4+4	8	Prostatectomy	1999-05-10	2004-09-28	2009-04-24
33	6	68,2	1	3a	2+3	5	Prostatectomy	1999-06-10	No progr.	2009-04-24
34	27,5	54,7	2	3a	3+4	7	Prostatectomy	1999-05-01	2000-03-23	2006-06-06
35	9,5	67,9	3	3b	5+4	9	Prostatectomy	1999-06-01	No progr.	2009-04-24
36	9,2	61,6	1	2	3+2	5	Prostatectomy	1999-04-14	No progr.	2009-04-24
37	11,5	55,6	2	3b	3+4	7	Prostatectomy	1999-05-28	No progr.	2006-06-06
38	11	69,6	1	3a	2+3	5	Prostatectomy	1999-07-09	No progr.	2009-04-24
39	13,5	62,7	3	3b	4+5	9	Prostatectomy	1999-06-18	2002-09-30	2009-04-24
40	5,8	63,7	2	3a	3+4	7	Prostatectomy	1999-07-23	2004-09-22	2009-04-24
41	7,5	67,3	2	2b	3+5	8	Prostatectomy	1999-07-01	2000-05-16	2009-04-24
42	7,3	58,8	1	ND 1.	3+3	6	Radiotherapy	1999-07-13	No progr.	2009-04-24
43	8,3	69,6	2	3a	3+4	7	Prostatectomy	1999-08-04	No progr.	2009-04-24
44	3,4	63,2	1	2b	3+2	5	Prostatectomy	1999-08-17	No progr.	2009-04-24
45	4,13	67,3	2	3b	3+4	7	Prostatectomy	1999-08-13	No progr.	2009-04-24
46	41,2	64,8	2	3b	4+3	7	Prostatectomy	1999-08-13	2002-10-28	2009-04-24
47	27	68,3	2	2b	ND	ND	Prostatectomy	1998-08-25	No progr.	2009-04-24
48	4,24	63,3	1	2b	3+4	7	Prostatectomy	1999-07-12	No progr.	2009-04-24
49	8,6	68,9	1	2b	3+3	6	Prostatectomy	1999-08-20	No progr.	2009-04-24
50	13,6	66,6	3	3b	5+4	9	Prostatectomy	1999-08-13	No progr.	2008-04-01
51	6,3	71,2	3	2b	4+4	8	Prostatectomy	1999-08-06	No progr.	1999-08-06
52	6	68,3	1	2	3+2	5	Prostatectomy	1999-07-13	No progr	2009-04-24
53	3,9	55,7	1	2b	2+2	4	Prostatectomy	1999-09-13	No progr.	2009-04-24
54	5,9	57,6	1	2b	2+3	5	Prostatectomy	1999-07-12	No progr.	2009-04-24
55	6,2	62,5	1	2b	3+3	6	Prostatectomy	1999-07-23	2004-11-24	2009-04-24
56	5,4	69,3	1	2b	3+2	5	Prostatectomy	1999-09-01	No progr.	2009-04-24

57	9,1	66,9	1	3a	3+2	5	Prostatectomy	1999-09-09	2007-10-10	2009-04-24
58	18,1	82,5	3	ND 1.	4+5	9	Horm. therapy 3.	1999-10-05	ND	2006-06-06
59	10,2	76,9	2	ND 1.	3+3	6	Horm. therapy 3.	1999-09-23	ND	2009-04-24
60	3	66,8	1	2b	2+3	5	Prostatectomy	1999-06-11	No progr.	2009-04-24
61	9,05	67,8	3	3a	5+4	9	Prostatectomy	1999-10-07	No progr.	2009-04-24
62	8,2	65,5	3	3b	4+4	8	Prostatectomy	1999-10-14	No progr.	2009-04-24
63	11	58,6	1	3a	3+2	5	Prostatectomy	1999-09-10	2008-11-13	2009-04-24
64	7,3	59,8	2	2b	3+3	6	Prostatectomy	1999-10-14	No progr.	2009-04-24
65	31,8	67,1	2	ND 1.	4+3	7	Horm. therapy 4.	1999-11-16	No progr.	1999-11-16
66	17	68	2	3b	4+3	7	Prostatectomy	1998-10-05	1999-07-19	1998-10-05
67	5,5	56,1	2	2b	3+3	6	Prostatectomy	1999-11-01	No progr.	2009-04-24
68	5,6	61,1	2	2b	3+3	6	Prostatectomy	1999-11-01	No progr.	2006-06-06
69	3	56,8	2	2b	3+2	5	Prostatectomy	1999-11-05	No progr.	2009-04-24
70	7,6	60,5	2	2b	3+3	6	Prostatectomy	1999-11-10	No progr.	2009-04-24
71	76	76,8	2	ND 1.	3+4	7	Horm. therapy 3.	2000-01-01	No progr.	2000-01-01
72	2480	74,7	2	ND 1.	3+5	8	Horm. therapy 3.	2000-01-04	No progr.	2006-06-06
73	21	67,1	3	3b	4+5	9	Prostatectomy	1999-12-13	2003-09-16	2009-04-24
74	8,9	60,8	2	2b	3+4	7	Prostatectomy	1999-11-01	No progr.	2009-04-24
75	9,6	67,4	2	2b	2+4	6	Prostatectomy	1999-12-13	No progr.	2009-04-24
76	5,7	59,8	2	2b	3+4	7	Prostatectomy	1999-12-17	2003-08-18	2009-04-24
77	4,4	63,3	2	2b	3+4	7	Prostatectomy	1999-12-29	No progr.	ND
78	4,2	60,9	2	2b	3+4	7	Prostatectomy	1999-12-29	2006-11-20	2009-04-24
79	5	63,3	3	3a	4+5	9	Prostatectomy	1999-12-30	2003-11-03	2009-04-24
80	4,57	62,8	2	2b	2+4	6	Prostatectomy	1999-06-09	No progr.	2009-04-24
81	6,7	61,8	1	2b	2+3	5	Prostatectomy	2000-01-31	No progr.	2009-04-24
82	13	64,9	2	2b	4+4	8	Prostatectomy	2000-01-12	No progr.	2009-04-24
83	10,9	59,3	2	ND 1.	4+3	7	Horm. therapy 4.	2000-02-01	ND	2000-02-01
84	12,9	65,8	2	3b	4+3	7	Prostatectomy	2000-02-15	2003-11-27	2000-02-15
85	9,5	59,8	2	3a	4+3	7	Prostatectomy	2000-02-11	2005-09-08	2009-04-24
86	6,2	58,2	2	ND 1.	3+3	6	Radiotherapy 5.	2000-01-13	ND	2006-06-06
87	9,7	58,7	2	2a	3+3	6	Prostatectomy	2000-01-28	No progr.	2009-04-24

88	4,1	71	2	3a	4+3	7	Prostatectomy	2000-03-21	2001-08-31	2009-04-24
89	7,9	65,9	1	2	3+2	5	Prostatectomy	2000-02-17	No progr.	2009-04-24
90	5,9	65,5	2	2b	3+4	7	Prostatectomy	1999-07-13	No progr.	2006-06-06
91	13,1	60,3	2	2b	3+4	7	Prostatectomy	2000-04-10	No progr.	2009-04-24
92	16,2	64,8	2	3a	3+4	7	Prostatectomy	2000-03-09	2007-08-01	2009-04-24
93	14,8	62,4	2	3a	3+4	7	Prostatectomy	2000-04-05	2001-07-13	2009-04-24
94	5,6	53,6	2	2	3+3	6	Prostatectomy	2000-04-04	2009-10-22	2009-04-24
95	412	69,3	2	ND 1.	4+3	7	Horm. therapy 4.	2000-06-30	ND	2009-04-24
96	12,4	58,6	2	3a	3+4	7	Prostatectomy	2000-05-15	No progr.	2009-04-24
97	8	67,3	3	3a	4+5	9	Prostatectomy	2000-05-18	2006-10-19	2009-04-24
98	8,2	67,3	2	2b	3+4	7	Prostatectomy	2000-05-16	2011-11-16	2009-04-24
99	16,7	55,2	2	2b	3+4	7	Prostatectomy	2000-04-27	2007-10-29	ND
100	7	56,8	3	3b	4+3	7	Prostatectomy	2000-05-15	2001-10-02	2009-04-24
101	3,1	68,8	2	2b	3+3	6	Prostatectomy	2000-07-20	2009-10-26	ND
102	2,8	63,2	2	2b	3+5	8	Prostatectomy	2000-06-21	2011-09-14	2009-04-24
103	6,6	66,8	2	2b	3+2	5	Prostatectomy	2000-06-20	No progr.	2009-04-24
104	1,1	47,8	1	2b	2+3	5	Prostatectomy	2000-06-08	No progr.	ND
105	3,3	58,6	1	2b	2+3	5	Prostatectomy	2000-03-24	2006-09-12	ND
106	3,9	60,1	2	2b	3+4	7	Prostatectomy	2000-05-29	No progr.	ND
107	12	67,1	1	2	2+2	4	Prostatectomy	2000-06-19	No progr.	ND
108	8,2	67,9	2	3a	3+2	5	Prostatectomy	2000-07-11	2002-12-09	ND
109	48,1	58,1	2	3a	3+4	7	Prostatectomy	2000-07-17	2003-11-21	2009-04-24
110	7,54	66,7	2	3a	3+3	6	Prostatectomy	2000-08-08	2009-04-28	2009-04-24
111	7	67,8	2	ND 1.	3+3	6	Radiotherapy	2000-08-31	ND	2009-04-24
112	7	72,9	2	2	3+3	6	Prostatectomy	2000-09-05	No progr.	2009-04-24
113	19	68,1	2	3b	4+3	7	Prostatectomy	2000-08-04	2002-01-31	2009-04-24
114	4,1	59,6	2	2b	3+4	7	Prostatectomy	2000-08-22	No progr.	2009-04-24
115	8,2	62,8	2	3a	4+3	7	Prostatectomy	2000-08-21	No progr.	2009-04-24
116	10,1	60,2	2	2b	4+3	7	Prostatectomy	2000-08-25	2006-11-09	2009-04-24
117	13,2	59,1	1	2b	3+2	5	Prostatectomy	2000-07-11	No progr.	2009-04-24
118	5,7	59,6	2	2b	3+4	7	Prostatectomy	2000-08-23	No progr.	2009-04-24

119	4,1	67	3	3a	3+5	8	Prostatectomy	2000-08-24	2007-10-09	2009-04-24
120	4,3	63,6	1	2b	3+2	5	Prostatectomy	2000-08-22	No progr.	2009-04-24
121	5,5	59,3	1	2b	3+3	6	Prostatectomy	2000-05-16	No progr.	ND
122	8,2	61,2	2	2b	3+4	7	Prostatectomy	2000-03-28	No progr.	ND
123	5,2	60,9	1	2b	3+2	5	Prostatectomy	2000-11-15	No progr.	2009-04-24
124	4,4	67,7	1	2b	3+2	5	Prostatectomy	2000-10-02	2006-02-21	2009-04-24
125	8,2	53,6	2	2	3+3	6	Prostatectomy	2000-10-05	No progr.	2009-04-24
126	6,1	67,5	2	2b	3+2	5	Prostatectomy	2000-08-17	No progr.	2009-04-24
127	15,8	66,5	2	2b	4+4	8	Prostatectomy	1998-09-24	2000-09-26	2009-04-24
128	29,4	80	2	ND 1.	4+5	9	Horm. therapy 3.	2000-11-14	ND	2000-11-14
129	13,4	65,2	2	2b	3+4	7	Prostatectomy	2000-12-21	2002-05-21	2009-04-24
130	4,8	53	3	3a	5+5	10	Prostatectomy	2001-01-01	No progr.	2006-06-06
131	4,1	49,6	2	2a	3+3	6	Prostatectomy	2001-02-05	No progr.	2009-04-24
132	10,3	67,3	2	3a	2+4	6	Prostatectomy	2001-02-06	No progr.	2009-04-24
133	10,1	62,6	2	3b	4+4	8	Prostatectomy	2000-12-13	2002-07-26	2009-04-24
134	7,5	60	2	2b	4+3	7	Prostatectomy	2001-02-16	2004-04-28	2009-04-24
135	9,3	63,3	2	2b	4+3	7	Prostatectomy	2001-01-05	No progr.	2009-04-24
136	8,48	54,8	2	2b	3+3	6	Prostatectomy	2001-02-08	No progr.	2009-04-24
137	9,7	59,5	2	3b	3+4	7	Prostatectomy	2001-01-22	2004-12-07	2009-04-24
138	9,9	67	2	2b	3+4	7	Prostatectomy	2000-06-19	No progr.	2009-04-24
139	11,4	74,8	2	ND 1.	3+4	7	Horm. therapy 3.	2001-05-15	ND	2001-05-15
140	12,3	59,1	1	2b	3+2	5	Prostatectomy 6.	2000-11-02	No progr.	2000-11-02
141	7,4	54,7	2	3a	3+3	6	Prostatectomy	2001-03-13	No progr.	2009-04-24
142	7,6	62,3	2	3a	3+4	7	Prostatectomy	2001-02-21	No progr.	2009-04-24
143	15,3	61,8	2	2b	4+3	7	Prostatectomy	2001-03-14	2002-05-20	2009-04-24
144	42,5	77,5	2	ND 1.	4+4	8	Horm. therapy 4.	2001-06-26	ND	2001-06-26
145	9,3	61,9	2	3a	4+3	7	Prostatectomy	2001-04-05	2002-12-13	2009-04-24
146	3,8	64,9	2	2b	3+3	6	Prostatectomy	2001-07-01	No progr.	2009-04-24
147	6,5	66,6	2	2b	3+4	7	Prostatectomy	2001-02-09	No progr.	2009-04-24
148	17	61,7	2	3a	4+4	8	Prostatectomy	2001-03-22	No progr.	2009-04-24
149	6,2	64	2	2b	3+4	7	Prostatectomy	2001-03-07	No progr.	2009-04-24

150	**3,7**	**61,9**	**2**	**2b**	**3+4**	**7**	**Prostatectomy**	**2001-04-01**	**No progr.**	**2009-04-24**
151	**17,4**	**67,6**	**2**	**3b**	**3+4**	**7**	**Prostatectomy**	**2001-03-28**	**2004-08-09**	**2009-04-24**
152	**6**	**69,7**	**2**	**2**	**4+3**	**7**	**Prostatectomy**	**2001-04-30**	**2004-09-28**	**2009-04-24**
153	**12**	**67,1**	**2**	**2**	**3+3**	**6**	**Prostatectomy**	**2000-03-24**	**2004-12-02**	**2009-04-24**
154	**4,48**	**62,3**	**2**	**3a**	**3+3**	**6**	**Prostatectomy**	**2001-03-13**	**No progr.**	**2009-04-24**
155	**95,3**	**71,7**	**3**	**ND 1.**	**5+5**	**10**	**Horm. therapy 4.**	**2001-07-10**	**ND**	**2001-07-10**
156	**7,5**	**71,8**	**2**	**3b**	**3+5**	**8**	**Prostatectomy**	**2001-05-04**	**2002-04-05**	**2009-04-24**
157	**4,2**	**67,2**	**2**	**2b**	**2+3**	**5**	**Prostatectomy**	**2001-05-08**	**No progr.**	**2009-04-24**
158	**5,3**	**58,7**	**2**	**2b**	**3+3**	**6**	**Prostatectomy**	**2001-05-03**	**2009-06-23**	**2009-04-24**
159	**8,6**	**62,7**	**2**	**2b**	**3+4**	**7**	**Prostatectomy**	**2001-05-02**	**No progr.**	**2006-06-06**
160	**8,4**	**62,6**	**2**	**2a**	**3+4**	**7**	**Prostatectomy**	**2001-05-03**	**No progr.**	**2009-04-24**
161	**13,3**	**68,8**	**2**	**2b**	**4+3**	**7**	**Prostatectomy**	**2000-11-29**	**No progr.**	**2009-04-24**
162	**2,4**	**61,9**	**2**	**2b**	**3+4**	**7**	**Prostatectomy**	**2001-05-15**	**No progr.**	**2009-04-24**
163	**7,8**	**59,4**	**2**	**3a**	**4+3**	**7**	**Prostatectomy**	**2001-06-04**	**2006-11-22**	**2009-04-24**

1. Pathological tumor stage (pT) not determined due to biopsy atypia; **2**. Pathological tumor stage (pT) not determined due to transurethral resection of the prostate gland (TURP); **3**. Surgical castration; **4**. Chemical castration; **5**. Total Androgen Blockade (TAB); **6**. Antiandrogen treatment; **ND**, not determined; **PSA**, prostate specific antigen; **WHO**, tumor stage according to World Health Organization; **pT**, pathological tumor stage; **GS**, Gleason score; **PC diagnosis**, prostate cancer diagnosis; **PSA progression**., prostate specific antigen progression (increase in PSA levels > 0.5 ng/ml after primary treatment); **No progr**., No prostate specific antigen progression after primary treatment.