



**CHARACTERIZATION OF FUSION ONCOGENES INVOLVING THE
ETS FAMILY OF TRANSCRIPTION FACTORS AND THEIR ROLE
AS DIAGNOSTIC AND PROGNOSTIC MARKERS IN PROSTATE
CANCER**

JOÃO DIOGO BARROS SILVA

Tese de doutoramento em Ciências Biomédicas

2013

JOÃO DIOGO BARROS SILVA

Characterization of fusion oncogenes involving the ETS family of transcription factors and their role as diagnostic and prognostic markers in prostate cancer

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador:

Doutor Manuel Teixeira

Categoria - Professor Catedrático Convidado

Afiliação - Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Co-orientador:

Doutor Rui Henrique

Categoria - Professor Auxiliar Convidado

Afiliação - Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Supervisores externos:

Doutora Ragnhild Lothe

Doutor Rolf Skotheim

Instituição de acolhimento - Department of Cancer Prevention and Centre for Cancer Biomedicine, University of Oslo, Norway

Publicações

Ao abrigo do disposto do n.º 2, alínea a) do artigo 31º do Decreto-Lei n.º 230/2009 fazem parte integrante desta tese de doutoramento os seguintes trabalhos já publicados:

Artigo I

Paulo P*, **Barros-Silva JD***, Ribeiro FR, Ramalho-Carvalho J, Jerónimo C, Henrique R, Lind GE, Skotheim RI, Lothe RA, Teixeira MR.

FLI1 is a novel ETS transcription factor involved in gene fusions in prostate cancer.

Genes Chromosomes Cancer. 2012 Mar;51(3):240-9

*** these authors contributed equally to this work**

Artigo II

Barros-Silva JD*, Paulo P*, Bakken AC, Cerveira N, Løvf M, Henrique R, Jerónimo C, Lothe RA, Skotheim RI, Teixeira MR.

Novel 5' fusion partners of ETV1 and ETV4 in prostate cancer.

Neoplasia. 2013 Jul;15(7):720-6.

*** these authors contributed equally to this work**

Artigo III

Barros-Silva JD, Ribeiro FR, Rodrigues A, Cruz R, Martins AT, Jerónimo C, Henrique R, Teixeira MR.

Relative 8q gain predicts disease-specific survival irrespective of the TMPRSS2-ERG fusion status in diagnostic biopsies of prostate cancer.

Genes Chromosomes Cancer. 2011 Aug;50(8):662-71.

“In the last few millennia we have made the
most astonishing and unexpected
discoveries [...].
They remind us that humans have evolved
to wonder, that understanding is a joy, that
knowledge is prerequisite to survival.”

Carl Sagan, *Cosmos*

Preface

Looking back, I can easily identify the two “hits” that started the cascade of events that lead to this thesis. The first dates back to the late 1980’s when I was an attentive spectator of the French animated series *Il était une fois... la vie* (Once upon a time... life) created by Albert Barillé. Without realizing, I was being introduced to basic concepts of human life sciences in a subtle yet accurate way. Years latter, while studying molecular and cell biology at the university’s library, I came across a final paragraph of a book chapter relating cell cycle deregulation and cancer. That spark irreversibly ignited my curiosity about the biological grounds of cancer. It was the second “hit”. After completing the Applied Biology course, applying for the MSc Programme in Molecular Medicine and Oncology was a natural step. The curriculum plan was appealing: lectures with national and guest international researchers, followed by a research project. During these lectures I was acquainted with the work of Professor Manuel Teixeira, who accepted to be my MSc thesis supervisor and, a couple of years latter, my PhD supervisor.

The work presented in this thesis was performed in the Department of Genetics of the Portuguese Oncology Institute – Porto, and in the Department of Cancer Prevention of the Institute for Cancer Research – Oslo. It is a result of a collaboration of a multidisciplinary and multinational team. Professionally, it was an enriching experience, allowing me to evolve, to learn new lab skills and writing skills. From a personal point of view it was equally rewarding. The months I spent in Norway were a test to my adaptation abilities. It turned out to be easier than I expected. The warm and friendly way everyone received me at the lab contributed greatly to this smooth adaptation. Excluding the obvious phenotypical and cultural differences, we are more alike than I thought.

I feel privileged to have as national and foreign supervisors such talented researchers. It’s a pleasure to work with intelligent, enthusiastic and hard-working people. Their example reminds me that there is always room for improvement.

Acknowledgements

I am deeply grateful to Professor Manuel Teixeira for his wise guidance throughout this work. Our discussions of the work in progress transmitted me confidence and motivation. Your thorough and accurate revisions were invaluable lessons.

I would like to express my gratitude to Professor Rui Henrique for his remarkable work of putting together the sets of samples studied in this work. Thank you for the pertinent inputs provided and for always being available to help with your expertise.

I am deeply thankful to Professor Ragnhild Lothe for allowing me to work in the Department of Cancer Prevention and to Rolf Skotheim for integrating me in his group (Genome Biology), constantly accompanying the performed work. Thank you Anne Cathrine for being the best lab host one can possibly wish for. I would like to thank everyone in the department for making me feel like home, especially Matthias, Torfinn, Kim, Stine and Gard.

I would like to show my gratitude to the members of the Cancer Genetics group that made an important contribution to this thesis, Ricardo and Paula. Likewise, I would like to thank the Cancer Epigenetics group leader Professor Carmen Jerónimo and João for their valuable contribution to this thesis.

I owe a deep thank to all the clinicians of IPO-Porto who dedicated some of their time to gather clinical and follow-up data, essential for the publications here presented.

I am thankful to everyone in the Department of Genetics particularly my fellow researchers Joana, Pedro, Márcia, Diogo, Filipa, “Júlio”, Elsa, Rui, Maria, Inês and also the cytogeneticists Susana, Joana, Lurdes and Cecília for keeping a good and friendly atmosphere.

Acknowledgements

I would like to thank the Fundação para a Ciência e Tecnologia and also the Liga Portuguesa Contra o Cancro for the financial support.

To my family, for their everlasting support.

To my un-inherited family, my friends. Those whom I see regularly and the ones scattered throughout the world. Every time we get together is a memorable event. Thanks for your genuine friendship.

Finally, thank you Rita for being by my side, for making me happy.

Summary

Prostate carcinoma (PCa) is one of the most common malignant neoplasms worldwide and a significant cause of cancer-related morbidity and mortality. The great variability of tumor behavior constitutes a major issue for clinical management of this disease. The current trend of world population ageing strongly suggests that the medical burden of this disease will become increasingly important in the near future. In order to face these challenges it is essential to deepen the knowledge of the molecular mechanisms of prostate carcinogenesis and also to find novel genetic biomarkers with prognostic value. The discovery of recurrent genomic rearrangements involving several members of the ETS transcription factor family constitutes an important step forward to unravel the mechanisms of prostatic carcinogenesis, but further studies are necessary to fully understand the biological and clinical impact of these rearrangements. Apart from gene fusions, other genomic rearrangements, particularly relative 8q chromosomal gains, have been deemed of clinical importance.

The aims of this thesis are divided in two major topics. One was to perform a comprehensive characterization of the fusion oncogenes affecting ETS transcription factors in PCa, and to identify new fusion oncogenes in the about 50% of PCa that do not harbor rearrangements of *ERG*, *ETV1*, *ETV4* or *ETV5*. Additionally, novel 5' partners involved in ETS gene fusions were sought. For this first topic, tumor samples from a cohort of 200 patients with clinically localized PCa consecutively diagnosed and treated with radical prostatectomy were studied. The other major aim was to evaluate the prognostic value of ETS rearrangements alone and in combination with relative 8q gain in PCa patients spanning all histological grades and clinical stages, including an additional series of archival diagnostic biopsies from 200 PCa patients with long follow up. The data generated by this work resulted in 3 publications that are part of this thesis.

In **Paper I**, the relative expression level of ETS family genes previously found rearranged in PCa (*ERG*, *ETV1*, *ETV4*, *ETV5*), Ewing sarcoma (*FLI1*, *ERG*, *ETV1*, *ETV4* and *FEV*) or leukemia (*ERG* and *ETV6*), and additional ETS genes found overexpressed (*ELK4*) or localized between two genes commonly rearranged (*ETS2*), was analyzed with TaqMan low-density arrays, followed with fluorescent in situ hybridization, RT-PCR and sequencing analyses in selected

cases. This approach allowed the identification of rearrangements involving previously described ETS genes in 51.5%, 7%, 1% and 0.5% for *ERG*, *ETV1*, *ETV4* and *ETV5*, respectively. Additionally, this strategy allowed also the identification of a novel ETS gene involved in PCa gene fusions, namely *FLI1*, which was found to be fused with *SLC45A3*. This work also unveiled a group of PCa with ETS rearrangements with unidentified 5' fusion partners. This subgroup of patients was further studied by 5' rapid amplification of cDNA ends and FISH, as described in **Paper II**. Two novel 5' fusion partners were identified (*OR51E2* and *UBTF*), as well as two novel gene fusion combinations of previously described genes (*SLC45A3-ETV4* and *HERVK17-ETV4*). Five previously described gene fusions involving *ETV1* and *ETV4* were also found. **Paper III** describes the study of a consecutive series of 200 diagnostic needle-biopsies with confirmed PCa with detailed clinicopathological data, including a 10-year disease-specific survival follow-up, in which the frequency of *ERG* rearrangements and relative 8q chromosomal gains were assessed by FISH. Whereas *ERG* rearrangement alone was not associated with clinical outcome, relative 8q gain predicted worse disease-specific survival in PCa patients both with and without *ERG* gene fusions, independently of Gleason score, clinical stage and treatment modality.

Resumo

O cancro da próstata é uma das neoplasias malignas mais frequentes e uma das principais causas de morte por cancro a nível mundial. A variabilidade do comportamento clínico desta doença constitui um importante desafio. Tendo em conta a tendência actual de envelhecimento da população mundial, é espectável que o cancro da próstata venha a assumir uma importância cada vez maior. Para que seja possível dar uma resposta eficiente a estes desafios, é fundamental aprofundar o conhecimento dos mecanismos moleculares da carcinogénese prostática, assim como procurar novos biomarcadores genéticos com valor prognóstico. A descoberta de rearranjos genómicos recorrentes envolvendo vários membros da família de factores de transcrição ETS representa um avanço importante na compreensão da carcinogénese prostática, sendo necessários estudos adicionais para clarificar o impacto clínico e biológico destes rearranjos. Para além dos genes de fusão, outros rearranjos genómicos, nomeadamente os ganhos relativos do braço cromossómico 8q, têm sido associados a comportamento clínico mais agressivo.

Os objectivos desta tese dividem-se em dois temas principais. Por um lado, pretendeu-se caracterizar detalhadamente os oncogenes de fusão envolvendo factores de transcrição ETS em carcinoma da próstata e identificar novos oncogenes de fusão nos 50% de carcinomas da próstata que não apresentam rearranjos com *ERG*, *ETV1*, *ETV4* ou *ETV5*. Adicionalmente, pretendeu-se descobrir novos genes parceiros 5' das fusões envolvendo genes ETS. De modo a dar resposta a estas questões, foram estudados carcinomas da próstata de uma série consecutiva de doentes tratados com prostatectomia radical. O outro tema principal consistiu na avaliação do potencial valor prognóstico dos rearranjos ETS isoladamente ou em combinação com os ganhos relativos do braço cromossómico 8q em pacientes com cancro da próstata abrangendo os diferentes graus histológicos e estádios clínicos. Para esse efeito, estudou-se uma série adicional de 200 biópsias com diagnóstico de cancro da próstata com vários anos de acompanhamento clínico (follow-up). Os resultados obtidos resultaram em 3 publicações em revistas internacionais indexadas, sendo aquelas parte integrante desta tese.

No **Artigo I** é descrito o estudo dos níveis relativos de expressão de factores de transcrição ETS previamente conhecidos como estando envolvidos em

rearranjos em cancro da próstata (*ERG*, *ETV1*, *ETV4* e *ETV5*), em Sarcoma de Ewing (*FLI1*, *ERG*, *ETV1*, *ETV4* e *FEV*) ou leucemias (*ERG*, *ETV6*), e ainda de genes ETS com sobre-expressão conhecida em carcinoma da próstata (*ELK4*) ou localizados entre dois genes frequentemente rearranjados (*ETS2*). A expressão do painel de genes mencionados foi estudada usando análise de expressão quantitativa em larga escala, seguida de hibridação fluorescente *in situ*, RT-PCR e análises de sequenciação de casos seleccionados. Com esta abordagem foi possível identificar rearranjos dos genes ETS previamente conhecidos, apresentando 51,5%, 7%, 1% e 0,5% dos casos estudados rearranjos dos genes *ERG*, *ETV1*, *ETV4* e *ETV5*, respectivamente. Esta estratégia possibilitou a identificação de um novo gene ETS envolvido em rearranjos em carcinoma da próstata, *FLI1*, que foi encontrado fundido com o gene *SLC45A3*. Adicionalmente, esta abordagem expôs um subgrupo de tumores com rearranjos dos genes ETS sem parceiro 5' conhecido. O **Artigo II** reporta o estudo deste subgrupo de carcinomas da próstata utilizando a técnica 5'- RACE (5' rapid amplification of cDNA ends), que possibilitou a clonagem e subsequente sequenciação dos parceiros de fusão desconhecidos. Análises de FISH em cortes de parafina validaram a identificação, pela primeira vez, dos genes *OR51E2* e *UBTF* como parceiros de fusão dos factores de transcrição *ETV1* e *ETV4*, respectivamente. Adicionalmente, este estudo permitiu encontrar novas combinações de genes de fusão envolvendo parceiros de fusão previamente descritos, nomeadamente *SLC45A3-ETV4* e *HERVK17-ETV4*, assim como 5 genes de fusão previamente descritos envolvendo os genes *ETV1* e *ETV4*. No **Artigo III** é descrito o estudo de uma série consecutiva de 200 biópsias prostáticas com diagnóstico confirmado de cancro da próstata, com informação clínica e patológica detalhada, incluindo o seguimento clínico de 10 anos com dados de sobrevivência específica de doença. Foram realizadas análises de FISH de modo a avaliar a frequência dos rearranjos genómicos envolvendo o gene *ERG*, assim como os ganhos relativos do braço cromossómico 8q. Não foi observada associação dos rearranjos envolvendo o gene *ERG* com sobrevivência específica de doença. Por outro lado, observou-se que os ganhos relativos do 8q estão associados com menor sobrevivência específica de doença independentemente da presença de rearranjos do *ERG*, do grau de Gleason, do estágio clínico e do tipo de terapia utilizada.

Table of contents

LIST OF ABBREVIATIONS	xix
CHAPTER 1	
Introduction	3
CHAPTER 2	
Aims	37
CHAPTER 3	
<i>Original publication # I</i>	41
<i>FLI1</i> is a Novel ETS Transcription Factor Involved in Gene Fusions in Prostate Cancer	
CHAPTER 4	
<i>Original publication # II</i>	59
Novel 5' Fusion Partners of <i>ETV1</i> and <i>ETV4</i> in Prostate Cancer	
CHAPTER 5	
<i>Original publication # III</i>	71
Relative 8q Gain Predicts Disease-Specific Survival Irrespective of the <i>TMPRSS2-ERG</i> Fusion Status in Diagnostic Biopsies of Prostate Cancer	
CHAPTER 6	
General Discussion	85
CHAPTER 7	
Conclusions	101
CHAPTER 8	
Future perspectives	105
REFERENCES	107

List of Abbreviations

AFMS	Anterior fibromuscular stroma
AJCC	American Joint Committee on Cancer
AR	Androgen receptor
ASAP	Atypical small acinar proliferation
BAC	Bacterial artificial chromosome
BPH	Benign prostatic hyperplasia
BR	Biochemical recurrence
CGH	Comparative genomic hybridization
CML	Chronic myelogenous leukemia
CNA	Copy number aberration
COPA	Cancer outlier profile analysis
CRPR	Castrate resistant prostate cancer
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
DSB	Double-strand break
EAU	European association of urology
EBRT	External-beam radiotherapy
ETS	Erythroblast-transformation specific
FDA	Food and Drug Administration
FDR	False discovery rate
FISH	Fluorescent <i>in situ</i> hybridization
GS	Gleason score
HGPIN	High grade prostatic intraepithelial neoplasia
HT	Hormone therapy
IHC	Immunohistochemistry
LHRH	Luteinizing hormone-releasing hormone
LOH	Loss of heterozygosity
NTT	N-terminal truncated
PCa	Prostate carcinoma
PCR	Polymerase chain reaction
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia

List of Abbreviations

PPV	Positive predictive value
PSA	Prostate-specific androgen
qRT-PCR	Quantitative real-time polymerase chain reaction
QT	Chemotherapy
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RP	Radical prostatectomy
RT	Radiotherapy
RT-PCR	Reverse transcriptase polymerase chain reaction
SNP	Single nucleotide polymorphism
TLDA	Taqman low density array
TNM	Tumor, Node, Metastasis (staging system)
TRUS	Transrectal ultrasonography

Chapter 1

Introduction

1. CANCER: A DISEASE OF THE GENOME

Cancer is a disease involving dynamic changes in the genome. Genetic and epigenetic alterations giving rise to oncogenes with dominant gain of function and tumor-suppressor genes with recessive loss of function are the driving force of human tumor pathogenesis. The transformation of normal cells into ultimately malignant and invasive tumor cells entails alterations in key cell physiology mechanisms resulting in self-sufficiency in growth signals and insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis) and limitless replicative potential, evading immune destruction, reprogramming of energy metabolism, sustained angiogenesis, and tissue invasion and metastasis. The acquisition of these distinctive and complementary capabilities enabling cancer cells to survive, proliferate and disseminate is, arguably, transversal to all tumor types.

Tumorigenesis follows a multistep progression pathway consisting on a succession of clonal expansions, each of which triggered by the chance acquisition of a mutant phenotype that confers selective advantage on subclones of cancer cells (Nowell, 1976; Vogelstein and Kinzler, 1993). This evolutionary process is expedited by mutations unbalancing the cellular mechanism of DNA damage response to endogenous and environmental carcinogenic agents, increasing mutation rates across the cancer genome (genomic instability), thus increasing genetic diversity. The inflammatory response in pre-malignant and malignant lesions also contributes to cancer pathogenesis. Inflammatory cells can supply bioactive molecules, such as growth factors, survival factors, pro-angiogenic factors and extra-cellular matrix-modifying enzymes, contributing to tumor progression. Additionally, inflammatory cells can release actively mutagenic agents (e.g. reactive oxygen species) favoring further mutations in cancer cells. The cancer genome is characterized by its heterogeneity. It is seen across tumor types, among cases of a particular tumor type and even within an individual tumor (Yates and Campbell, 2012).

It is often stated that prostate cancer is a heterogeneous disease. This heterogeneity is apparent at several levels, including epidemiology, clinical presentation, pathological features, and biologic behavior of the tumor. It seems obvious that this wide variability derives not only from the genetic background of the host (i.e. the patient) but also from the biologic characteristics of each tumor. Thus, it is likely that the pathways that lead to malignant transformation in the

prostate epithelium are not uniform across the whole spectrum of disease (Emmert-Buck et al., 1995; Bostwick et al., 1998; Macintosh et al., 1998). Furthermore, many same-stage tumors that are considered to be histologically identical lead to different clinical outcomes, ranging from latent to highly aggressive disease (Humphrey, 2004). This clinical heterogeneity could, at least partly, be explained by an underlying genetic heterogeneity.

2. PROSTATE CANCER EPIDEMIOLOGY

Prostate cancer (PCa) is the second most common cancer and the sixth leading cause of cancer death among men worldwide, with an estimated 903,500 new cases and 258,400 new deaths in 2008 (Jemal et al., 2011). The worldwide PCa burden is expected to grow to 1.7 million new cases and 499,000 new deaths by 2030 simply due to the growth and aging of the global population (Ferlay et al., 2010b). In Portugal, data from 2008 shows that PCa is the most common malignant disease in men (21.4%) and the third cause of cancer death (13.8%) after colorectal (14.4%) and lung (18.2%) carcinomas (Ferlay et al., 2010a).

The worldwide distribution of incidence and mortality rates displays a heterogeneous pattern. In 2008, the PCa incidence rates varied 24-fold with the highest estimated rates in Australia/New Zealand, Western Europe, North America, and the Caribbean and the lowest in South Central Asia, Northern Africa, and Eastern Asia (**Figure 1a**). In contrast, estimated PCa mortality rates varied 10-fold with the highest rates in the Caribbean, but also in a number of countries in Southern and Western Africa and in South America. Most parts of Asia, Northern Africa, as well as North America displayed the lowest mortality rates (**Figure 1b**) (Center et al., 2012).

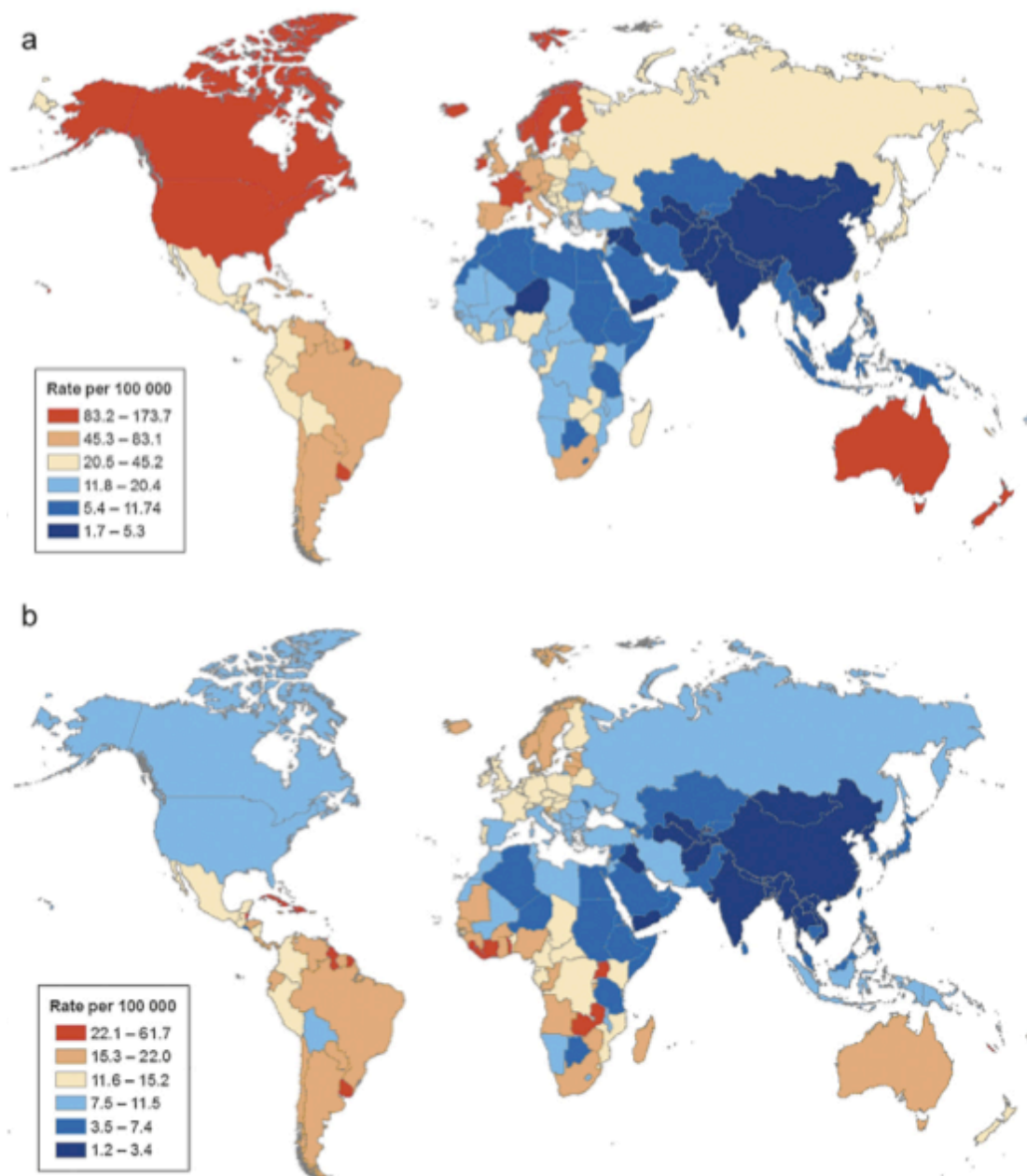


Figure 1. International variation in age-standardized prostate cancer incidence (a) and mortality rates (b) (adapted from Center et al., 2012).

2.1. Risk factors of Prostate Carcinoma

There are three well-established risk factors for PCa, namely age, family history, and ethnic origin. Age is the risk factor with biggest impact. Prostate cancer rarely occurs before the age of 40, but the incidence rises rapidly thereafter. Indeed, men with up to 39 years old have a probability of developing PCa of 0.01% (1 in 8,499), but this probability increases to 2.63% (1 in 38) for

men with 40 to 59 years, 6.84% (1 in 15) in men with 60 to 69 years, and 12.54% (1 in 8) in men with 70 years old or older (Siegel et al., 2012).

Family history of PCa is an important risk factor for this malignant disease. Individuals who have a first-degree relative with history of PCa have a two-fold risk of developing the disease compared with the general population (Carter et al., 1992; Edwards and Eeles, 2004) and it increases to 5-11 fold if two or more first-line relatives are affected (Hemminki, 2012; Jansson et al., 2012). Patients with hereditary PCa, which accounts for about 9% of all prostate tumors, usually have an age of onset 6-7 years prior to sporadic cases (Hemminki, 2012).

There is also consistent data implicating ethnic origin as a prostate cancer risk factor (**Table 1**). Black patients are not only diagnosed at a younger age but have higher tumor burdens within each stage category (Sanchez-Ortiz et al., 2006), a 1.5-fold higher frequency of metastatic disease at presentation, and lower survival rates. Asians have lower prostate cancer rates both in the United States and in Asia.

Table 1. Incidence and mortality rates of PCa by ethnicity in United States from 2004 to 2008 (adapted from Siegel et al., 2012).

Prostate cancer rates	Ethnicity				
	<i>White</i>	<i>African american</i>	<i>Asian american or pacific islander</i>	<i>American indian or Alaska native</i>	<i>Hispanic / latino</i>
Incidence rate (per 100,000)	142.8	230.8	79.7	101.2	126.7
Mortality rate (per 100,000)	22.4	54.9	10.5	20.7	18.5

The frequency of autopsy-detected cancers is roughly the same in different parts of the world (Breslow et al., 1977). This finding is in sharp contrast to the incidence of clinical PCa, which differs widely between different geographical areas, being high in the USA and Northern Europe and low in Southeast Asia (Kheirandish and Chinegwundoh, 2011). However, if Japanese men move from Japan to Hawaii, their risk of PCa increases. If they move to California their risk increases even more, approaching that of American men (Kheirandish and Chinegwundoh, 2011). These findings indicate that exogenous factors affect the risk of progression from so-called latent PCa to clinical PCa.

3. ANATOMY OF THE PROSTATE GLAND

The prostate gland is a partly glandular and muscular walnut-sized organ located within the lower pelvis directly below the internal urethral orifice and around the beginning of the urethra (**Figure 2**). This exocrine gland secretes an alkaline fluid that forms part of the ejaculate, which aids in motility and nourishment of the sperm (Marandola et al., 2004).

Three major anatomical zones compose the prostate gland: peripheral, transitional, and central (McNeal, 1981). The peripheral zone comprises all the prostatic glandular tissue at the apex, as well as all of the tissue located posteriorly near the capsule (**Figure 2**). In this zone, carcinoma, chronic prostatitis, and post-inflammatory atrophy are more common compared to the other zones. The central zone is a cone-shaped area of the adult gland, with the apex of the cone at the confluence of the ejaculatory ducts and the prostatic urethra at the verumontanum (**Figure 2**). The transition zone consists of two equal portions of glandular tissue lateral to the urethra in the midgland (**Figure 2**). Age-related benign prostatic hyperplasia (BPH) is the most frequent lesion in this zone, although adenocarcinomas may also develop. The anterior fibromuscular stroma (AFMS) forms the convexity of the anterior external surface. The apical half of the area is rich in striated muscle, which blends into the gland and the muscle of the pelvic diaphragm (**Figure 2**). The distal portion of the AFMS is important in voluntary sphincter functions, whereas the proximal portion plays a central role in involuntary sphincter functions.

The histologic architecture of the prostate is that of a branched duct gland. Two cell layers, a luminal secretory columnar cell layer and an underlying basal cell layer, line each gland or duct. Additionally, the prostate epithelium also includes transient cells, an intermediate between secretory and basal cells (Isaacs and Coffey, 1989), and neuroendocrine cells. The prostate gland is supported by a stroma and extracellular matrix composed of smooth muscle cells, fibroblasts, lymphocytes, and neurovascular tissue.

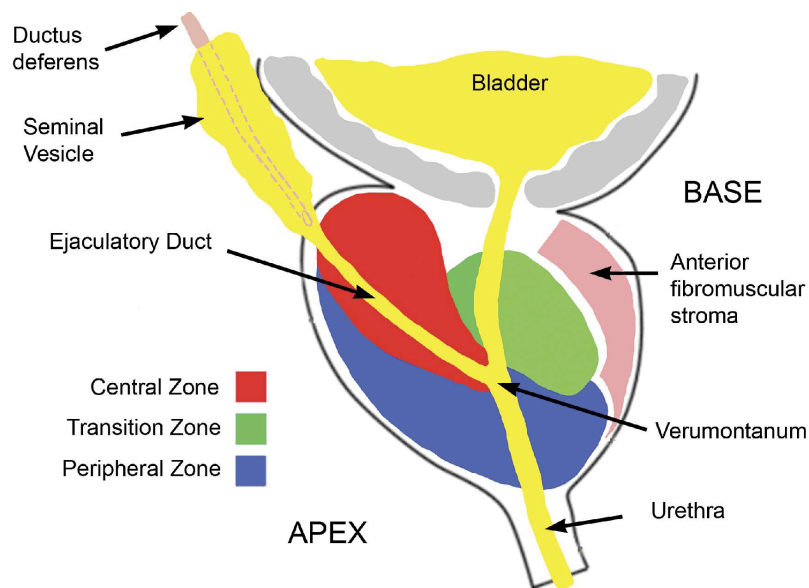


Figure 2. Sagittal schematic view of the normal prostate illustrating the different zones of the gland as described by McNeal (adapted from (Cohen et al., 2008).

4. PROSTATE GLAND PATHOLOGY

Pathologic conditions affecting the prostate are very diverse and include benign, pre-malignant and malignant lesions. The incidence of all these conditions increases with advancing age.

4.1. Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is a pathologic process that consists of androgen-dependent overgrowth of epithelial and stromal cells in the peri-urethral area (transition zone) of the prostate, contributing to the development of lower urinary tract symptoms in aging men (Sandhu and Te, 2004). While the number of luminal epithelial cells increases in BPH, the columnar appearance and nuclear cytology remain normal. This lesion is very common in older males, with up to 90% of men affected by BPH as they reach the age of 80 (Bushman, 2009), and is not considered to be the precursor to prostate cancer (Kristal et al., 2010).

4.2. Pre-malignant lesions of the prostate

In pathological terms, pre-malignancy equates to morphologically altered tissue in which cancer is more likely to occur than its apparently normal counterpart (Berney and Warren, 2013). Histological lesions of the epithelial cell compartment, such as high-grade prostatic intraepithelial neoplasia (HGPIN) and, to a lesser extent, proliferative inflammatory atrophy (PIA), are considered to be precursor lesions of prostate cancer.

Prostatic intraepithelial neoplasia (PIN) is defined as a neoplastic proliferation of the prostatic acinar cells, with nuclear and nucleolar enlargement similar to that seen in prostate cancer. The growth is confined by the basement membrane and is therefore intraepithelial and, unlike prostatic adenocarcinoma, does not display invasion of the stroma (Berney and Warren, 2013). Histologically, PIN is dichotomized into low- and high-grade PIN, where only the later is considered a precursor of invasive carcinomas (Epstein, 2009). Shared common features between HGPIN and PCa supports this hypothesis. As PCa, HGPIN lesions are usually multi-centric (Qian et al., 1997; Bostwick et al., 1998) and heterogeneous, occurring mostly on the peripheral zone of the prostate (Bostwick and Cheng, 2012). Early stromal invasion, the earliest evidence of carcinoma, occurs at sites of acinar outpouching and basal cell disruption in acini with PIN. Such microinvasion is present in about 2% of high-power microscopic fields of PIN (Bostwick, 1996). HGPIN has a high predictive value as a marker for adenocarcinoma when multifocal or extensive. There is a 30.2% risk of cancer in the following biopsy after a HGPIN diagnosis in one or two sample cores, increasing to 40% with HGPIN in three cores, and 75% with HGPIN in more than three cores (Kronz et al., 2001).

Proliferative changes of the epithelium are linked to carcinogenesis in almost all epithelial malignancies. Glandular atrophy associated with glandular proliferation, named proliferative inflammatory atrophy (PIA), has been postulated as a pre-malignant lesion. The hypothesis is that cellular injury and regeneration characteristic of PIA is induced by inflammation and release of reactive oxygen species (oxidative stress) resulting from insult owing to chemicals (e.g., dietary carcinogens), physical factors (e.g., arteriosclerosis-induced ischemia), or bacteria. The regenerating cells are at increased risk of mutation that, in turn, predisposes them to cancerous initiation, promotion, and progression (Bostwick and Cheng, 2012). Evidence to date is inconclusive as to

whether PIA is a precursor of PIN, direct precursor of cancer that bypasses PIN, or simply an epiphenomenon (Bostwick and Cheng, 2012). Furthermore, the finding of PIA on biopsy material is very frequent in the absence of carcinoma and does not increase the risk of cancer on repeat biopsy (Postma et al., 2005).

Lesions suspicious for carcinoma known as atypical small acinar proliferation (ASAP) are found in 2 to 5% of prostate biopsies (Cheville et al., 1997; Borboroglu et al., 2001; Vis et al., 2001). Although there is no consensus that it is an actual entity, the risk of prostate cancer on repeat biopsy is substantially higher following a single ASAP focus than for PIN with an average risk of 45% (Berney and Warren, 2013). In cases with PIN and adjacent atypical small acinar proliferation (PIN + ASAP), all men need re-biopsy within 3 to 6 months of the diagnosis, as the risk of subsequent cancer is high regardless of number of biopsies obtained (Kronz et al., 2001).

4.3. Malignancy in the prostatic gland

Adenocarcinoma of the prostate comprises 95% of the malignant neoplasms in this organ. The histopathologic diagnosis is made on the basis of a combination of architectural and cytological features with supportive cytoplasmic/luminal data (**Table 2**). Most prostate cancers arise in the peripheral zone (70-75%), with only 10-15% occurring in the transition zone and ~10% in the central zone (Schulz et al., 2003). The clinical course of the disease is variable and difficult to predict, as some tumors remain localized for years, whereas others may metastasize early without evidence of local dissemination.

Several autopsy studies of people dying from different causes have shown that 60-70% of older men have histological PCa (Haas et al., 2008), but that a large proportion of these tumors will not progress. Prostate cancer is diagnosed in only 15-20% of men during their lifetime, with a 3% lifetime risk of death (Jemal et al., 2006). Approximately 50% of PCa diagnosed in population-based studies show the pathological features of the incidental cancers found at autopsy (Gosselaar et al., 2005). This implies that a subset of men diagnosed with prostate cancer do not require any active, invasive treatment (Bangma et al., 2012; Bangma and Roobol, 2012). Only a small proportion of patients with cancer diagnosed at an early stage progress to metastatic disease and die from PCa within 10 – 15 yr (Chodak et al., 1994; Johansson et al., 2004). However, extended follow-up suggests that even disease with an initially indolent course

may become aggressive and eventually result in lethal outcome. A recently published update from a randomized trial (Bill-Axelsson et al., 2011) reported PCa deaths among men in the watchful-waiting arm with presumed low-risk tumors.

Table 2. Criteria for histopathologic diagnosis of prostate adenocarcinoma (adapted from Shevchuk and Robinson, 2013).

Primary criteria
<i>Architectural—diagnostic</i>
1. Small glands (microacini)
2. Crowded glands
3. Haphazardly arranged glands, not in lobules
4. Fused glands
5. Infiltrative pattern
6. Small glands around/between benign glands
7. Perineural invasion
8. Mucinous fibroplasia
9. Glomerulations
Secondary criteria
<i>Cytological — diagnostic</i>
1. Absence of basal cells
2. Large nucleoli
3. Large hyperchromatic nuclei, with an increased nucleus-cytoplasm ratio
Tertiary criteria
<i>Cytoplasmic/luminal—supportive</i>
1. Luminal blue mucin
2. Luminal pink amorphous secretions
3. Crystalloids
4. Sharp/rigid luminal borders
5. Amphophilic or foamy cytoplasm

5. DIAGNOSIS OF PROSTATE CANCER

Local symptoms from prostate cancer usually do not manifest until invasion of the periprostatic tissue has occurred, at which point curative therapy is usually not possible (discussed in 9.1 Treatment of prostate cancer). Therefore, detection of curable prostate cancer depends on diagnosis prior to onset of symptoms.

The main diagnostic tools to obtain evidence of PCa include digital rectal examination (DRE), serum concentration of prostate-specific antigen (PSA), and transrectal ultrasonography (TRUS). Its definite diagnosis depends on the histopathologic verification of adenocarcinoma in transrectal ultrasound-guided needle-biopsy cores of the prostate or operative specimens.

5.1. Digital rectal examination (DRE)

The sensitivity of DRE for diagnosing prostate cancer depends both on the stage of the tumor and the experience of the examiner. While DRE is of limited value in detecting tumors confined to the prostate gland, it remains a useful adjunct to PSA testing in identifying higher-risk cancer when the PSA is low (Yates and Anderson, 2013). Twenty percent (20 %) of tumors detected by DRE when the PSA is less than 2 ng/ml are not organ confined (Okotie et al., 2007). Moreover, in a trial involving 986 PCa patients with PSA levels lower than 4.0ng/ml the positive predictive value of an abnormal DRE was 8.8% (Bozeman et al., 2005).

5.2. Prostate-specific antigen (PSA)

Prostate glands in humans consist of a single layer of secretory epithelial cells, which are surrounded by a continuous layer of basal cells and a basement membrane. PSA is a serine protease produced by these secretory epithelial cells in the acini and ducts, and it is secreted directly into the lumen. A characteristic early feature of PCa is the disruption of the basal cell layer and basement membrane, which allows the migration of PSA into the more vascularized stromal component of the gland, and hence enter the blood stream (Brawer, 2000; Balk et al., 2003). Apart from prostate cancer, there are several major causes of increased serum PSA, including BPH, prostate inflammation or infection, and prostate or perineal trauma, so PSA expression is organ-specific rather than tumor-specific.

Traditionally, a serum PSA concentration of 4 ng/mL has been used as a common threshold above which a prostate biopsy should be considered, since approximately 70% of cancers will be detected using this cut-off (Catalona et al., 1994). This established threshold has some drawbacks. Up to 30 % of men with a PSA in the range 4–10 ng/ml will have PCa on biopsy (Gann et al., 1995), but it has been shown that a high prevalence of PCa exists among men with a $PSA \leq 4$

ng/ml and that at these PSA levels many men can harbor clinically significant disease (Thompson et al., 2004). In fact, regarding men with PSA levels ranging from 3.1 to 4.0 ng/ml, 26.9 % had PCa and, of these, 25 % were high-grade cancers (Thompson et al., 2004).

In order to increase sensitivity and/or specificity of the standard PSA test, several modifications have been described, including assessment of PSA kinetics (PSA doubling time and PSA velocity), PSA density, age-specific reference ranges for serum PSA, and ratio of free to total (free plus complexed) PSA. Because of potential trade-offs between sensitivity and specificity, there is at present no consensus on optimal strategies for using the different modifications of PSA testing. However, using a combination of factors as described above may better detect higher risk cancers compared to using single PSA cut off and DRE alone (Greene et al., 2013).

5.3. Transrectal ultrasonography (TRUS)

On TRUS, cancers appear hypoechoic compared with the normal-appearing peripheral zones of the prostate. In addition to its function to establish tissue diagnosis, TRUS is also useful in assessing the volume of the prostate and calculating the PSA density. Limitations of this imaging modality include the difficulty to characterize the integrity of prostatic capsule and to visualize early extra-capsular extension or seminal vesicle involvement. TRUS has a well established role as a tool for guiding biopsy in the diagnosis of prostate cancer.

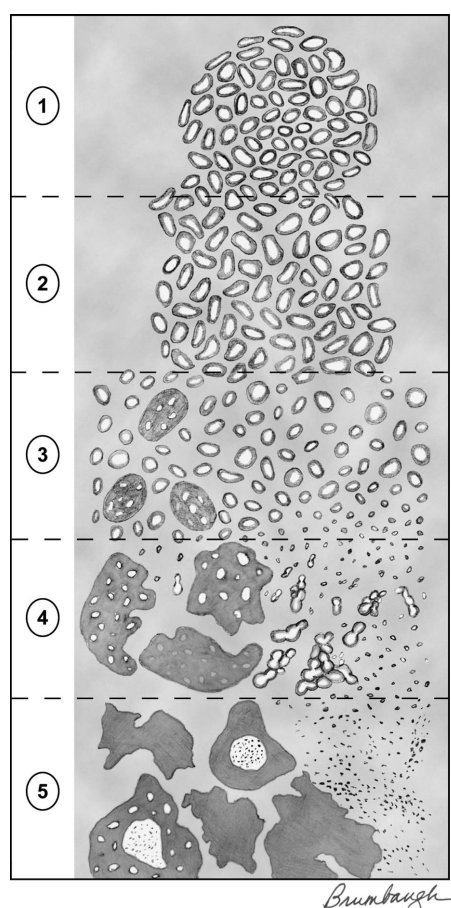
6. GRADING AND STAGING OF PROSTATE ADENOCARCINOMAS

Assessing the degree of dedifferentiation and the level of tumor spreading is an important tool to clinical practice, providing valuable prognostic information about the behavior of the disease and treatment response.

6.1. Histological grading

Grading of prostatic adenocarcinoma using the modified Gleason score system (Epstein et al., 2005) is the single strongest prognostic factor for clinical behavior and treatment response. This grading system is based entirely on the histologic pattern of arrangement of carcinoma cells observed at low

magnification in H&E-stained prostatic tissue sections, defining five patterns or grades (from 1 to 5) with decreasing degree of differentiation (**Figure 3**). The morphological heterogeneity of prostate cancer is addressed combining the primary and secondary Gleason grades. Gleason score (GS) is, thus, the sum of the most dominant and second most dominant Gleason grades. Both the primary and secondary grade are reported in addition to the GS. Ranging from 2 to 10, this grading system can be used to stratify PCa patients according to prognosis. In a multi-institutional radical prostatectomy cohort, the 15-year prostate cancer-specific mortality rates ranged from 0.2 to 1% for pathological GS 6 or less, 4 to 6% for GS 3+4 tumors, 6 to 11% for GS 4+3 tumors, and 26 to 37% for GS 8 or higher cancers (Eggener et al., 2011).



2005 ISUP Modified Gleason System

Pattern 1:

- Circumscribed nodule of closely packed but separate, uniform, rounded to oval, medium-sized acini (larger glands than pattern 3)

Pattern 2:

- Like pattern 1, fairly circumscribed, yet at the edge of the tumor nodule there may be minimal infiltration
- Glands are more loosely arranged and not quite as uniform as Gleason pattern 1

Pattern 3:

- Discrete glandular units
- Typically smaller glands than seen in Gleason pattern 1 or 2
- Infiltrates in and amongst nonneoplastic prostate acini

- Marked variation in size and shape
- Smoothly circumscribed small cribriform nodules of tumor

Pattern 4:

- Fused microacinar glands
- Ill-defined glands with poorly formed glandular lumina
- Large cribriform glands
- Cribriform glands with an irregular border
- Hypernephromatoid

Pattern 5:

- Essentially no glandular differentiation, composed of solid sheets, cords, or single cells
- Comedocarcinoma with central necrosis surrounded by papillary, cribriform, or solid masses

Figure 3. Gleason patterns according to 2005 ISUP Consensus Conference (Epstein et al., 2005).

6.2. Tumor staging

Accurate and uniform staging is vital for prediction of tumor behavior, treatment selection and evaluation of response to treatment. The tumor, node and metastasis (TNM) staging system for prostatic carcinoma was first introduced in 1992, when the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) adopted a unified TNM staging system for prostate cancer (Beahrs and Henson, 1992). This system, lastly revised in 2010 (Edge and Compton, 2010), comprise two types of staging considering timing of data collection, namely clinical and pathological staging (**Table 3**).

Clinical staging is based on data obtained prior to the first definite treatment. The extent of clinically evident tumor is determined by digital rectal examination (DRE), transrectal ultrasonography (TRUS), and/or other imaging techniques. Pathological staging requires histological identification of the extent of tumor within the prostate and in surrounding tissues. Agreement between clinical and pathological stages would facilitate assessment of patient prognosis and guide appropriate therapy. However, clinical staging is an inaccurate tool with which to predict final pathological stage (Montie, 1994). None of the current imaging modalities, including TRUS and magnetic resonance imaging, is reliable for predicting extra-prostatic extension or seminal vesical invasion. Furthermore, over 80% of PCas are multifocal, which makes accurate clinical staging even more difficult and unreliable (Andreoiu and Cheng, 2010). Clinical tumor under-staging has consistently been shown to range from 40% to 60% (Grignon and Sakr, 1996; Campbell et al., 2001; Abdellaoui et al., 2011; Heidenreich et al., 2011).

Table 3. American Joint Committee on Cancer clinical and pathological classification of prostatic tumors (adapted from Cheng et al., 2012).

Clinical tumor staging		
T1		Clinically unapparent tumor neither palpable nor visible by imaging
	T1a	Tumor incidental, histological finding in $\leq 5\%$ of tissue resected
	T1b	Tumor incidental, histological finding in $> 5\%$ of tissue resected
	T1c	Tumor identified by needle biopsy (e.g. Because of elevated PSA)
T2		Tumor confined within the prostate
	T2a	Tumor involves $\leq$ one-half of one lobe
	T2b	Tumor involves $>$ one-half of one lobe but not both lobes
	T2c	Tumor involves both lobes
T3		Tumor extends through the prostate capsule
	T3a	Extracapsular extension (unilateral or bilateral)
	T3b	Tumor invades seminal vesicles(s)
T4		Tumor is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
NX		Regional lymph nodes were not assessed
N0		No regional lymph node metastasis
N1		Metastasis in regional lymph nodes
M0		No distant metastasis
M1		Distant metastasis
	M1a	Non-regional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s) with or without bone disease
Pathological tumor staging		
pT2		Organ confined
	pT2a	Unilateral, one-half of one side or less
	pT2b	Unilateral, involving more than one-half of side but not both sides
	pT2c	Bilateral disease
pT3		Extraprostatic extension
	pT3a	Extraprostatic extension or microscopic invasion of bladder neck
	pT3b	Seminal vesicle invasion
pT4		Invasion of rectum, levator muscle, and/or pelvic wall
pNX		Regional nodes not sampled
pN0		No positive regional nodes
pN1		Metastasis in regional node(s)
pM0		No distant metastasis
pM1		Distant metastasis
	pM1a	Non-regional lymph node(s)
	pM1b	Bone(s)
	pM1c	Other site(s) with or without bone disease. When more than one site of metastasis is present, the most advanced category is used. pM1c is most advanced

7. TREATMENT OF PROSTATE CANCER

The therapeutic approaches for prostate adenocarcinomas are to a great extent dependent on the stage of disease progression, but also on patient's physical status and life expectancy (Aus et al., 2005). For organ confined PCa, treatment alternatives include active surveillance, radical prostatectomy and radiation therapy (Heidenreich et al., 2011). For tumors displaying extra-prostatic invasion or metastatic prostate cancer treatment, options include hormone therapy, radiation therapy, the combination of the previous two, and chemotherapy. In the near future more treatment options may be available as some targeted therapies are currently undergoing clinical trials (Corcoran and Gleave, 2012).

7.1. Active surveillance

This therapeutic strategy consists in submitting patients to a strict clinical surveillance plan to postpone invasive treatment until it is required (Heidenreich et al., 2008). Men with PCa deemed low risk of disease progression, low PSA ($<10\text{ng/ml}$), biopsy Gleason score ≤ 6 , cT1c-cT2a, life expectancy <10 years, are good candidates for this treatment modality. Several studies show a very consistent disease-specific survival at 10 years, ranging from 82-87% (Johansson et al., 1989; Chodak et al., 1994; Sandblom et al., 2000; Bill-Axelsson et al., 2005; Jonsson et al., 2006; Adolfsson et al., 2007), and up to 80-95% if T1-T2 and GS ≤ 7 (Lu-Yao et al., 2009).

7.2. Radical prostatectomy

This surgical procedure involves removal of the entire prostate gland, both seminal vesicles, and is often accompanied by bilateral pelvic lymph node dissection (Heidenreich and Pfister, 2013). Generally, this procedure is indicated for patients with low and intermediate risk localized PCa (cT1a-T2b and Gleason score 2-7 and PSA $\leq 20\text{ng/mL}$) and life expectancy > 10 years.

7.3. Radiotherapy

As an alternative to radical prostatectomy, radiotherapy is a valid curative treatment for men with clinical localized prostate tumors. Its efficacy is deemed equivalent to RP, with the advantage of being a minimally invasive therapy, with less inconvenient side effects (Jani and Hellman, 2003). However, this approach implies forgoing the pathological staging that radical prostatectomy provides, hampering prognosis estimation. There are two main types of radiation therapy, external beam radiation and brachytherapy (internal radiation).

The efficacy of complete irradiation of the prostate by external-beam radiotherapy (EBRT) has been highly increased by technical improvements. Three-dimensional conformal radiotherapy allows visualization of the clinical target volume and adds a surrounding safety margin. Subsequently, intensity-modulated treatment planning capabilities enabled highly conformal dose escalation for improved outcomes without added toxicity (Jani et al., 2003).

Brachytherapy consists in the placement of a small radioactive source into or near the tumor. As a monotherapy, it is most effective in low-risk prostate cancer (T1-T2a N0, M0; GS $\leq$ 6; PSA < 10ng/mL). Other eligibility criteria include prostate size smaller than 60 cm³, none or mild urinary obstructive symptoms, and no history of previous pelvic radiotherapy or transurethral resection of the prostate (Koontz and Lee, 2013).

Radiation therapy improves treatment results of patients with high-risk prostate cancer (T3 or GS > 7 or PSA > 20ng/mL) when combined with hormone therapy (Milecki et al., 2007).

7.4. Hormone therapy

Men with advanced prostate tumors presenting extra-prostatic or metastatic spreading are candidates for hormonal therapy. Although not curative, this approach is effective at prolonging progression-free survival, minimizing complications, and maintaining quality of life.

Prostate cells are physiologically dependent on androgens to stimulate growth, function and proliferation. Likewise, androgens are essential for the growth and perpetuation of tumor cells (Walsh, 1975). Suppression of androgen activity is the basis of hormonal therapy. Androgen deprivation can be achieved either by arresting the secretion of testicular androgens through the use of

luteinizing hormone-releasing hormone (LHRH) agonists or antagonists (chemical castration) or by orchiectomy (surgical castration). Additionally, androgen deprivation can also be achieved by inhibiting the action of circulating androgens at the level of their receptor in prostate cells using competing compounds known as anti-androgens. Although these approaches consistently result in androgen activity suppression, virtually all the patients will develop castration-resistant prostate cancer (CRPC) after a median response time of 18-24 months (Hamberg et al., 2008; Sternberg, 2008).

7.5. Chemotherapy

Cytotoxic chemotherapy is the standard treatment option for patients with metastatic castration-resistant prostate cancer (mCRPC). Until recently, taxanes were the only cytotoxic chemotherapy agents, namely docetaxel, which significantly prolonged overall survival (Petrylak et al., 2004; Tannock et al., 2004). In the next section novel therapeutic approaches will be presented, whose effectiveness has been recently validated by clinical trials that may in a near future improve mCRPC treatment.

7.6. Novel targeted therapies

Prostate cancer was one of the first solid tumors benefiting from targeted therapy, with the insight that surgical castration could induce regression of advanced disease being made 40 years before the target, the androgen receptor (AR), was cloned (Huggins and Hodges, 1972; Lubahn et al., 1988). The development of modern molecular biological techniques has dramatically increased the understanding of the key events underpinning malignant transformation, and yielded an increasing array of potential targets that can be manipulated to therapeutic advantage.

Androgen regulation plays a critical role in prostate cancer initiation and progression. The U.S. Food and Drug Administration (FDA) have recently approved two novel therapeutic agents for treatment of mCRPC, targeting different steps of the androgen receptor pathway, namely abiraterone and enzalutamide. The first is an inhibitor of the cytochrome P450 enzyme CYP17A1 that catalyses two steps of testosterone biosynthesis (Rehman and Rosenberg, 2012). Clinical trials have shown that administration of this drug prolongs overall

survival in patients with mCRPC in the postchemotherapy setting (de Bono et al., 2011; Fizazi et al., 2012). The latter, enzalutamide, is a nonsteroidal antiandrogen that binds to the androgen receptor, reducing the efficiency of its nuclear translocation and impairing both DNA binding to androgen response elements and recruitment of co-activators (Tran et al., 2009). It has been shown that this drug significantly prolongs the survival of men with mCRPC after chemotherapy (Scher et al., 2012).

Molecular chaperones are important during times of cellular stress, such as that induced by radiation, androgen withdrawal, and chemotherapy, to prevent protein misfolding, aggregation, and denaturation. Increased expression of molecular chaperones is important in stabilizing signaling and transcriptional regulatory networks during oncogenic transformation and proliferation, and in response to cell damage, and is an important cause of treatment resistance in prostate cancer (Whitesell and Lindquist, 2005). This makes the targeting of molecular chaperones an attractive therapeutic option. The clusterin gene, *CLU*, encodes a cytoprotective chaperone that plays an antiapoptotic role and is involved in progression to androgen independence in PCa (Miyake et al., 2000). Moreover, the expression of this chaperone is significantly increased in PCa following androgen withdrawal therapy (July et al., 2002). Custirsen (OGX-011) is an antisense oligonucleotide drug complementary to the *CLU* translation initiation site, thus inhibiting its translation. Chi and co-workers observed that patients with mCRPC treated with OGX-011 showed evidence of biological effect and was associated with improved survival (Chi et al., 2010).

Activation of the insulin-like growth factor 1 receptor (IGF-IR) is implicated in prostate cancer development and progression. In a study evaluating the clinical and biological effects of the human monoclonal IGF-IR antibody, figitumumab, in patients with localized PCa, a decrease in IGF-IR was observed. This effect was also associated with a decrease in prostate cancer androgen receptor expression and a prostate-specific antigen decline in the majority of patients (Chi et al., 2012).

The antibody Denosumab® targets the tumor necrosis factor cytokine family member RANKL, which is a key factor for osteoclast differentiation. In a large randomized study, castrate-resistant prostate cancer patients treated with this antibody showed a delay in bone metastasis (Smith et al., 2012).

8. GENOMIC CHANGES IN PROSTATE CANCER

Over the last decades, there have been intensive efforts to elucidate the underlying genetic and epigenetic alterations that lead to prostate cancer development and progression. There have been tremendous advances in our understanding of the molecular basis of prostate cancer, facilitated by continuous improvements in laboratory technology and bioinformatics. This increasing knowledge will be crucial to establish efficient novel markers for early detection of PCa, to timely distinguish indolent from aggressive tumors, and also to develop novel targeted therapies.

The following sections present a brief introduction of the current knowledge on germline mutations and the wide spectrum of somatic alterations in prostate cancer, with emphasis on copy number alterations and recurrent ETS rearrangements.

8.1. Germline mutations

As previously discussed in this thesis (see Risk factors of prostate cancer), it is estimated that about 9% of prostate cancers are of hereditary origin. Hereditary prostate cancer is associated with an early onset of the disease and family history of prostate cancer. To identify the genetic basis for hereditary prostate cancer, linkage analysis of cancer-prone families have been preformed, resulting in the discovery of putative susceptibility loci (**Table 4**).

Genome-wide linkage analyses of prostate cancer families have implicated many loci, however, no definitive gene that causes a significant proportion of the familial prostate cancers has been identified. More recently, high-throughput genotyping techniques are being used to fine map the location of familial cancer genes. So, to date multiple loci and genes with moderate effects associated with susceptibility to prostate cancer have been identified (**Table 4**), but none account for a large proportion of susceptibility to the disease (Boyd et al., 2012).

Table 4. Prostate cancer predisposition loci and associated genes described by linkage analysis.

Chromosomal position	Susceptibility locus	Associated genes
1p36	CAPB	
1q23-25	HPC1	<i>RNASEL</i>
1q42-43	PCAP	
8p22-23	-	<i>MSR1</i>
13q12.3		<i>BRCA2</i>
17p	HPC2	<i>ELAC2</i>
17q21-22	-	<i>HOXB13</i>
17q21	-	<i>BRCA1</i>
20q13	HPC20	
22q12.3-13.1	-	<i>APOL3</i>
Xq27-28	HPCX	

8.2. Epigenetic changes in PCa

Epigenetic changes contribute to the development and progression of prostate cancer. Furthermore, there is evidence that particular epigenetic changes are associated with different stages of the disease (Chin et al., 2011).

The term epigenetic refers to changes in a cell's phenotype that are stably inherited through cell division, but that have not resulted from a change in DNA sequence. Epigenetic mechanisms include methylation of deoxycytidine residues within CpG dinucleotides, chromatin remodeling and histone modifications, and miRNA deregulation (Gurel et al., 2008; Jeronimo et al., 2011).

Hypermethylation of the CpG island within the *GSTP1* promoter region occurs in over 90% of PCa, causing loss of expression of the *GSTP1* gene that encodes an enzyme involved in the metabolism, detoxification and elimination of reactive chemical compounds, thus with the function of protecting cells from DNA damage and cancer (Jeronimo et al., 2001; Lee, 2007). DNA hypomethylation has also been reported in PCa. For instance, repetitive DNA regions like *LINE1*, are hypomethylated in around 50% of PCa samples (Jeronimo et al., 2011).

Evidence indicates that chromatin remodeling and histone post-translational modifications are important for the deregulation of gene expression in PCa. In fact, gene repression in PCa commonly occurs in domains of inactive

chromatin (Coolen et al., 2010). In addition, several histone-modifying enzymes, including HDACs, HMTs, and HDMs, are altered in this tumor. Among these, the best studied is EZH2, a histone methyltransferase polycomb protein that catalyses the trimethylation of histone H3K27 and, occasionally, dimethylates H3K9 (Cao et al., 2002). Moreover, EZH2 may be also directly involved in DNA methylation through physical contact with DNA methyl-transferase (Vire et al., 2006). *EZH2* upregulation is associated with high proliferation rate and tumor aggressiveness in PCa (Bachmann et al., 2006) and can lead to silencing of developmental regulators and tumor suppressor genes.

So far 50 miRNAs have been reported as abnormally expressed in PCa, although only a few have been experimentally proven to contribute to the disease (Jeronimo et al., 2011). In PCa, miRNA deregulation affects epigenetic reprogramming, blockade of apoptosis, promotion of cell cycle, migration, and invasion and is an alternative mechanism sustaining androgen-independent growth (Coppola et al., 2010).

8.3. Commonly altered genes in prostatic carcinogenesis

Down-regulation of the transcription factor NK3 homeobox 1, *NKX3.1*, represents a frequent and critical event in prostate cancer initiation (Abate-Shen et al., 2008). This regulator of prostate epithelial differentiation (Shen and Abate-Shen, 2010) is localized within a 150-Mb minimal deleted region of chromosome 8p21.2 that often displays loss-of-heterozygosity (LOH) in high-grade PIN lesions and adenocarcinomas (Emmert-Buck et al., 1995; Vocke et al., 1996; Haggman et al., 1997; Swalwell et al., 2002; Bethel et al., 2006). However, although LOH of 8p21 progressively increases in frequency with cancer grade, the remaining allele of *NKX3.1* remains unmutated (Vocke et al., 1996; Voeller et al., 1997; Ornstein et al., 2001; Bethel et al., 2006). Functional studies have led to a model in which *NKX3.1* represents a haploinsufficient tumor suppressor gene that acts as a “gatekeeper” gene for prostate cancer initiation (Kim et al., 2002; Gelmann, 2003; Magee et al., 2003).

The phosphatase and tensin homolog gene, *PTEN*, is another relevant tumor suppressor gene in prostate carcinogenesis. Loss of this gene, located at 10q23, leads to upregulation of PI3K/AKT, which is a pathway associated with progression of prostate cancer (Ayala et al., 2004; Ohigashi et al., 2005; Sarker et al., 2009; Sircar et al., 2009; Wang et al., 2010). Heterozygous and, less

commonly, homozygous deletions at the *PTEN* locus occur in approximately 40% of primary PCas and in 70-80% in advanced PCa (reviewed in (Squire et al., 2011). Although less frequently, loss of *PTEN* can also be caused by inactivating point mutations (Cairns et al., 1997; Barbieri et al., 2012; Grasso et al., 2012; Weischenfeldt et al., 2013). Heterozygous knockout of *PTEN* in mice causes HGPIN (Kwak et al., 2013) and is correlated with progression to aggressive, castration-resistant disease (Shen and Abate-Shen, 2010). In fact, disruption of *PTEN* expression in human prostate cancer cell lines or targeted deletion of *PTEN* in mouse prostate cancers is sufficient for the development of castration resistance (Lin et al., 2004; Bertram et al., 2006; Gao et al., 2006). While this may reflect the ability of *PTEN* to interact directly with the androgen receptor, the mechanistic details by which *PTEN* loss promotes castration resistance remain to be resolved.

The androgen receptor (*AR*) gene, located at Xq12, encodes a steroid-hormone activated transcription factor essential for development and maintenance of the normal prostate (Dehm and Tindall, 2006). The *AR* binds to androgens in the cell cytoplasm and translocates to the nucleus, where it dimerizes and binds to specific sequences named androgen-responsive elements (*AREs*) found in the regulatory regions of *AR*-target genes (Yuan et al., 2013). Androgen receptor is mutated or amplified in 20–30% of castration-resistant prostate cancers (Koivisto et al., 1997; Feldman and Feldman, 2001). Although somatic *AR* mutations are rarely detected in early-stage prostate cancer, mutation frequency is significantly increased in advanced androgen-independent tumors, suggesting that *AR* mutations have a role in tumor progression (Taplin et al., 1995; Marcelli et al., 2000; Dong, 2006). Androgen receptor (*AR*) expression is often heterogeneous in prostate cancer, perhaps reflecting an underlying genomic instability.

8.4. Copy number alterations

Current comparative genomic hybridization technologies enable the characterization of losses and gains of both large chromosome regions as well as more subtle genetic aberrations, such as gene duplications and deletions (Boyd et al., 2012). Global analyses of copy number profiles of primary tumors and metastases identified recurrent aberrations associated with PCa development and progression, including broad losses of 1p, 6q, 8p and 9p and losses of large

regions of chromosomes 13q, and 16q (Boyd et al., 2012). The most frequently found chromosomal gains in PCa include 1q, 3q, 7q and 8q (Boyd et al., 2012). Additionally, focal amplifications of the androgen receptor (Xq12) and focal deletions of *PTEN* (10q) and *NKX3.1* (8p) are also frequent in PCa (Demichelis et al., 2009; Taylor et al., 2010; Lonigro et al., 2011; Grasso et al., 2012). As described above, these genes play an important role in prostatic carcinogenesis. The **Figure 4** represents the profile of copy number alterations (CNA) in PCa. A recent comprehensive CNA study of 218 primary and metastatic tumors by Taylor et al. confirmed the earlier data, and added a significant role for somatic copy number increases of the *NCOA2* gene, which encodes an *AR* coactivator (Taylor et al., 2010).

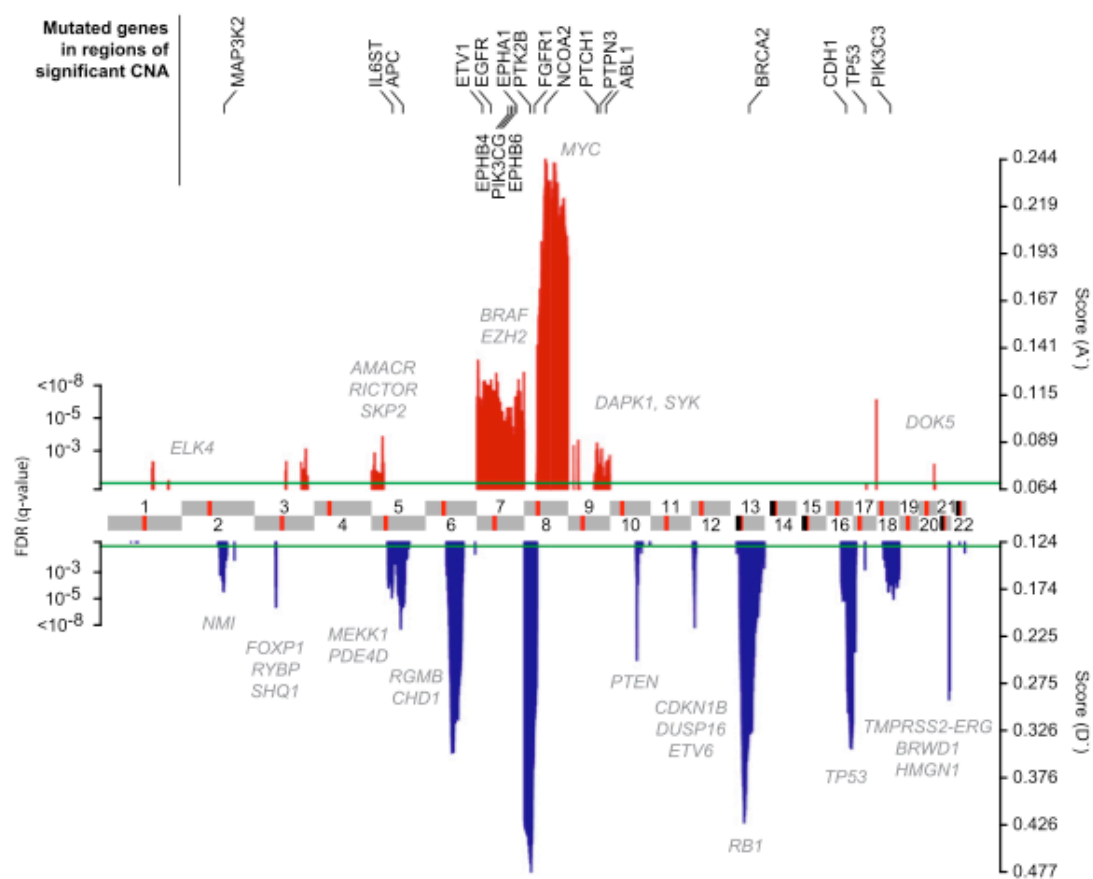


Figure 4. Significant genomic aberrations in the prostate oncogene. Regions of amplification (red) or deletion (blue) with $FDR \leq 10\%$ are plotted, with chromosomes indicated at the center and centromeres in red. Genes with somatic non-synonymous mutations are listed on top (black). Additional genes of interest targeted by copy-number alterations alone are also indicated (gray) (adapted from Taylor et al., 2010).

Multiple authors have shown that the number of CNAs correlates with high Gleason grade, advanced tumor stage, and other poor prognostic features (Ribeiro et al., 2006b; Lapointe et al., 2007b; Taylor et al., 2010). This finding may reflect the impact of the degree of genomic instability in these tumors or may represent the accumulation of driving events, with prognosis worsening as the tumor accumulates additional “hits” (Barbieri et al., 2013). A meta-analysis applying multivariate statistical methods on a total of 294 PCa with detailed CGH and clinicopathological data suggests a genetic model of prostate carcinogenesis (Ribeiro et al., 2006a). The proposed model revealed that losses at 8p and 13q are independent initiating events in prostate carcinogenesis, followed by a group of four intermediate alterations (8q gain and 6q, 16q, and 18q losses) and then by a cluster of late events that includes all the other frequently gained regions and also losses at 2q, 5q, and 10q (**Figure 5**).

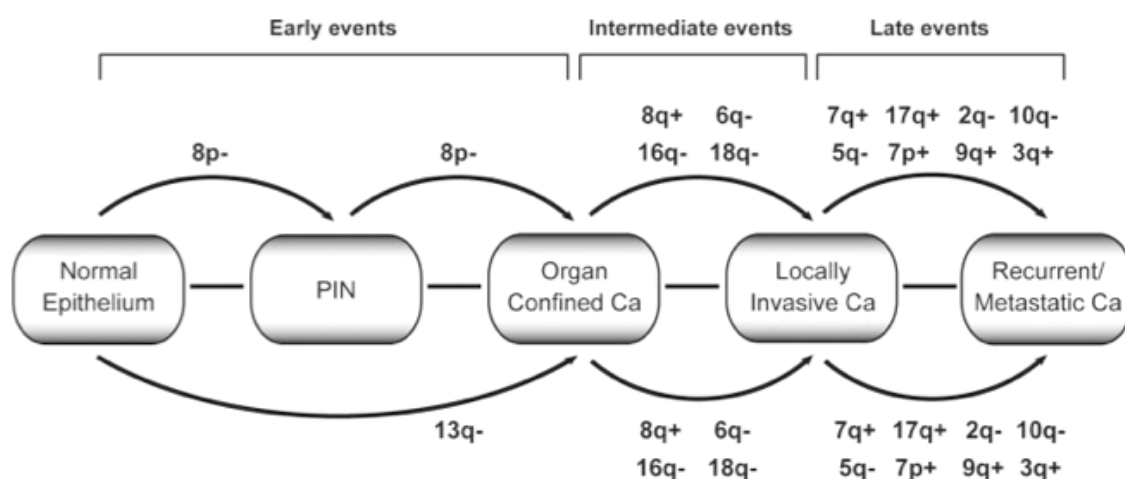


Figure 5. Genetic model of prostate cancer progression based on genomic imbalances detected by comparative genomic hybridization. DNA copy number changes detected are categorized as early, intermediate, or late events. Two preferential pathways of genetic progression are proposed, one starting with 8p loss and the other starting with 13q loss (adapted from Ribeiro et al., 2006a).

The specific genomic imbalances 8q gain and 13q loss are associated with extraprostatic disease independently of Gleason grade, which indicates that genetic features may add significant prognostic information to standard histopathological analysis of prostate cancer specimens (Ribeiro et al., 2006a). In

fact, previous studies have associated gains of chromosome 8q detected in PCa samples either by FISH (Takahashi et al., 1994; Sato et al., 1999; Ribeiro et al., 2007) or CGH (Alers et al., 2000; Fu et al., 2000; Steiner et al., 2002; van Dekken et al., 2003; Ribeiro et al., 2006b) with poor prognosis. Interestingly, a recent *in silico* analysis of 8227 human cancer copy number profiles from all the common solid tumors, and also sarcomas, leukemia and lymphomas, has reported that chromosome 8q gains ranks as one of the most frequent chromosomal alterations of all tumor genomes (Kim et al., 2013) and is also related with bad prognosis in several tumor types (Ghadimi et al., 2003; De Bortoli et al., 2006; Klatte et al., 2012; van den Bosch et al., 2012).

8.5. Recurrent ETS rearrangements in PCa

The discovery of recurrent gene fusions in prostate carcinomas involving the androgen-regulated gene *TMPRSS2* with members of the ETS transcription factor family genes (*ERG* and *ETV1*) constituted a major breakthrough in the understanding of the molecular biology of prostatic carcinomas (Tomlins et al., 2005), therefore raising a vast new set of interesting questions.

With the goal of identifying genes exhibiting the characteristic expression pattern of causal cancer genes (*i.e.* very high expression in a subset of cancer samples) on DNA microarrays data, Tomlins and co-workers developed a statistical method, termed cancer outlier profile analysis (COPA), that successfully identified, amongst others, *ERBB2* in breast cancer and *RUNX1T1* in acute myeloid leukemia when applied to the Oncomine database of cancer microarray studies (<http://www.oncomine.com>). Using this strategy, two members of the ETS transcription factor family gene, *ERG* and *ETV1*, were identified as high-ranking outliers in multiple prostate cancer profiling studies. Using exon-walking quantitative PCR, the authors demonstrated differential expression of the 5' and the 3' exons in *ERG* or *ETV1*, in cases with outlier expression of the respective ETS family member. Subsequent 5'-RNA ligase-mediated rapid amplification of cDNA ends (5'-RACE) revealed a replacement of the 5' ends of the ETS family member with sequences from the 5' untranslated region of the androgen regulated gene *TMPRSS2*. *ERG* expression is ~2,000 fold higher in the VCaP cell line, which harbors *TMPRSS2-ERG*, than in the LNCaP cell line, which does not harbor this gene fusion. Additionally, upon androgen stimulation *ERG* expression increases significantly in VCaP, whereas no effect is observed in LNCaP.

Furthermore, qPCR experiments in 22 prostate carcinomas showed evidence of fusion with *TMPRSS2* in >90% of cases that overexpress *ERG* or *ETV1*. Altogether, this data strongly suggests that the genomic rearrangements underlie the marked overexpression of *ERG* and *ETV1*, where the 5' promoter elements normally driving androgen-mediated expression of *TMPRSS2* in prostate shift to drive aberrant expression of the ETS genes rearranged. In this seminal study, *ERG* or *ETV1* were markedly overexpressed in 95 of 167 (57%) prostate cancer cases included in three independent microarray data sets, whereas overexpression was not observed across 54 benign prostate tissue samples. A recent study summarizing rearrangements involving *TMPRSS2* and/or *ERG* across published studies has reported a prevalence of 47% of 10,779 cases (Pettersson et al., 2012).

8.5.1. Diversity of ETS rearrangements in prostate cancer

Shortly after the initial description of recurrent *ERG* and *ETV1* gene fusions in PCa subsequent studies provided additional data revealing the vast diversity that the ETS rearrangements encompass.

TMPRSS2 and *ERG* genes are both located in the long arm of chromosome 21, about 3Mb (megabases) apart. FISH studies aiming to detect *TMPRSS2-ERG* in tissue sections showed different genomic mechanisms underlying the fusion of the two neighboring genes. A dual-color, break-apart FISH strategy employing two probes flanking *ERG* shows that the gene fusion can arise either from an insertion into another chromosome or a deletion of the DNA segment between the two genes (Perner et al., 2006; Teixeira, 2008). Perner and co-workers have documented these two alternative mechanisms and confirmed the interstitial deletion between *TMPRSS2* and *ERG* using 100kb oligonucleotide SNP arrays (Perner et al., 2006). Apart from *ERG* and *ETV1*, it has been shown that other members of the ETS transcription factor family may also be involved, although less frequently, in gene fusions in prostate cancer, namely *ETV4* (Tomlins et al., 2006) and *ETV5* (Helgeson et al., 2008). Based on these findings, it is plausible that other rearrangement partners may be found (Rubin and Chinnaiyan, 2006).

After the initial report of *TMPRSS2-ETV1* fusions, follow-up studies detected these rearrangements in 2 of 205 (1%) using RT-PCR PCa (Cerveira et al., 2006; Hermans et al., 2006; Perner et al., 2006; Soller et al., 2006; Yoshimoto et al., 2006; Mehra et al., 2007; Winnes et al., 2007). However, microarray studies

showed that about 8.5% (19 of 224) of PCa overexpress *ETV1* (Glinsky et al., 2004; Lapointe et al., 2004; Yu et al., 2004; Tomlins et al., 2007). This observed discrepancy triggered the characterization of *ETV1* transcripts in cases with outlier expression of this ETS gene, leading to the discovery of 5' fusion partners of *ETV1* other than *TMPRSS2*, namely *HERV-K_22q11.23*, *SLC45A3*, *C15orf21* and *HNRPA2B1* (Tomlins et al., 2007). Up until now, four different fusion partners have been described for *ERG*, ten for *ETV1*, four for *ETV4* and two for *ETV5* (Table 5).

Table 5. Known ETS genes and 5' partners involved in gene fusions.

Translocation partner	Locus	Androgen response ^a	Structure of encoded protein ^b	Gene function
<i>ERG</i>				
<i>TMPRSS2</i>	21q22	Upregulated	NTT; Fusion (5aa); WT	Transmembrane serine protease
<i>SLC45A3</i>	1q32	Upregulated	NS	Solute carrier protein
<i>HERPUD1</i>	16q13	Upregulated	Fusion (49aa)	Ubiquitin-like member
<i>NRDG1</i>	8q24.3	Upregulated		Alfa/beta hydrolase
<i>ETV1</i>				
<i>TMPRSS2</i>	21q22	Up-regulated	NTT; Fusion (5aa)	Transmembrane serine protease
<i>SLC45A3</i>	1q32	Up-regulated	NTT	Solute carrier protein
<i>ACSL3</i>	2q36.1	Up-regulated	NTT	Acetyl-CoA synthetase
<i>HERV-K22</i>	22q11.23	Up-regulated	NTT	Endogenous retrovirus
<i>HERV-K17 (FLJ35294)</i>	17p13.1	Up-regulated	NTT	Endogenous retrovirus
<i>FOXP1</i>	3p13	Up-regulated	Fusion (19aa)	Forkhead box P1
<i>EST14</i>	14q21.1	Up-regulated	NTT; Fusion (5aa)	Non-coding RNA
Chr 14 (q13.3-14q21.1)	14q13.3-21.1	Up-regulated	WT	NA
<i>C15orf21</i>	15q21.1	Down-regulated	NTT	Unidentified open reading frame
<i>HNRPA2B1</i>	7p15	No effect	Fusion (2aa)	Ribonuclear protein
<i>ETV4</i>				
<i>TMPRSS2</i>	21q22	Up-regulated	NTT; Fusion (5aa)	Transmembrane serine protease
<i>KLK2</i>	19q13.33	Up-regulated	NTT	Kallikrein peptidase
<i>CANT1</i>	17q25.3	Up-regulated	NTT	Calcium activated nucleotidase
<i>DDX5</i>	17q24.1	No effect	Fusion (102aa)	RNA helicase
<i>ETV5</i>				
<i>TMPRSS2</i>	21q22	Up-regulated	WT; Fusion (84aa)	Transmembrane serine protease
<i>SLC45A3</i>	1q32	Up-regulated	NTT	Solute carrier protein

NTT, N-terminal truncated; WT, Wild type; NS, not specified; NA, not applicable.

Among these 5'partners there is diversity regarding tissue specificity and androgen responsiveness. Fusion partners like the *SLC45A3* gene or the endogenous retroviral element (*HERVK17*) display similar tissue specificity as *TMPRSS2* and are equally androgen-induced. Contrarily, the fusion partner *C15orf21*, despite being overexpressed in PCa, is repressed by androgens. However, there are ubiquitously expressed 5' fusion partners, such as the *HNRPA2B1* gene, displaying no evidence of androgen regulation. Finally, ETS family genes may be rearranged with prostate-specific enhancers in chromosomal regions such as 14q13.3-14q21.1. It is generally accepted that the main effect of the 5' fusion partner is to promote an abnormal expression of the ETS gene. In fact, in a recent IHC expression analysis it has been demonstrated that ERG protein expression was extremely high and at similar levels regardless of *ERG* being rearranged with *TMPRSS2*, *SLC45A3* or *NDRG1* (Park et al., 2010). Other study reported decreased expression of *SLC45A3* protein expression in cases with *SLC45A3-ERG* gene fusions, in contrast to *TMPRSS2* that showed no correlation with protein expression and rearrangement status (Perner et al., 2013)

The described genomic diversity of ETS rearrangements leads to an even higher complexity of the derived fusion transcripts originated. Each ETS rearrangement may generate several fusion transcripts. For example, regarding the *TMPRSS2-ERG* fusion, several expressed variants have been reported differing with respect to the break point sites in *TMPRSS2* (exons 1-5) and *ERG* (exons 2-5). For the most common variant *TMPRSS2* (exon 1)-*ERG* (exon 4), protein translation is predicted to initiate from an in-frame ATG codon in exon 4 of *ERG*, thus yielding a N-terminal truncated (NTT) ERG protein. Fusion transcript variants may also encode full-length ERG protein and chimeric proteins. Regarding rearrangements involving *ETV1* and *ETV4*, overexpression of a NTT ETS protein is also the most frequent outcome, however fusion transcripts encoding for chimeric proteins have also been found in tumors with *ETV1* and *ETV4* rearrangements. Additionally, rearrangements involving *ETV1* with a region of chromosome 14 (14q13.3-14q21.1) have been described resulting in overexpression of wild type (wt) *ETV1* (Gasi et al., 2011). Rearrangements involving *ETV5* are the least common so far described, but fusion transcripts encoding wt *ETV5*, NTT *ETV5* and chimeric protein have already been found (Helgeson et al., 2008).

There is evidence in the literature indicating that the reported spectrum of 5' partners is incomplete (Attard et al., 2008b). For example, Attard and colleagues found that only 8 of 23 (34%) cases with *ETV1* rearrangements by FISH harbored 5' partners known at that time and were able to identify *ACSL3* as a new 5' fusion partner of *ETV1* (Attard et al., 2008b). Numerous rare novel 5' partners will likely continue to be identified, particularly with the advent of RNA-seq, which allows for the identification of the 5' end of gene fusions far more expediently than 5'-RACE or similar techniques.

8.5.2. ETS transcription factor family

The *ERG*, *ETV1*, *ETV4* and *ETV5* genes belong to the E26 transformation-specific (ETS) transcription factor family, one of the largest families of transcription regulators including a total of 28 genes (**Figure 6**). The coded ETS proteins contain an evolutionary conserved ETS domain of 85 amino acids that recognizes 5'-CGAA/T-3' core sequences, known as ETS binding sites, present in promoter regions of target genes (Donaldson et al., 1994; Sharrocks et al., 1997). The second conserved domain found in a subset of ETS genes is the pointed (PNT) domain. This 65–85 amino acid domain is found in 11 of 28 human ETS genes and has been shown to function homo-oligomerization, hetero-dimerization and transcriptional repression (Seth and Watson, 2005). These proteins may also have additional domains, such as repressor domain and transcription activation domain, or rarer ones present in only one subfamily, such as B-box that is exclusively found in the TCF subfamily or the OST domain that is only present in GABPa (Wasylyk et al., 1998; Sharrocks, 2001; Seth and Watson, 2005; Hollenhorst et al., 2011).

Genome wide occupancy studies reveal that a single ETS protein can bind to hundreds to thousands of ETS binding sites in a particular cell type, but these studies also show that multiple ETS factors can occupy any given single ETS binding site within a particular cell type, indicating redundant binding (Hollenhorst et al., 2007; Barski and Zhao, 2009; Farnham, 2009; Hollenhorst et al., 2011). Importantly, many of the redundant binding sites are characterized by the consensus ETS sequence ACCGGAAGT and are mostly found on the proximal promoters of housekeeping genes (Hollenhorst et al., 2007; Barski and Zhao, 2009; Farnham, 2009). By contrast, non-redundant ETS sites are more often characterized by atypical, low affinity ETS binding site sequences that are often

flanked by binding sites of other transcription factors (Wheat et al., 1999; Hollenhorst et al., 2011). Differences in consensus DNA binding sequences for most ETS proteins are minimal, and specificity is often determined by the amino acids surrounding the ETS domain in the ETS protein and by DNA sequences flanking the central GGAA/T core in the genome. Based on in vitro binding affinities, the ETS binding profiles for the 12 ETS subfamilies fall into four distinct classes (Hollenhorst et al., 2007; Barski and Zhao, 2009; Farnham, 2009).

Human ETS family



Figure 6. Structural and functional domains of the ETS family of transcription factors. Nomenclature (HUGO) and domains of the 28 paralogous human ETS proteins are sorted by subfamily. Boxes identify the DNA-binding ETS domain (red), PNT domain (green), OST domain (blue), and B-box (magenta) of the ETS factors (adapted from Hollenhorst et al., 2011).

ETS factors are trans-acting phosphoproteins known to act as positive or negative regulators of the expression of genes that are involved in various biological processes, including those that control cellular proliferation, differentiation, hematopoiesis, apoptosis, metastasis, tissue remodeling, angiogenesis, and transformation. While most ETS factors were initially characterized as transcriptional activators or repressors, it has become evident that several ETS factors can function as either activators or repressors depending upon the type of promoter and cellular context (Seth and Watson, 2005).

8.5.3. Prognostic value of ETS rearrangements in prostate carcinoma

Several clinical features of PCa, including tumor stage (Jewett, 1975), degree of tumor cell differentiation or Gleason score (Gleason, 1966), and the serum PSA (Stamey et al., 1987), are used in routine clinical practice to separate men into groups at low, intermediate, and high risk for tumor recurrence following local therapy. Men with tumors of higher grade (Gleason 8-10), stage (T3-T4), or PSA level (e.g., >20ng/mL) are deemed to have high-risk disease and thus present worse prognosis. However, the majority of patients undergoing prostatectomy have low to intermediate-risk clinical features, and determining the prognosis for these patients remain difficult (Singh et al., 2002). The utility of existing prognostic factors might be limited because they largely measure tumor differentiation and bulk, but do not otherwise sample the underlying biological properties that likely drive tumor behavior.

To prove clinical utility of any new potentially important prognostic marker, including molecular markers, it will need to provide added independent value in multivariate analysis beyond established prognostic indicators (Humphrey, 2004). The prognostic value of the *TMPRSS2-ERG* gene fusion is a controversial topic. In an early study, prior to the discovery of the *TMPRSS2-ERG* gene fusion, Petrovics and co-workers observed that patients with high levels of ERG overexpression had better biochemical recurrence-free survival (Petrovics et al., 2005). Similarly, Saramaki and co-workers found an association of the *TMPRSS2-ERG* fusion gene with favorable biochemical recurrence-free survival (Saramaki et al., 2008). However, there are studies in which no association was found between *TMPRSS2-ERG* and biochemical recurrence (Lapointe et al., 2007a) or disease-specific survival (FitzGerald et al., 2008; Gopalan et al., 2009). Finally, some authors have found associations of the gene fusion with biochemical recurrence (Nam et al.,

2007) or with disease progression (Demichelis et al., 2007; Attard et al., 2008a). Attard and co-workers described a subgroup with copy number increase and rearrangement by deletion that tended to be clinically more aggressive (Attard et al., 2008a). However, this association has been contested by Gopalan and co-workers, who, by assessing whole DNA ploidy by flow cytometry and ploidy of an independent chromosome (#9) by FISH, concluded that the aggressive phenotype is associated with aneuploidy and not with the duplication of *TMPRSS2-ERG* itself, therefore indicating that other genomic changes may be better prognostic markers (Gopalan et al., 2009).

Differences in study design, gene fusion assessment method, and clinical end point may be contributing to this disparity. Clinical end point is especially relevant. In fact, although all patients with lethal prostate cancer experience biochemical recurrence, only a minority of patients with biochemical recurrence will succumb to the disease (Narod et al., 2008).

Chapter 2

Aims

Aims

The diversity of genomic alterations found in prostate adenocarcinomas is, arguably, related to the documented clinical outcome heterogeneity of this disease. Given the recent findings showing the pathogenetic relevance of ETS transcription factors in prostate cancer, the aims of this thesis were:

- To characterize the pattern of fusion oncogenes affecting ETS transcription factors, and to identify new fusion oncogenes involving other ETS genes in the about 50% of prostate carcinomas that do not harbor rearrangements of *ERG/ETV1/ETV4/ETV5*;
- To identify novel genes acting as 5' fusion partners of ETS transcription factors in prostate carcinomas;
- To evaluate the extent of intratumor heterogeneity of ETS fusions in prostate carcinomas;
- To define the role of ETS rearrangements in the pathogenetic pathways of prostate carcinogenesis, namely, their association with the recurrent copy number change 8q gain;
- To evaluate the clinicopathological associations and the prognostic value of ETS fusion oncogenes, alone or in combination with relative 8q copy number gain, in diagnostic needle biopsies from prostate cancer patients with long follow up.

Chapter 3

Original publication # I

FLI1 is a Novel ETS Transcription Factor Involved in Gene Fusions in Prostate Cancer

Paula Paulo^{*,1,3,6,7}, **João D. Barros-Silva**^{*,1,3}, Franclim R. Ribeiro^{1,3,6,7}, João Ramalho-Carvalho^{1,4}, Carmen Jerónimo^{1,4,5}, Rui Henrique^{2,4,5}, Guro E. Lind^{6,7}, Rolf I. Skotheim^{6,7}, Ragnhild A. Lothe^{6,7} and Manuel R. Teixeira^{1,3,5,7}

Departments of ¹Genetics and ²Pathology, Portuguese Oncology Institute, Porto, Portugal; Groups of ³Cancer Genetics and ⁴Cancer Epigenetics, Research Centre of the Portuguese Oncology Institute, Porto, Portugal; ⁵Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal; ⁶Department of Cancer Prevention, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway; ⁷Centre for Cancer Biomedicine, University of Oslo, Oslo, Norway;

* PP and JDBS contributed equally to this work and should be regarded as joint first authors.

FLII Is a Novel ETS Transcription Factor Involved in Gene Fusions in Prostate Cancer

Paula Paulo,^{1,2,3,4†} João D. Barros-Silva,^{1,2†} Francim R. Ribeiro,^{1,2,3,4} João Ramalho-Carvalho,^{1,5} Carmen Jerónimo,^{1,5,6} Rui Henrique,^{5,6,7} Guro E. Lind,^{3,4} Rolf I. Skotheim,^{3,4} Ragnhild A. Lothe,^{3,4} and Manuel R. Teixeira^{1,2,4,6*}

¹Department of Genetics, Portuguese Oncology Institute, Porto, Portugal

²Group of Cancer Genetics, Research Centre of the Portuguese Oncology Institute, Porto, Portugal

³Department of Cancer Prevention, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway

⁴Centre for Cancer Biomedicine, University of Oslo, Oslo, Norway

⁵Group of Cancer Epigenetics, Research Centre of the Portuguese Oncology Institute, Porto, Portugal

⁶Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal

⁷Department of Pathology, Portuguese Oncology Institute, Porto, Portugal

To characterize the pattern of ETS rearrangements and to uncover novel ETS fusion genes, we analyzed 200 prostate carcinomas (PCa) with TaqMan low-density arrays (TLDA), followed by selective analyses with fluorescence in situ hybridization (FISH), RT-PCR, and sequencing. Besides confirming the recurrent presence of *ERG*, *ETV1*, *ETV4*, and *ETV5* rearrangements, we here report *FLII* as the fifth ETS transcription factor involved in fusion genes in prostate cancer. Outlier expression of the *FLII* gene was detected by TLDA in one PCa that showed relative overexpression of *FLII* exons 4:5 as compared with *FLII* exons 2:3. A structural rearrangement was found using FISH probes flanking the *FLII* gene and RT-PCR and sequencing analyses showed fusion of *SLC45A3* exon 1 with *FLII* exon 3. Interestingly, we found four cases with two different ETS rearrangements in the index tumor, thus revealing intratumor genetic heterogeneity. Correlation analysis with clinico-pathological data showed association of *ERG* rearrangements with locally advanced disease (pT3, $P = 0.007$) and *MYC* overexpression ($P = 0.001$), and association of *ETV1* rearrangements with *PTEN* downregulation ($P = 0.015$). We report that *FLII* is a novel ETS transcription factor involved in gene fusions in prostate cancer and that intratumor genetic heterogeneity of ETS rearrangements can occasionally be found in index primary tumors. © 2011 Wiley Periodicals, Inc.

INTRODUCTION

Rearrangement of *TMPRSS2* with *ERG* has been recurrently found in around 50% of prostate adenocarcinomas (PCa) and 20% of precursor high-grade prostatic intraepithelial neoplasia (HGPIN) lesions (Tomlins et al., 2005; Cerveira et al., 2006; Tomlins et al., 2009). *TMPRSS2* is a prostate specific gene regulated by androgens and the most common *TMPRSS2-ERG* fusion (*TMPRSS2* exon 1 with *ERG* exon 4) results in overexpression of a 5' truncated *ERG* transcript (Wang et al., 2006). Three other members of the ETS family of transcription factors, namely, *ETV1*, *ETV4*, and *ETV5*, have also been found to be involved in gene fusions in a smaller proportion of PCa (Tomlins et al., 2006; Tomlins et al., 2007; Helgeson et al., 2008). Whereas *TMPRSS2* is the most common 5' partner, several different alternative fusion partners have been described in ETS rearrangements (Kumar-Sinha et al., 2008; Clark and Cooper, 2009). Furthermore, different

ETS rearrangements have been found in spatially separated tumor foci in multifocal PCa (Perner et al., 2006; Barry et al., 2007; Mehra et al., 2007; Clark et al., 2008; Furusato et al., 2008). Considering that close to 50% of the PCa have no known ETS changes, Han et al. (2008) conducted a fluorescence in situ hybridization (FISH) screen using break-apart probes to interrogate all ETS family members in clinically localized PCa, but

Additional Supporting Information may be found in the online version of this article.

Supported by: Fundação para a Ciência e a Tecnologia (FCT), Grant numbers: PTDC/SAU/OBD/70543/2006; SFRH/BD/27669/2006; SFRH/BD/46574/2008; SFRH/BPD/26492/2006.

[†]Paula Paulo and João D. Barros-Silva contributed equally to this work and should be regarded as joint first authors.

*Correspondence to: Manuel R. Teixeira, Department of Genetics, Portuguese Oncology Institute—Porto, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal.

E-mail: manuel.teixeira@ipporto.min-saude.pt

Received 17 July 2011; Accepted 10 October 2011

DOI 10.1002/gcc.20948

Published online 12 November 2011 in

Wiley Online Library (wileyonlinelibrary.com).

TABLE 1. Correlation Analysis of the Presence of ETS, *ERG*, or *ETV1* Rearrangements with Clinico-Pathological Parameters

Clinico-pathological parameters	Group	ETS rearrangement		<i>ERG</i> rearrangement		<i>ETV1</i> rearrangement	
		Positive N (%)	Negative N (%)	Positive N (%)	Negative ^a N (%)	Positive N (%)	Negative ^a N (%)
Age	<65	65 (54.6)	37 (45.7)	57 (55.3)	37 (45.7)	7 (50.0)	37 (45.7)
	≥65	54 (45.4)	44 (54.3)	46 (44.7)	44 (54.3)	7 (50.0)	44 (54.3)
	P value	0.136		0.124		0.494	
PSA at diagnosis	<10	82 (68.9)	57 (70.4)	68 (66.0)	57 (70.4)	13 (92.9)	57 (70.4)
	≥10	37 (31.1)	24 (29.6)	35 (34.0)	24 (29.6)	1 (7.1)	24 (29.6)
	P value	0.476		0.320		0.068	
Pathological stage	pT2	57 (47.9)	55 (67.9)	49 (47.6)	55 (67.9)	6 (42.9)	55 (67.9)
	pT3	62 (52.1)	26 (32.1)	54 (52.4)	26 (32.1)	8 (57.1)	26 (32.1)
	P value	0.006		0.007		0.128	
Gleason score	≤7 (3 + 4)	99 (83.2)	57 (70.4)	88 (85.4)	57 (70.4)	9 (64.3)	57 (70.4)
	≥7 (4 + 3)	20 (16.8)	24 (29.6)	15 (14.6)	24 (29.6)	5 (35.7)	24 (29.6)
	P value	0.037		0.018		0.755	

^aETS-negative cases only. For statistical analysis, cases with Gleason grades 3 + 4 and 4 + 3 were lumped with cases with Gleason score <7 and >7, respectively.

no additional ETS members were found rearranged (Han et al., 2008).

Although ETS gene fusions are now recognized as primary genetic events in prostate carcinogenesis, secondary chromosome changes such as 8q gain and 10q loss have been shown to be more relevant as prognostic factors. Whereas we have recently shown that relative 8q gain around *MYC* is associated with poor disease-specific survival irrespective of the *TMPRSS2-ERG* fusion gene status (Barros-Silva et al., 2011), others have shown that *MYC* is a downstream target of the overexpressed *ERG* transcription factor (Sun et al., 2008). On the other hand, an association has been shown between the *TMPRSS2-ERG* fusion gene and 10q deletions including the *PTEN* gene, a combination that is also associated with poor prognosis (Yoshimoto et al., 2008).

In this work, we used the expression pattern of nine ETS family members as a screening strategy to characterize a series of 200 patients with clinically localized prostate cancer for known and new ETS rearrangements. The pattern of ETS rearrangements was then correlated with clinico-pathological parameters and with *MYC* and *PTEN* expression levels.

MATERIALS AND METHODS

Prostate Cancer Patients and Samples

Primary tumor samples were collected from 200 patients with clinically localized PCa consecutively diagnosed and treated with radical prostatectomy at the Portuguese Oncology Institute—Porto, Portugal. After histological identification of the tumor areas by an experienced pathologist,

the tissue fragment was trimmed to maximize the yield of target cells of the index tumor and an average of fifty 12-micron thick sections was cut for RNA extraction. RNA was extracted from each sample using TRIzol[®] Reagent following purification with PureLink[™] RNA Mini Kit columns (Invitrogen SA, Spain). Only the index tumor was tested by the molecular genetic and molecular cytogenetic methods described below. Relevant clinical data were obtained from medical records. This study was approved by the institutional review board.

TaqMan Low-Density Arrays

To study the expression level of the genes of interest, 100 µg of RNA were converted to cDNA using the High-Capacity RNA-to-cDNA kit, according to the manufacturer's instructions (Applied Biosystems, Carlsbad, CA). The TaqMan low-density array (TLDA) gene panel included the ETS transcription factors *ERG*, *ETV1*, *ETV4*, *ETV5*, *ELK4*, *FLI1*, *FEV*, *ETV6*, and *ETS2*, as well as assays for *MYC*, *PTEN*, and for the most common Type III *TMPRSS2-ERG* fusion transcript. The ETS family members were selected because they had previously been found rearranged in PCa (*ERG*, *ETV1*, *ETV4*, and *ETV5*), Ewing sarcoma (*FLI1*, *ERG*, *ETV1*, *ETV4*, and *FEV*) or leukemia (*ERG* and *ETV6*). Additionally, we included *ELK4* because it has been found overexpressed in PCa and *ETS2* for its localization in 21q22.2 between *ERG* and *TMPRSS2*. TaqMan assays selected for the TLDA card are listed in Supporting Information Table 1. Relative expression of the genes of

interest was obtained by the comparative Ct method, using *18S* levels as control. Expression data obtained for *ERG* was previously reported (Ribeiro et al., 2011).

Fluorescent In Situ Hybridization

To look for genomic rearrangements involving the ETS transcription factors showing outlier expression patterns in PCa, BAC probes flanking *ERG*, *ETV1*, *ETV4*, *ETV5*, and *FLII* were used (Supporting Information Table 2) on tissue sections from paraffin blocks of the index tumor (Ribeiro et al., 2011). For clarification of the *TMPRSS2-ERG* rearrangement, a triple-color commercial probe flanking the *TMPRSS2* and *ERG* genes at 21q22 (Poseidon *TMPRSS2-ERG* probe, Kretech Diagnostics, Amsterdam, The Netherlands) was used. In addition to the red and green probes flanking the *TMPRSS2* gene, it includes a third blue probe covering most of the *ERG* gene (including the marker RH123924) and up to marker DS21S1439. The probe design allows identification of *TMPRSS2-ERG* fusions by deletion and by insertion, but also possible rearrangements of each gene with other partners. Samples showing *ERG* rearrangement with a different 5' partner were screened for rearrangement with *SLC45A3* using specific 5' BAC probes (Supporting Information Table 2). An abnormal signal pattern was considered representative when present in a minimum of 50 morphologically intact, non-overlapping nuclei.

Quantitative RT-PCR

To look for *FLII* exon-level differential expression in the PCa with outlier expression (see below), we used TaqMan probes targeting exon 2-3 and exon 4-5 transitions (Supporting Information Table 3). For this purpose, whole transcriptome cDNA was used as previously described (Ribeiro et al., 2011). Briefly, 200 ng of RNA were converted into cDNA using the TransPlex Whole Transcriptome Amplification Kit (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions. *FLII* exon-level expression was normalized to the expression of the *GUSB* housekeeping gene, using the comparative Ct method. Three rearrangement-negative PCa and three normal prostate tissue samples were included as controls.

RT-PCR and Sequencing

To evaluate the type of transcripts derived from the different *TMPRSS2-ERG* rearrange-

ments, RT-PCR was performed as previously described (Cerveira et al., 2006). Primer sequences are described in Supporting Information Table 3. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Deutschland) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the manufacturer's recommendations.

Statistical Analysis

Expression data was analyzed using the Statistical Package for Social Sciences software, version 15.0 (SPSS, Chicago, IL). For correlation analysis with clinico-pathological parameters, the Pearson Chi-Square test was used. To compare mRNA expression values in the different sample groups, we used the Mann-Whitney non-parametric test. Associations between the expression of pairs of genes in the same samples were assessed by the Spearman non-parametric correlation (r_s). A *P* value smaller than 0.05 was considered statistically significant.

RESULTS

Identification and Classification of *ERG* Rearrangement-Positive PCa

According to the bimodal distribution of the threshold cycle (C_{tERG}) for *ERG* expression, we used FISH to clarify the rearrangement status in samples that showed intermediate *ERG* expression levels ($n = 22$, $C_{tERG} = 27.00 \pm 1.00$) and in samples with high *ERG* expression but low *TMPRSS2-ERG* Type III expression ($n = 9$, $C_{tERG} < 26.5$ and $C_{tTMPRSS2-ERG} > 27$; Supporting Information Fig. 1). FISH analysis allowed the stratification of PCa as *ERG* rearrangement positive and *ERG* rearrangement negative, either with *TMPRSS2* or with a different 5' partner (Fig. 1A, Supporting Information Table 4). The *TMPRSS2-ERG* fusion gene was additionally validated by FISH in five randomly selected PCa with both *ERG* and *TMPRSS2-ERG* Ct values less than 27. For validation purposes, the 11 samples with *TMPRSS2-ERG* rearrangement found by FISH and a $C_{tTMPRSS2-ERG} > 27$ were evaluated by RT-PCR and the presence of a *TMPRSS2-ERG* transcript was confirmed by sequencing (Supporting Information Fig. 2). All four PCa with an *ERG* rearrangement with a 5' partner other than *TMPRSS2* were shown by FISH to harbor the *SLC45A3-ERG* rearrangement

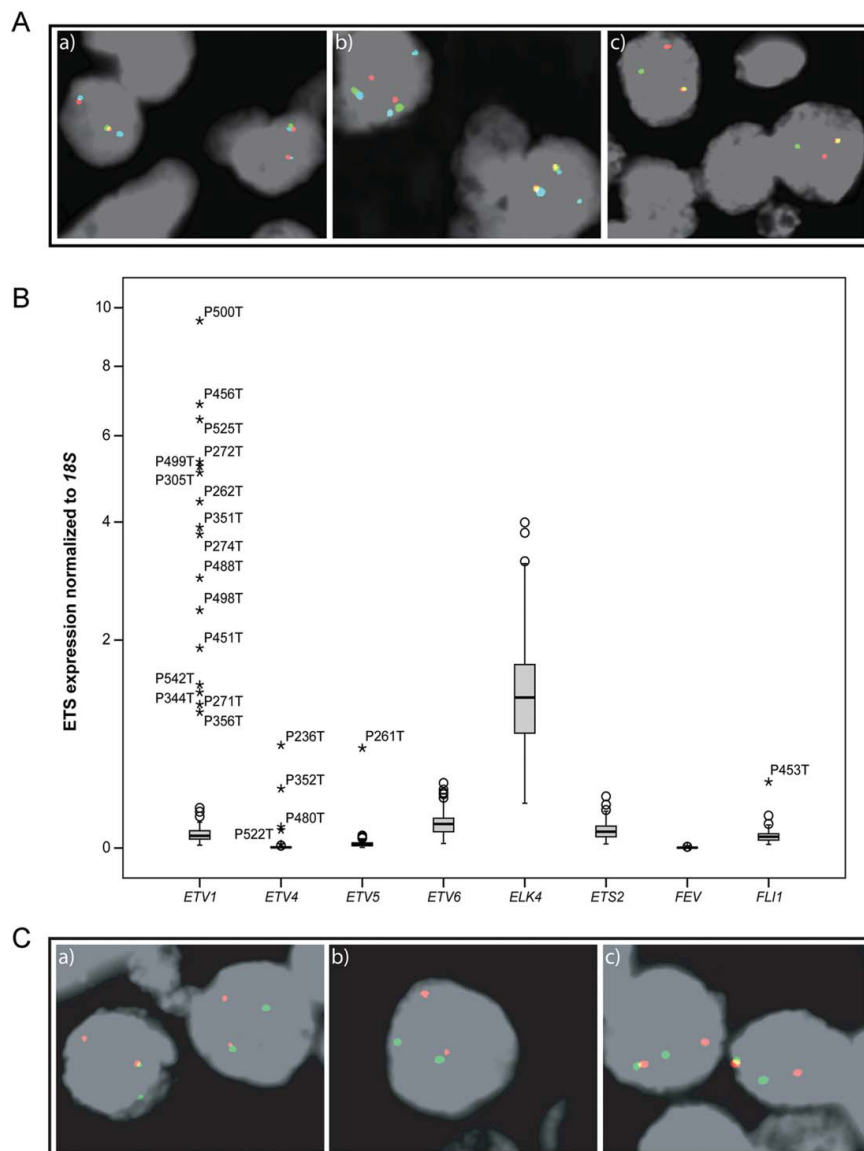


Figure 1. Identification of PCa with ETS rearrangements. A. Representative pictures of the FISH pattern obtained for *ERG* rearrangement positive PCa either with the *TMPRSS2-ERG* tricolor probe (a and b) or with BAC probes (c). (a) Case P555T showing the *TMPRSS2-ERG* fusion by deletion. (b) Case P236T showing a pattern consistent with *ERG* rearrangement with a different 5' partner. (c) Case P467T showing the *SLC45A3-ERG* rearrangement; 5'-*SLC45A3* (red), 3'-*ERG* (green). B. Boxplot showing distribution of 200 prostate

carcinomas according to ETS expression. Asterisks show cases with extreme outlier expression and circles show cases with outlier expression. C. Representative pictures of FISH analysis obtained for *ETV1*, *ETV4*, and *ETV5* with break-apart BAC probes. (a) Case P451T showing break-apart of *ETV1*. (b) Case P522T showing break-apart of *ETV4*. (c) Case P261T showing break-apart of *ETV5*. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

244

PAULO ET AL.

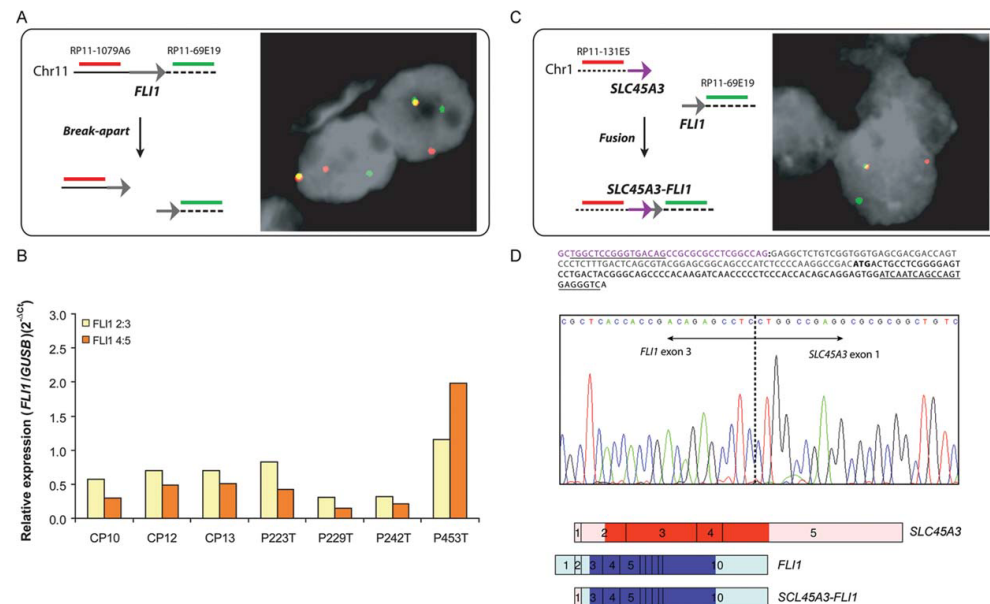


Figure 2. Identification of the novel *SLC45A3-FLI1* rearrangement in case P453T. A. FISH analysis using *FLI1* break-apart probes showing a split of probes flanking the *FLI1* gene. B. qRT-PCR analysis showing relative overexpression of *FLI1* exons 4:5 as compared with *FLI1* exons 2:3 in sample P453T. C. FISH analysis combining a probe directed to the 5' region of *SLC45A3* (red) and the probe directed to the 3' region of *FLI1* (green) shows a fusion signal indicative of a

SLC45A3-FLI1 rearrangement. D. *SLC45A3-FLI1* rearrangement seen at the transcript level revealing fusion of *SLC45A3* exon 1 with *FLI1* exon 3 (sequencing analysis with the reverse primer is shown). Schematic representation of the fusion is shown in the bottom panel. Protein coding regions are colored in dark. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Fig. 1A). The combined approach therefore allowed the identification of 99 PCa (49.5%) with the *TMPRSS2-ERG* fusion gene and five cases (2.5%) with the *SLC45A3-ERG* fusion gene.

Identification of *ETV1*, *ETV4*, and *ETV5* Rearrangement-Positive PCa

Analysis of the TLDA data on the remaining eight ETS transcription factors revealed extreme outlier expression of *ETV1* in 16 cases, of *ETV4* in four cases, of *ETV5* in one case and of *FLI1* in one case, whereas *ETV6*, *ELK4*, *ETS2*, *FEV*, and *ELK4* did not show extreme outlier expression values in any PCa (Fig. 1B). FISH validation using break-apart BAC probes confirmed an *ETV1* genomic rearrangement in 14 of the 16 cases with *ETV1* outlier expression (Fig. 1C), representing 7% of all PCa. Genomic rearrangement was confirmed in three of the four PCa showing outlier expression of *ETV4* and in the single PCa with *ETV5* overexpression (Fig. 1C), thus representing a frequency of 1.5% and 0.5%, respectively.

Identification of a New Rearrangement Involving *FLI1*

In the single case showing *FLI1* outlier expression by the TLDA analysis, BAC probes flanking this gene showed a break-apart signal that confirmed the presence of a genomic rearrangement (Fig. 2A). Expression quantification using TaqMan gene expression assays revealed a 2.6 and a 3.2 fold-increase in expression of exons 4:5 relative to exons 2:3 (Fig. 2B) in this case as compared to controls and to tumors without *FLI1* outlier expression, respectively, as expected for a gene fusion originating overexpression of the 3' partner gene. To identify the 5' fusion partner of *FLI1*, we followed a candidate gene approach by using BAC probes targeting the two most common ETS 5' fusion partners *TMPRSS2* and *SLC45A3*. Using BAC probes targeting 5' *SLC45A3* and 3' *FLI1*, a co-localization signal was consistently observed, thus revealing the presence of an *SLC45A3-FLI1* rearrangement (Fig. 2C). Finally, RT-PCR followed by sequencing allowed the identification of the fusion transcript

SLC45A3-FLI1, where *SLC45A3* exon 1 is fused with *FLI1* exon 3 (Fig. 2D). Because the *FLI1* translation start codon is localized on exon 3, this rearrangement is expected to lead to overexpression of a structurally normal FLI1 protein.

Intratumor Genetic Heterogeneity

Crossing *ERG* rearrangement positive with other ETS positive carcinomas, we found three cases with rearrangements of two different ETS genes. Careful examination of the correspondent H&E sections by an experienced pathologist (RH) showed that the different ETS rearrangements were present in different parts of the index tumor in all three cases (Supporting Information Table 4). One case presented both a *SLC45A3-ERG* fusion gene and an *ETV4* rearrangement (Fig. 3A), another had the new *SLC45A3-FLI1* fusion gene and the *TMPRSS2-ERG* rearrangement (Fig. 3B), and the third case showed a *TMPRSS2-ERG* rearrangement and an *ETV4* rearrangement (not shown). Additionally, one of the five samples randomly selected for validation of the *TMPRSS2-ERG* status by FISH (having *ERG* and *TMPRSS2-ERG* Ct values less than 27) showed both a *TMPRSS2-ERG* and a *SLC45A3-ERG* rearrangement in the index tumor (not shown). Thus, altogether, a total of four of the 200 index tumors presented more than one ETS rearrangement.

ETS Rearrangements and MYC and PTEN Expression

MYC expression was higher ($P = 0.001$) and *PTEN* expression was lower ($P = 0.017$) in PCa with ETS rearrangements than in those without (Supporting Information Fig. 3). Looking at *ERG* and *ETV1* rearrangements separately, *MYC* was significantly upregulated only in PCa harboring *ERG* rearrangement ($P = 0.001$), whereas *PTEN* was significantly downregulated only in PCa harboring *ETV1* rearrangement ($P = 0.015$; Supporting Information Fig. 3). Spearman correlation also showed a significant positive association between *ERG* and *MYC* expression ($r_s = 0.450$, $P = 0.0000019$) in PCa with *ERG* rearrangement (Supporting Information Fig. 4).

ETS Rearrangements and Clinico-Pathological Associations

PCa with ETS rearrangements were associated with locally advanced disease (pT3, $P = 0.006$)

and with lower Gleason score ($P = 0.037$) (Table 1). When PCa patients were further stratified by the main ETS rearrangements, these clinico-pathological associations were significant only for *ERG* ($P = 0.007$ and $P = 0.018$, respectively; Table 1). *MYC* and *PTEN* expression were not significantly associated with any clinico-pathological parameter.

DISCUSSION

We characterized the expression pattern of nine ETS family members as a screening strategy to uncover new and known ETS fusion genes in a series of 200 patients with clinically localized prostate cancer. By using TLDA analysis, a high-throughput strategy of expression analysis based on quantitative real-time PCR, followed by selective analyses with FISH, RT-PCR, and sequencing, we found 103 (51.5%) PCa with an *ERG* fusion gene, namely, 99 (49.5%) with the *TMPRSS2-ERG* fusion gene and five (2.5%) with the *SLC45A3-ERG* fusion gene (one of them with both rearrangements). Regarding the other ETS members previously known to be involved in gene fusions in PCa, our combined cytogenetic and molecular approach allowed the identification of 14 cases with *ETV1* genomic rearrangement and overexpression, representing 7% of all cases. Genomic rearrangement was confirmed also in three (1.5%) PCa showing outlier expression of *ETV4* and in one (0.5%) PCa with *ETV5* overexpression, respectively. The overall representation of rearrangements of *ERG*, *ETV1*, *ETV4*, and *ETV5* in our series of 200 PCas is in accordance with what was described by others (Attard et al., 2008; Kumar-Sinha et al., 2008; Clark and Cooper, 2009; Esgueva et al., 2010; Svensson et al., 2011). Despite of the outlier expression of *ETV1* or *ETV4* in two and one additional cases, respectively, we could not find a genomic rearrangement with FISH using a break-apart strategy in these PCa. This may be explained by a genetic change below the resolution of the FISH analysis or by the existence of an upstream regulatory mechanism that drove *ETV1* or *ETV4* overexpression in these cases (Hermans et al., 2006; Tu et al., 2007; Hermans et al., 2008). Further analyses are ongoing to characterize the mechanism underlying *ETV1* and *ETV4* overexpression in these three cases, as well as to identify the 5' fusion partners of *ETV1*, *ETV4*, and *ETV5* in the cases with genomic rearrangements of these ETS genes.

In addition to find in our series all ETS members previously known to be implicated in fusion

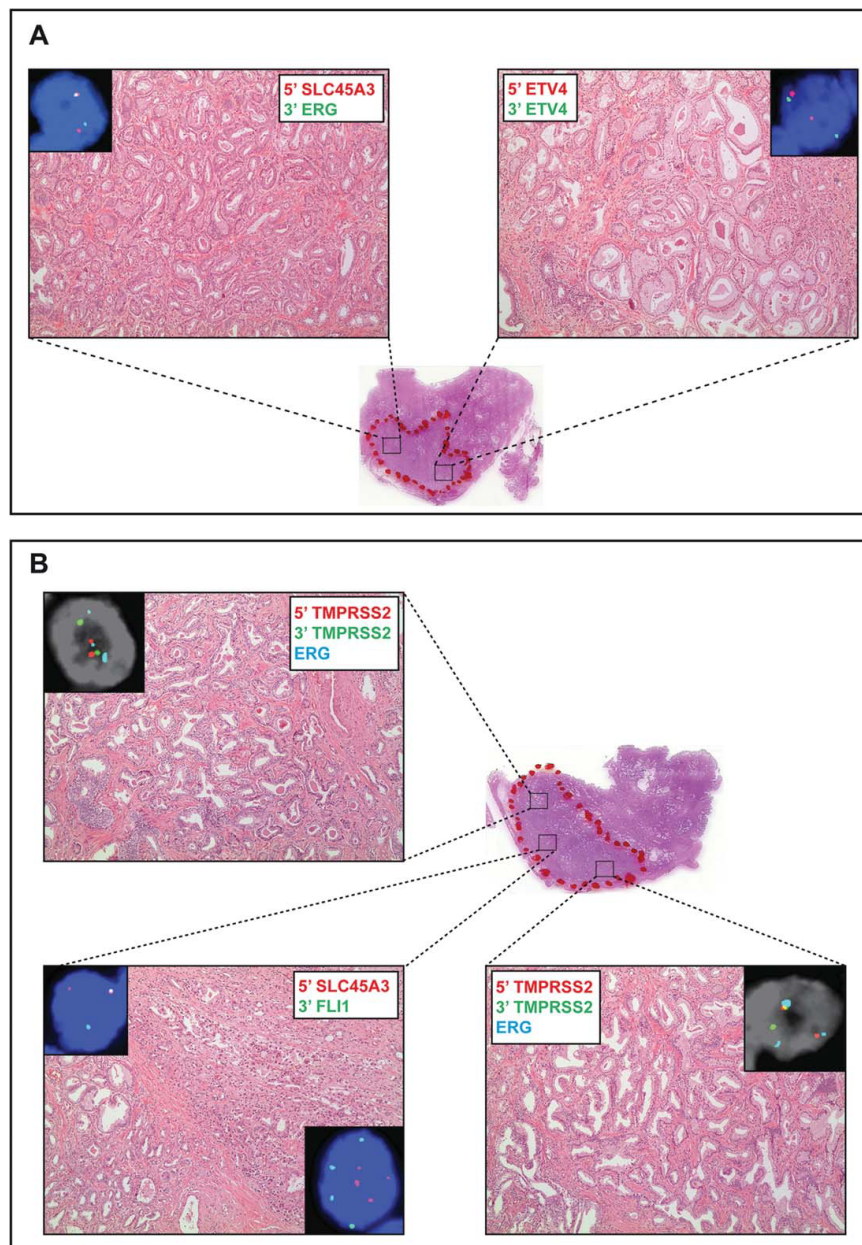


Figure 3. Prostate carcinomas showing intratumor genetic heterogeneity assessed by FISH, evidencing that index tumors may result from collision of independent clones, each with different ETS rearrangements. A. Case P236T showing that the index tumor is made of two clonal populations, one harboring the *SLC45A3-ERG* rearrangement and other harboring an *ETV4* rearrangement. B. Case P453T showing that the index tumor is made of three clonal populations, namely, two clones harboring the *TMPRSS2-ERG* rearrangement (with

different morphological features) flanking a third clone harboring the *SLC45A3-FLI1* rearrangement. The bottom left panel shows a transition zone between an tumor area (bottom left) with the *SLC45A3-FLI1* fusion gene and a tumor area (upper right) showing four copies of both *SLC45A3* and *FLI1* but no fusion (this area has the *TMPRSS2-ERG* rearrangement). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

genes in PCa, we here report the identification of a new ETS transcription factor involved in a gene fusion in this disease. In fact, in the PCa showing the highest *FLI1* expression by the TLDA analysis, using a combination of BAC probes, exon-level quantitative RT-PCR and qualitative RT-PCR followed by sequencing, we were able to demonstrate the presence of the *SLC45A3-FLI1* fusion gene, in which *SLC45A3* exon 1 is fused with *FLI1* exon 3. The data at hand therefore indicates that *SLC45A3-FLI1* is an alternative primary genetic event in prostate carcinogenesis, making *FLI1* the fifth ETS family member involved in gene fusions in PCa. *FLI1* is the member of the ETS family of transcription factors that is most frequently involved in gene fusions in Ewing sarcomas, as the *EWSR1-FLI1* fusion gene is present in about 85% of the cases (Riggi and Stamenkovic, 2007). Conversely, the ETS transcription factor most commonly rearranged in PCa, *ERG*, is the second most recurrent 3' fusion partner of *EWSR1* in Ewing sarcoma (up to 10%), whereas *ETV1* and *ETV4* fusions are more infrequently found in both PCa and Ewing sarcoma (Riggi and Stamenkovic, 2007; Tomlins et al., 2009). The pathognomonic chimeric protein EWSR1-FLI1 in Ewing sarcoma acts as a potent transcriptional activator due to the transactivation domain of EWSR1 and the DNA-binding domain of FLI1 (Welford et al., 2001; Uren et al., 2004; Uren and Toretsky, 2005). On the other hand, in the novel *SLC45A3-FLI1* rearrangement we here report in PCa, the 5' partner *SLC45A3* is not expected to contribute to the protein product since the breaks affect untranslated exons of both genes. This is a common feature of ETS rearrangements in PCa, which typically results in overexpression of a structurally normal or a 5' truncated ETS transcription factor (Tomlins et al., 2009). Moreover, as *ERG* and *FLI1* belong to the same ETS subfamily and have 98% of homology in the DNA binding domain (Wei et al., 2010; Hollenhorst et al., 2011), it is likely that similar target genes are affected by their overexpression in PCa, as described for *EWSR1-FLI1* and *EWSR1-ERG* in Ewing's sarcoma (Ginsberg et al., 1999; Im et al., 2000). As the currently available prostate cancer cell lines do not harbor *FLI1* overexpression (Rahim et al., 2011), the development of a specific biological model of the *SLC45A3-FLI1* rearrangement is needed to further clarify the oncogenic role of *FLI1* in prostate carcinogenesis.

Interestingly, four of the 200 PCa presented two different ETS rearrangements in the index tumor, therefore representing intratumor genetic heterogeneity. One case presented both a *SLC45A3-ERG* fusion gene and an *ETV4* rearrangement, whereas three *TMPRSS2-ERG* positive cases showed also an *ETV4* rearrangement, the *SLC45A3-ERG* rearrangement or the new *SLC45A3-FLI1* fusion gene, respectively. We found similar expression levels of the co-expressed ETS family members, thus confirming that the two ETS rearrangements had the expected biological corollary (Supporting Information Fig. 5). The fact that we did not find the different ETS rearrangements in the same tumor cells but in discrete tumor regions of the index tumors suggests that collision of clonally independent clones occurred during tumor growth. Earlier reports of multiple ETS rearrangements were focused on the analysis of spatially separated tumor foci from the same prostatectomy specimen and reported intertumoral heterogeneity with intratumoral homogeneity (Perner et al., 2006; Barry et al., 2007; Mehra et al., 2007; Clark et al., 2008; Furusato et al., 2008), although one case has been previously reported to harbor unspecified *ERG* and *ETV1* rearrangements in the same tumor focus (Svensson et al., 2011). Because evaluation of ETS rearrangement status in this study was initially guided by ETS overexpression, the degree of intratumor heterogeneity is likely to be underestimated. In fact, although *ERG* overexpression might originate from rearrangement with different fusion partners and by different mechanisms, these can only be detected by systematic, cell based genetic analysis (Svensson et al., 2011). Furthermore, because the mechanisms originating the *TMPRSS2-ERG* fusion gene, deletion and insertion, are both so common, they are not good clonality markers.

Looking for associations between genetic changes and clinico-pathological parameters, we found that PCa with *ERG* rearrangements were associated with locally advanced disease, but also with lower Gleason scores. Because both *MYC* gain and *PTEN* loss have previously been associated with poor prognosis, we looked for expression changes of these genes and correlated them with ETS rearrangements and clinico-pathological features. Although *MYC* and *PTEN* expression were not significantly associated with any clinico-pathological parameter, we found that *MYC* expression was significantly upregulated only in PCa harboring *ERG* rearrangement while *PTEN*

expression was significantly downregulated specifically in PCa harboring *ETV1* rearrangement. The strong positive correlation we found between *ERG* and *MYC* expression in PCa with *ERG* rearrangement is in agreement with earlier reports showing that *MYC* is a downstream target of the overexpressed *ERG* transcription factor (Sun et al., 2008). The data we present also support the previously reported association between loss of *PTEN* (10q) and PCa with *ERG* rearrangement (Sun et al., 2008), but further show that *PTEN* downregulation is particularly striking in PCa with *ETV1* rearrangements. Future studies are warranted to find out how similar the target genes of the various aberrant ETS transcription factors are in prostate cancer and whether or not they are associated with different disease-specific survival.

ACKNOWLEDGMENTS

J. Ramalho-Carvalho is a research fellow from Liga Portuguesa Contra o Cancro, Núcleo Regional do Norte.

REFERENCES

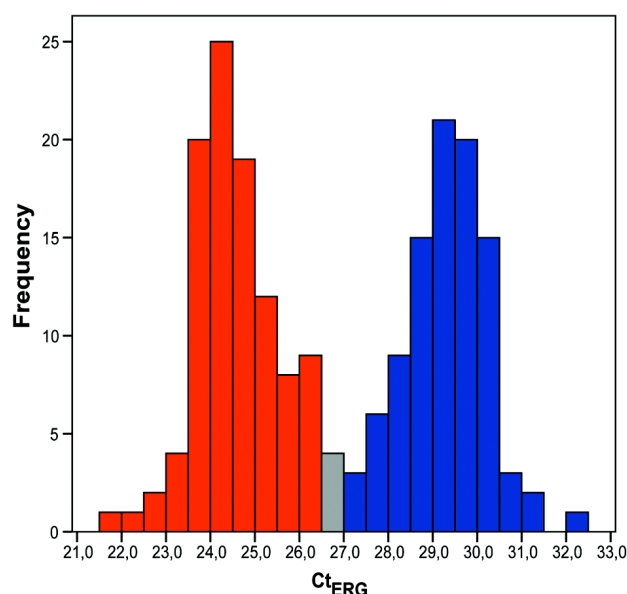
- Attard G, Clark J, Ambrosine L, Mills IG, Fisher G, Flohr P, Reid A, Edwards S, Kovacs G, Berney D, Foster C, Massie CE, Fletcher A, De Bono JS, Scardino P, Cuzick J, Cooper CS. 2008. Heterogeneity and clinical significance of *ETV1* translocations in human prostate cancer. *Br J Cancer* 99:314–320.
- Barros-Silva JD, Ribeiro FR, Rodrigues A, Cruz R, Martins AT, Jeronimo C, Henrique R, Teixeira MR. 2011. Relative 8q gain predicts disease-specific survival irrespective of the *TMPRSS2-ERG* fusion status in diagnostic biopsies of prostate cancer. *Genes Chromosomes Cancer* 50:662–671.
- Barry M, Perner S, Demichelis F, Rubin MA. 2007. *TMPRSS2-ERG* fusion heterogeneity in multifocal prostate cancer: Clinical and biologic implications. *Urology* 70:630–633.
- Cerveira N, Ribeiro FR, Peixoto A, Costa V, Henrique R, Jeronimo C, Teixeira MR. 2006. *TMPRSS2-ERG* gene fusion causing *ERG* overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions. *Neoplasia* 8:826–832.
- Clark JP, Cooper CS. 2009. ETS gene fusions in prostate cancer. *Nat Rev Urol* 6:429–439.
- Clark J, Attard G, Jhavar S, Flohr P, Reid A, De Bono J, Eccles R, Scardino P, Cuzick J, Fisher G, Parker MD, Foster CS, Berney D, Kovacs G, Cooper CS. 2008. Complex patterns of ETS gene alteration arise during cancer development in the human prostate. *Oncogene* 27:1993–2003.
- Esgueva R, Perner S, LaFargue CJ, Scheble V, Stephan C, Lein M, Fritzsche FR, Dietel M, Kristiansen G, Rubin MA. 2010. Prevalence of *TMPRSS2-ERG* and *SLC45A3-ERG* gene fusions in a large prostatectomy cohort. *Mod Pathol* 23:539–546.
- Furusato B, Gao CL, Ravindranath L, Chen Y, Cullen J, McLeod DG, Dobi A, Srivastava S, Petrovics G, Sesterhenn IA. 2008. Mapping of *TMPRSS2-ERG* fusions in the context of multifocal prostate cancer. *Mod Pathol* 21:67–75.
- Ginsberg JP, de Alava E, Ladanyi M, Wexler LH, Kovar H, Paulussen M, Zoubek A, Dockhorn-Dwornczak B, Juergens H, Wunder JS, Andrulis IL, Malik R, Sorensen PH, Womer RB, Barr FG. 1999. *EWS-FLI1* and *EWS-ERG* gene fusions are associated with similar clinical phenotypes in Ewing's sarcoma. *J Clin Oncol* 17:1809–1814.
- Han B, Mehra R, Dhanasekaran SM, Yu J, Menon A, Lonigro RJ, Wang X, Gong Y, Wang L, Shankar S, Laxman B, Shah RB, Varambally S, Palanisamy N, Tomlins SA, Kumar-Sinha C, Chinnaiyan AM. 2008. A fluorescence in situ hybridization screen for E26 transformation-specific aberrations: identification of *DDX5-ETV4* fusion protein in prostate cancer. *Cancer Res* 68:7629–7637.
- Helgeson BE, Tomlins SA, Shah N, Laxman B, Cao Q, Prensner JR, Cao X, Singla N, Montie JE, Varambally S, Mehra R, Chinnaiyan AM. 2008. Characterization of *TMPRSS2:ETV5* and *SLC45A3:ETV5* gene fusions in prostate cancer. *Cancer Res* 68:73–80.
- Hermans KG, van Marion R, van Dekken H, Jenster G, van Weerden WM, Trapman J. 2006. *TMPRSS2:ERG* fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. *Cancer Res* 66:10658–10663.
- Hermans KG, van der Korput HA, van Marion R, van de Wijngaert DJ, Ziel-van der Made A, Dits NF, Boormans JL, van der Kwast TH, van Dekken H, Bangma CH, Korsten H, Kraaij R, Jenster G, Trapman J. 2008. Truncated *ETV1*, fused to novel tissue-specific genes, and full-length *ETV1* in prostate cancer. *Cancer Res* 68:7541–7549.
- Hollenhorst PC, McIntosh LP, Graves BJ. 2011. Genomic and biochemical insights into the specificity of ETS transcription factors. *Annu Rev Biochem* 80:437–471.
- Im YH, Kim HT, Lee C, Poulin D, Welford S, Sorensen PH, Denny CT, Kim SJ. 2000. *EWS-FLI1*, *EWS-ERG*, and *EWS-ETV1* oncoproteins of Ewing tumor family all suppress transcription of transforming growth factor beta type II receptor gene. *Cancer Res* 60:1536–1540.
- Kumar-Sinha C, Tomlins SA, Chinnaiyan AM. 2008. Recurrent gene fusions in prostate cancer. *Nat Rev Cancer* 8:497–511.
- Mehra R, Han B, Tomlins SA, Wang L, Menon A, Wasco MJ, Shen R, Montie JE, Chinnaiyan AM, Shah RB. 2007. Heterogeneity of *TMPRSS2* gene rearrangements in multifocal prostate adenocarcinoma: Molecular evidence for an independent group of diseases. *Cancer Res* 67:7991–7995.
- Perner S, Demichelis F, Beroukhir R, Schmidt FH, Mosquera JM, Setlur S, Tchinda J, Tomlins SA, Hofer MD, Pienta KG, Kuefer R, Vessella R, Sun XW, Meyerson M, Lee C, Sellers WR, Chinnaiyan AM, Rubin MA. 2006. *TMPRSS2:ERG* fusion-associated deletions provide insight into the heterogeneity of prostate cancer. *Cancer Res* 66:8337–8341.
- Rahim S, Beauchamp EM, Kong Y, Brown ML, Toretzky JA, Uren A. 2011. *YK-4-279* inhibits *ERG* and *ETV1* mediated prostate cancer cell invasion. *PLoS One* 6:e19343.
- Ribeiro FR, Paulo P, Costa VL, Barros-Silva JD, Ramalho-Carvalho J, Jeronimo C, Henrique R, Lind GE, Skotheim RI, Lothe RA, Teixeira MR. 2011. Cysteine-rich secretory protein-3 (*CRISP3*) is strongly up-regulated in prostate carcinomas with the *TMPRSS2-ERG* fusion gene. *PLoS One* 6:e22317.
- Riggi N, Stamenkovic I. 2007. The biology of Ewing sarcoma. *Cancer Lett* 254:1–10.
- Sun C, Dobi A, Mohamed A, Li H, Thangapazham RL, Furusato B, Shaheduzzaman S, Tan SH, Vaidyanathan G, Whitman E, Hawksworth DJ, Chen Y, Nau M, Patel V, Vahey M, Gutkind JS, Sreenath T, Petrovics G, Sesterhenn IA, McLeod DG, Srivastava S. 2008. *TMPRSS2-ERG* fusion, a common genomic alteration in prostate cancer activates C-MYC and abrogates prostate epithelial differentiation. *Oncogene* 27:5348–5353.
- Svensson MA, LaFargue CJ, MacDonald TY, Pflueger D, Kitabayashi N, Santa-Cruz AM, Garsha KE, Sathyanarayana UG, Riley JP, Yun CS, Nagy D, Kosmeder JW, Pestano GA, Tewari AK, Demichelis F, Rubin MA. 2011. Testing mutual exclusivity of ETS rearranged prostate cancer. *Lab Invest* 91:404–412.
- Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM. 2005. Recurrent fusion of *TMPRSS2* and ETS transcription factor genes in prostate cancer. *Science* 310:644–648.
- Tomlins SA, Mehra R, Rhodes DR, Smith LR, Roulston D, Helgeson BE, Cao X, Wei JT, Rubin MA, Shah RB, Chinnaiyan AM. 2006. *TMPRSS2:ETV4* gene fusions define a third molecular subtype of prostate cancer. *Cancer Res* 66:3396–3400.
- Tomlins SA, Laxman B, Dhanasekaran SM, Helgeson BE, Cao X, Morris DS, Menon A, Jing X, Cao Q, Han B, Yu J, Wang L, Montie JE, Rubin MA, Pienta KJ, Roulston D, Shah RB, Varambally S, Mehra R, Chinnaiyan AM. 2007. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature* 448:595–599.

SLC45A3-FLII FUSION IN PROSTATE CANCER

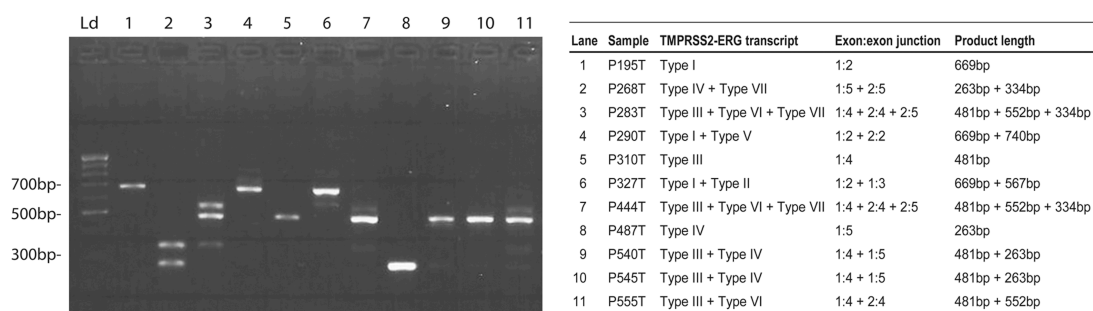
249

- Tomkins SA, Bjartell A, Chinnaiyan AM, Jenster G, Nam RK, Rubin MA, Schalken JA. 2009. ETS gene fusions in prostate cancer: From discovery to daily clinical practice. *Eur Urol* 56:275–286.
- Tu JJ, Rohan S, Kao J, Kitabayashi N, Mathew S, Chen YT. 2007. Gene fusions between TMPRSS2 and ETS family genes in prostate cancer: frequency and transcript variant analysis by RT-PCR and FISH on paraffin-embedded tissues. *Mod Pathol* 20:921–928.
- Uren A, Toretzky JA. 2005. Ewing's sarcoma oncoprotein EWS-FLII: The perfect target without a therapeutic agent. *Future Oncol* 1:521–528.
- Uren A, Tcherkasskaya O, Toretzky JA. 2004. Recombinant EWS-FLII oncoprotein activates transcription. *Biochemistry* 43:13579–13589.
- Wang J, Cai Y, Ren C, Ittmann M. 2006. Expression of variant TMPRSS2/ERG fusion messenger RNAs is associated with aggressive prostate cancer. *Cancer Res* 66:8347–8351.
- Wei GH, Badis G, Berger MF, Kivioja T, Palin K, Enge M, Bonke M, Jolma A, Varjosalo M, Gehrke AR, Yan J, Talukder S, Turunen M, Taipale M, Stunnenberg HG, Ukkonen E, Hughes TR, Bulik ML, Taipale J. 2010. Genome-wide analysis of ETS-family DNA-binding in vitro and in vivo. *EMBO J* 29:2147–2160.
- Welford SM, Hebert SP, Deneen B, Arvand A, Denny CT. 2001. DNA binding domain-independent pathways are involved in EWS/FLII-mediated oncogenesis. *J Biol Chem* 276:41977–41984.
- Yoshimoto M, Joshua AM, Cunha IW, Coudry RA, Fonseca FP, Ludkovski O, Zielenska M, Soares FA, Squire JA. 2008. Absence of TMPRSS2:ERG fusions and PTEN losses in prostate cancer is associated with a favorable outcome. *Mod Pathol* 21:1451–1460.

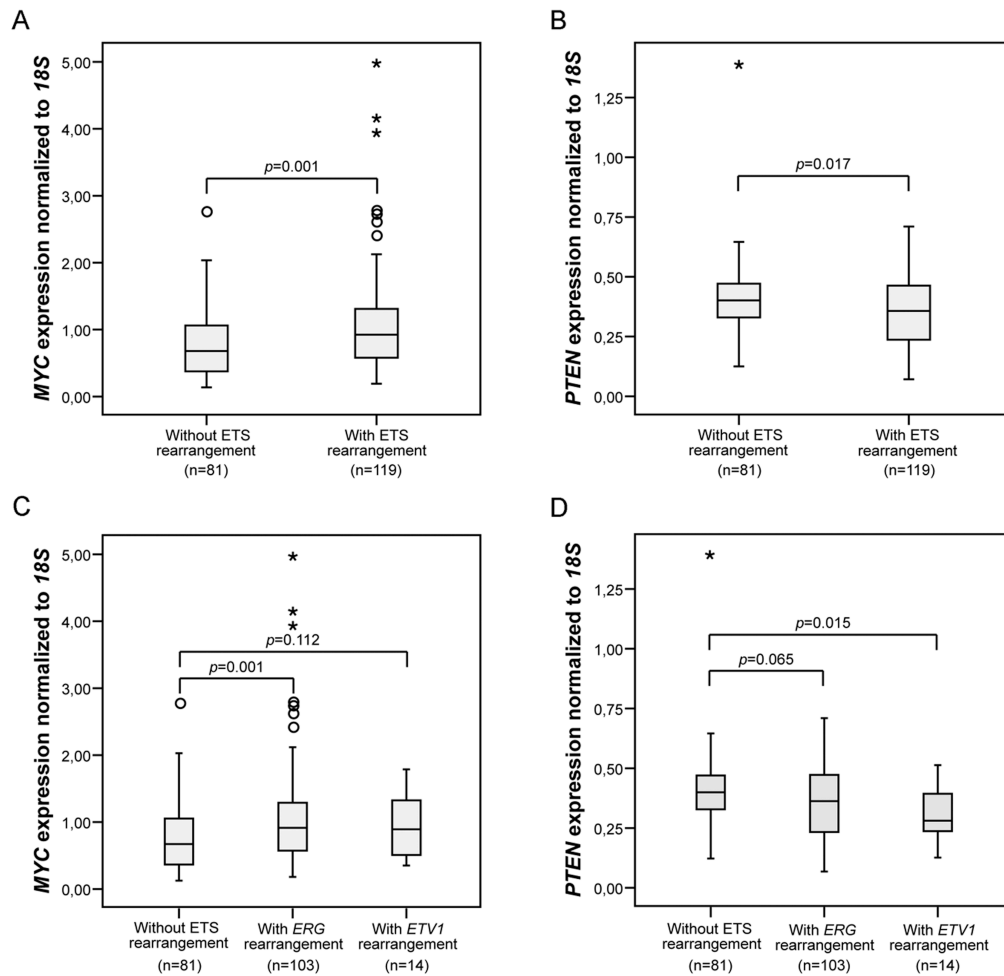
Supporting information



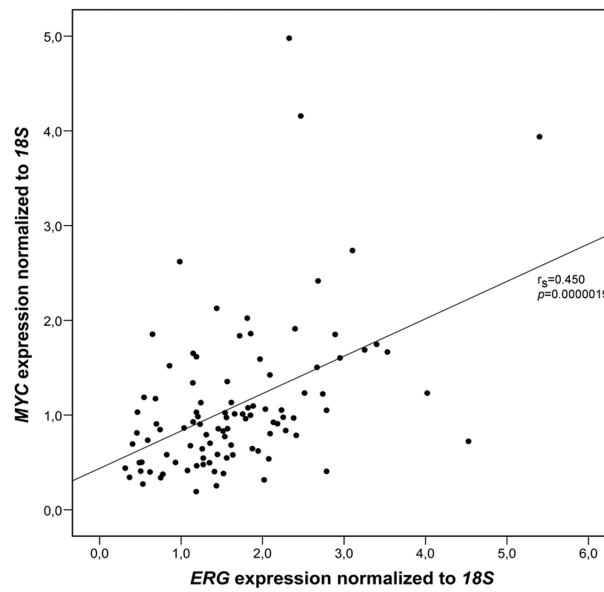
Supplementary Figure 1. Sample distribution according to the Ct values obtained for *ERG* expression. With the *TMPRSS2-ERG* tricolor FISH probe we showed that all nine PCa with $Ct_{ERG} = 27.5 \pm 0.5$ were *ERG* fusion negative. Of the four PCa with $Ct_{ERG} = 26.75 \pm 0.5$, only two were shown to have an *ERG* rearrangement by FISH. Red and blue bars represent samples considered positive and negative for *ERG* rearrangement, respectively; grey bar represents the cut-off interval of Ct values where classification of samples cannot be defined based on *ERG* expression only.



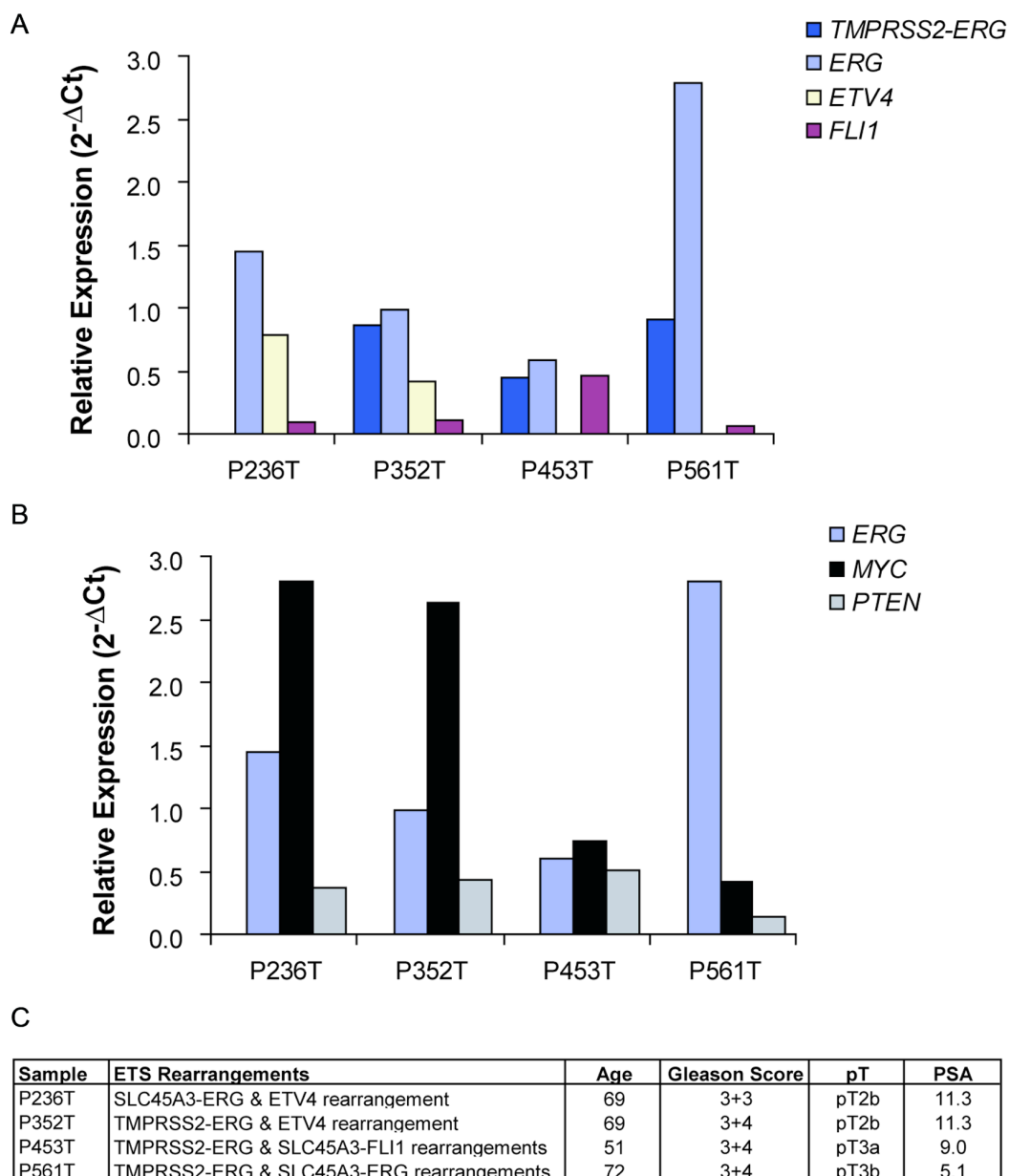
Supplementary Figure 2. RT-PCR analysis of *TMPRSS2-ERG* cases with low expression of the Type III transcript ($Ct_{TMPRSS2-ERG} > 27$). The Type III transcript was identified in samples 3, 7, 9, 10 and 11, in which $Ct_{TMPRSS2-ERG} = 27.5 \pm 0.5$ and in sample 5 ($Ct_{TMPRSS2-ERG} = 31.5$). In the remaining samples, a different transcript was detected, justifying the even lower Ct values obtained for these samples. Ld - 100bp ladder (Invitrogen).



Supplementary Figure 3. Box-plots showing distribution of MYC and PTEN expression in ETS rearrangement groups. **A.** MYC expression is significantly increased in PCa with ETS rearrangement ($p=0.001$). **B.** PTEN expression is significantly decreased in PCa with ETS rearrangement ($p=0.017$). **C.** MYC expression is significantly increased in PCa with ERG rearrangement ($p=0.001$). **D.** PTEN expression is significantly decreased in PCa with ETV1 rearrangement ($p=0.015$).



Supplementary Figure 4. Distribution of samples with ERG rearrangement according to MYC and ERG expression. A significant correlation is shown ($r_s=0.453$, $p=0.0000025$).



Supplementary Figure 5. Relevant expression and clinic-pathological data of the four prostate carcinomas showing intratumor heterogeneity regarding ETS rearrangements. **A.** Comparative expression levels of the ETS genes involved in the genomic rearrangements. **B.** Expression levels of *MYC* and *PTEN*. **C.** Clinicopathological information.

Supplementary Table 1. TaqMan gene expression assays selected for the TLDA card

Gene Symbol	Assay ID/Primer sequence
<i>ERG</i>	Hs01554635_m1
<i>ETV1</i>	Hs00951941_m1
<i>ETV4</i>	Hs00385910_m1
<i>ETV5</i>	Hs00231790_m1
<i>ETV6</i>	Hs01045746_m1
<i>ELK4</i>	Hs01111811_m1
<i>FEV</i>	Hs00232733_m1
<i>FLI1</i>	Hs00956711_m1
<i>ETS2</i>	Hs00232009_m1
<i>MYC</i>	Hs00153408_m1
<i>PTEN</i>	Hs00829813_s1
<i>TMPRSS2-ERG</i>	Hs03063375_ft

Supplementary Table 2. 5' and 3' break-apart BAC probes used to assess genomic rearrangements of ETS transcription factors. NA, not applicable

Gene Symbol	5' Probe (red)	3' Probe
<i>ERG</i>	RP11-137I13	RP11-24A11
<i>ETV1</i>	RP11-643C13	RP11-790J2
<i>ETV4</i>	RP11-436J4	RP11-831F13
<i>ETV5</i>	RP11-822O23	RP11-1144N13
<i>FLI1</i>	RP11-1079A6	RP11-69E19
<i>SLC45A3</i>	RP11-131E5	NA
<i>TMPRSS2</i>	RP11-35C4	NA

Supplementary Table 3. Sequence of primers pairs and TaqMan expression assays used in RT-PCR and qRT-PCR. F-forward primer, R-reverse primer

Primer	5'-3' sequence
<i>TMPRSS2</i> -ex1-F	TAGGCGCGAGCTAAGCAGGAG
<i>ERG</i> -ex6-R	CTGCCGCACATGGTCTGTAC
<i>SLC45A3</i> -ex1-F	TGGCTCCGGGTGACAG
<i>FLI1</i> -ex3-R	ACCCTCACTGGCTGATTGAT
<i>FLI1</i> -ex2:3	Hs00231107_m1*
<i>FLI1</i> -ex4:5	Hs00956709_m1*
<i>GUSB</i>	4333767F**

* Assay ID acquired from Applied Biosystems

** TaqMan® Endogenous Control acquired from Applied Biosystems

Chapter 4

Original publication # II

Novel 5' Fusion Partners of *ETV1* and *ETV4* in Prostate Cancer

JD Barros-Silva^{1,3,6,7,*}, P Paulo^{1,3,6,7,*}, AC Bakken^{6,7}, N Cerveira¹,
M Løvf^{6,7}, R Henrique^{2,4,5}, C Jerónimo^{1,4,5}, RA Lothe^{6,7}, RI
Skotheim^{6,7} and MR Teixeira^{1,3,5,7}

Departments of ¹Genetics and ²Pathology, Portuguese Oncology Institute, Porto, Portugal; Groups of ³Cancer Genetics and ⁴Cancer Epigenetics, Research Centre of the Portuguese Oncology Institute, Porto, Portugal; ⁵Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal; ⁶Department of Cancer Prevention, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway; ⁷Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, Oslo, Norway.

*JDBS and PP contributed equally to this work and should be regarded as joint first authors.

Novel 5' Fusion Partners of *ETV1* and *ETV4* in Prostate Cancer^{1,2}

João D. Barros-Silva^{*,†,‡,§,3}, Paula Paulo^{*,†,‡,§,3},
Anne Cathrine Bakken^{‡,§}, Nuno Cerveira^{*},
Marthe Løvfi^{‡,§}, Rui Henrique^{†,§,**,§},
Carmen Jerónimo^{*,#,**,§}, Ragnhild A. Lothe^{‡,§},
Rolf Inge Skotheim^{‡,§} and Manuel R. Teixeira^{*,†,§,**,§}

*Department of Genetics, Portuguese Oncology Institute, Porto, Portugal; †Group of Cancer Genetics, Research Centre of the Portuguese Oncology Institute, Porto, Portugal; ‡Department of Cancer Prevention, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway; §Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, Oslo, Norway; ¶Department of Pathology, Portuguese Oncology Institute, Porto, Portugal; #Group of Cancer Epigenetics, Research Centre of the Portuguese Oncology Institute, Porto, Portugal; **Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto, Portugal

Abstract

Gene fusions involving the erythroblast transformation-specific (ETS) transcription factors *ERG*, *ETV1*, *ETV4*, *ETV5*, and *FLI1* are a common feature of prostate carcinomas (PCas). The most common upstream fusion partner described is the androgen-regulated prostate-specific gene *TMPRSS2*, most frequently with *ERG*, but additional 5' fusion partners have been described. We performed 5' rapid amplification of cDNA ends in 18 PCas with *ETV1*, *ETV4*, or *ETV5* outlier expression to identify the 5' fusion partners. We also evaluated the exon-level expression profile of these ETS genes in 14 cases. We identified and confirmed by fluorescent *in situ* hybridization (FISH) and reverse transcription-polymerase chain reaction the two novel chimeric genes *OR51E2-ETV1* and *UBTF-ETV4* in two PCas. *OR51E2* encodes a G-protein-coupled receptor that is overexpressed in PCas, whereas *UBTF* is a ubiquitously expressed gene encoding an HMG-box DNA-binding protein involved in ribosome biogenesis. We additionally describe two novel gene fusion combinations of previously described genes, namely, *SLC45A3-ETV4* and *HERVK17-ETV4*. Finally, we found one PCa with *TMPRSS2-ETV1*, one with *C15orf21-ETV1*, one with *EST14-ETV1*, and two with *14q133-q21.1-ETV1*. In nine PCas (eight *ETV1* and one *ETV5*), exhibiting ETS outlier expression and genomic rearrangement detected by FISH, no 5' fusion partner was found. Our findings contribute significantly to characterize the heterogeneous group of ETS gene fusions and indicate that all genes described as 5' fusion partners with one ETS gene can most likely be rearranged with any of the other ETS genes involved in prostate carcinogenesis.

Neoplasia (2013) 15, 720–726

Abbreviations: PCa, prostate carcinoma; FISH, fluorescence *in situ* hybridization; 5'RACE, 5' rapid amplification of cDNA ends

Address all correspondence to: Manuel R. Teixeira, MD, PhD, Department of Genetics, Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal. E-mail: manuel.teixeira@ipporto.min-saude.pt

¹This work was supported by research grants Pest-OE/SAU/UI0776/2011 and PTDC/SAU/OBD/70543/2006 awarded by Fundação para a Ciência e a Tecnologia (FCT). J.D.B.-S. (SFRH/BD/46574/2008) and P.P. (SFRH/BD/27669/2006) are or were research fellows from FCT. The authors have no conflict of interest to declare.

²This article refers to supplementary materials, which are designated by Tables W1 to W3 and Figures W1 and W2 and are available online at www.neoplasia.com.

³These authors contributed equally to this work and should be regarded as joint first authors.

Received 16 January 2013; Revised 12 April 2013; Accepted 15 April 2013

Copyright © 2013 Neoplasia Press, Inc. All rights reserved 1522-8002/13/\$25.00
DOI 10.1593/neo.13232

Introduction

Gene fusions involving the erythroblast transformation-specific (ETS) transcription factor family of genes are a recurrent feature of prostate adenocarcinomas (PCas). These gene aberrations, caused by chromosomal structural abnormalities, originate fusion transcripts that lead to overexpression of N-truncated ETS proteins or, more rarely, to full-length ETS proteins or chimeric fusion proteins [1–3]. Fusion of the androgen-regulated promoter region of the *TMPRSS2* gene with *ERG* is the most common ETS rearrangement, being present in about 50% of PCa and in 20% of high-grade prostatic intraepithelial neoplasia lesions [1,4]. Other rarer fusion events can occur involving the PEA3 subfamily of ETS members, namely, *ETV1*, *ETV4*, and *ETV5* [1,5,6] or the ERG subfamily member *FLI1* [3].

Besides the prostate-specific and androgen-induced *TMPRSS2*, several ETS fusion partners have been described, namely, *HERPUD1*, *NDRG1*, *SLC45A3*, *ACSL3*, *HERV-K_22*, *HERVK17*, *CANT1*, *DDX5*, *KLK2*, *FOXP1*, *EST14*, *HNRPA2B1*, *C15orf21*, and the chromosomal region 14q13.3–14q21.1 [2,7,8], presenting heterogeneous tissue specificities and androgen responsiveness. Fusion partners like the *SLC45A3* gene or the endogenous retroviral element (*HERVK17*) display similar tissue specificity as *TMPRSS2* and are equally androgen-induced. Contrarily, the fusion partner *C15orf21*, despite being overexpressed in PCas, is repressed by androgens. However, there are ubiquitously expressed 5' fusion partners, such as the *HNRPA2B1* gene, displaying no evidence of androgen regulation. Finally, ETS family genes may be rearranged with prostate-specific enhancers in chromosomal regions such as 14q13.3–14q21.1.

We have previously performed a comprehensive characterization of ETS rearrangements on a series of 200 clinically localized PCas and found rearrangements involving *ERG*, *ETV1*, *ETV4*, *ETV5*, and *FLI1* in 52%, 7%, 1.5%, 0.5%, and 0.5%, respectively [3]. In the present work, we focused on the 18 PCas that showed outlier expression levels of *ETV1*, *ETV4*, or *ETV5* and a genomic rearrangement of the corresponding ETS locus. The combined use of exon-level expression profiles from exon microarrays, 5' rapid amplification of cDNA ends (5' RACE), and fluorescence *in situ* hybridization (FISH) with bacterial artificial chromosome (BAC)-specific probes allowed us to identify novel 5' fusion partners for *ETV1* and *ETV4*, as well as to describe novel combinations of genes known to be involved in PCa gene fusions.

Materials and Methods

PCa Samples

We studied a set of 18 PCas with outlier mRNA expression levels of *ETV1* ($n = 14$), *ETV4* ($n = 3$), and *ETV5* ($n = 1$) and with a genomic rearrangement previously demonstrated by FISH but with yet unknown fusion partners [3]. These samples were selected from a cohort of 200 patients with clinically localized PCa consecutively diagnosed and treated with radical prostatectomy that were previously typed for ETS rearrangements [3]. This study was approved by the Institutional Review Board, and informed consent was obtained from all subjects.

Gene Expression Microarrays

RNA was extracted from tissue samples using TRIzol (Invitrogen by Life Technologies, Carlsbad, CA), as previously described [3], and 1 μ g of RNA was processed into cDNA and hybridized to Affymetrix GeneChip Human Exon 1.0 ST arrays, following the manufacturer's

recommendations. The Affymetrix Expression Console v1.1 software was used to obtain exon-level robust multi-array average (RMA)-normalized expression values for the core probe sets only.

5' Rapid Amplification of cDNA Ends

The 5' RACE was performed using the SMARTer RACE cDNA amplification kit and protocol (Clontech Laboratories, Inc, Saint-Germain-en-Laye, France). Briefly, first-strand cDNA was reverse transcribed from 1 μ g of total RNA using the SMARTScribe Reverse Transcriptase with the 5' RACE cDNA synthesis primer (5'-CDS) and the SMARTer IIA oligo from the kit. An aliquot of the cDNA was then amplified using a forward gene-specific primer (GSP) and a universal primer mix. Polymerase chain reaction (PCR) conditions used were as described by the manufacturer. Nested PCRs using the nested universal primer as the reverse primer and a nested gene-specific primer (NGSP) were performed to increase the specificity and product yields of 5' RACE. Primers used on 5' RACE and nested PCR are listed in Table W1. Nested PCR products were analyzed on a 2% agarose gel (SeaKem LE Agarose; Lonza, Basel, Switzerland) and the bands were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

Cloning and Sequencing

Purified 5' nested race PCR products were cloned into pCR4-TOPO plasmids using the TOPO TA Cloning Kit for sequencing (Invitrogen). Colonies were picked and the plasmids were purified using the Qiagen Plasmid Miniprep Kit (Qiagen) and subsequently sequenced using the M13 forward and T3 primers using BigDye Terminator V3.1 sequencing chemistry on a 3730 DNA Analyzer (Applied Biosystems by Life Technologies, Foster City, CA) according to the manufacturer's recommendations. Genomic alignment of the resulting sequences was performed using BLAST (<http://www.ncbi.nlm.nih.gov/blast/>) and BLAT (<http://genome.ucsc.edu/>). All exons identified were numbered according to the longest matching transcripts of the Ensembl database (<http://www.ensembl.org/>).

Reverse Transcription-PCR

The reverse transcription (RT)-PCR assay for detection of prostate fusion transcripts was performed with the following primer combinations: *TMPRSS2_F* and *ETV1_R* (*TMPRSS2-ETV1*), *C15orf21_S* and *ETV1_AS1* (*C15orf21-ETV1*), *SLC45A3_S* and *ETV4_AS* (*SLC45A3-ETV4*), *UBTF-S* and *ETV4_AS* (*UBTF-ETV4*), *OR51E2_S* and *ETV1_AS2* (*OR51E2-ETV1*), *EST14_S* and *ETV1_AS2* (*EST14-ETV1*), and *HERVK17_S* and *ETV4_AS* (*HERVK17-ETV4*; Table W2). PCRs were performed with the Qiagen OneStep RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Amplified products were analyzed on a 2% agarose gel and further validated by sequencing.

Fluorescence In Situ Hybridization

To validate the 5' RACE findings, we performed FISH using BAC clones targeting the 5' fusion partner and the ETS gene on tissue sections from paraffin blocks of the index tumor (Table W3). BAC clones were selected using the University of California, Santa Cruz (UCSC) Human Genome Browser and obtained from the BACPAC Resources Centre (Oakland, CA). BAC DNA was extracted, amplified, labeled, and prepared for hybridization as previously reported [9]. Adequate mapping and probe specificity of all BAC clones was confirmed by hybridization onto human metaphase spreads of normal

lymphocytes. An abnormal signal pattern was considered representative when present in a minimum of 50 morphologically intact, non-overlapping nuclei.

In Silico Analysis of Androgen Effect on Gene Expression

To study the effect of androgen stimulus on *UBTF* and *OR51E2* expression, we used the expression data of the LNCaP cell line that can be accessed from the Gene Expression Omnibus (GSE32875) [10]. The normalized expression values of *OR51E2* and *UBTF* in four replicates of the LNCaP cell line treated with a synthetic androgen (R1881) and four replicates of the LNCaP cell line with no androgen stimulus were compared using the paired sample *t* test.

Results and Discussion

The starting point of this study was a subset of 18 PCas with evidence of rearrangement of *ETV1*, *ETV4*, and *ETV5* and unknown 5' fusion partner from a larger cohort previously typed for ETS rearrangements [3]. Of these 18 cases, there was evidence for involvement of *ETV1* in 14 cases, of *ETV4* in 3 cases, and of *ETV5* in 1 case, in every instance presenting outlier expression and genomic rearrangement by FISH [3]. After analyzing the exon-level expression data of 51 PCas [11], including 14 of the 18 cases included in this report, we here show that in samples P262T, P274T, P451T, and P542T the exons of *ETV1* are differentially expressed between the 5' and 3' ends, pinpointing the breakpoint before exons 4, 7, 8, and 7, respectively (Figure 1). Differences in the exon expression profile of *ETV4* and *ETV5* are present in samples P522T and P261T, respectively, indicating breakpoints before exon 5 of *ETV4* and before exon 6 of *ETV5* (Figure 1). In the remaining cases, all exons of the rearranged ETS gene were similarly overexpressed. To unveil the 5' fusion partners involved in these ETS gene fusions, we initially performed RT-PCR in all 18 PCas with primers targeting the most commonly involved 5' fusion partners, namely, *TMPRSS2* and *SLC45A3*. The *TMPRSS2-ETV1* fusion transcript was detected in one of the 14 PCas with *ETV1* rearrangement (P500T), and the *SLC45A3-ETV4* fusion transcript was found in one of the three samples with *ETV4* rearrangement (P236T; Figure W1).

Given our recent findings of occasional heterogeneity of PCa gene fusions in different areas of the same tumor [3], all samples were further investigated by 5'RACE PCR. Sequencing of the 5'RACE PCR products of cases P451T and P522T unveiled *OR51E2* and *UBTF* as novel 5' fusion partners of *ETV1* and *ETV4*, respectively. The *OR51E2-ETV1* transcript was composed by the untranslated exon 1 of *OR51E2* (ENST00000396950), with an additional untranslated 292-bp sequence downstream of exon 2 of *OR51E2*, fused to exon 8 of *ETV1* (Figure 2), which includes an in-frame ATG start codon (amino acid 132). As previously described [12,13], gene fusions involving noncoding regions of the 5' partner with exon 8 of *ETV1* results in an N-truncated ETV1 protein with 345 amino acids. An alternative transcript fusing exon 9 of *ETV1* with the same *OR51E2* sequences was also found, with the predicted resulting protein starting at an internal ATG in exon 9 of *ETV1* (amino acid 203) and consisting of 274 amino acids. The presence of both *OR51E2-ETV1* fusion transcripts was confirmed by RT-PCR. The *OR51E2* and *ETV1* genes map to chromosome bands 11p15 and 7p21, respectively, and have the same transcriptional orientation, which indicates that this novel fusion gene was originated by a chromosomal translocation. The *OR51E2* (alias *PSGR*) is named as olfactory receptor, family 51, subfamily E, member 2, and encodes a member of a large family of G-protein-coupled receptors arising from single coding exon

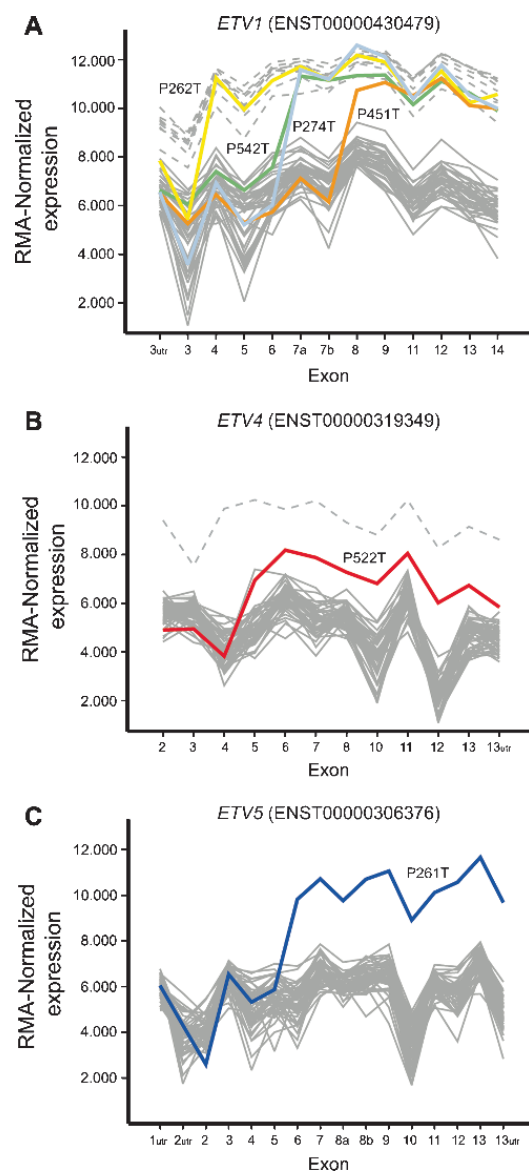


Figure 1. Exon-level expression profiles. RMA-normalized exon-level expression profile of *ETV1*, *ETV4*, and *ETV5* in 51 PCas obtained using Affymetrix GeneChip Human Exon 1.0 ST arrays. Differential expression of exons at the 5' and 3' ends of *ETV1* (A) was found in four samples (P262T, P275T, P451T, and P542T), indicating breakpoint before exons 4, 7, 8, and 7, respectively, whereas samples P522T and P261T displayed differential expression of exons at the 5' and 3' ends of *ETV4* (B) and *ETV5* (C), respectively, with a breakpoint before exon 5 of *ETV4* and exon 6 of *ETV5*. Gray lines represent samples with normal ETS expression, whereas gray dashed lines represent samples with overexpression of the whole transcript. For all genes, exons are numbered according to the indicated Ensembl transcript identifiers. The array included two probe sets targeting proximal and distal regions of exon 7 of *ETV1* and exon 8 of *ETV5* depicted in the plot as 7a and 7b and 8a and 8b, respectively.

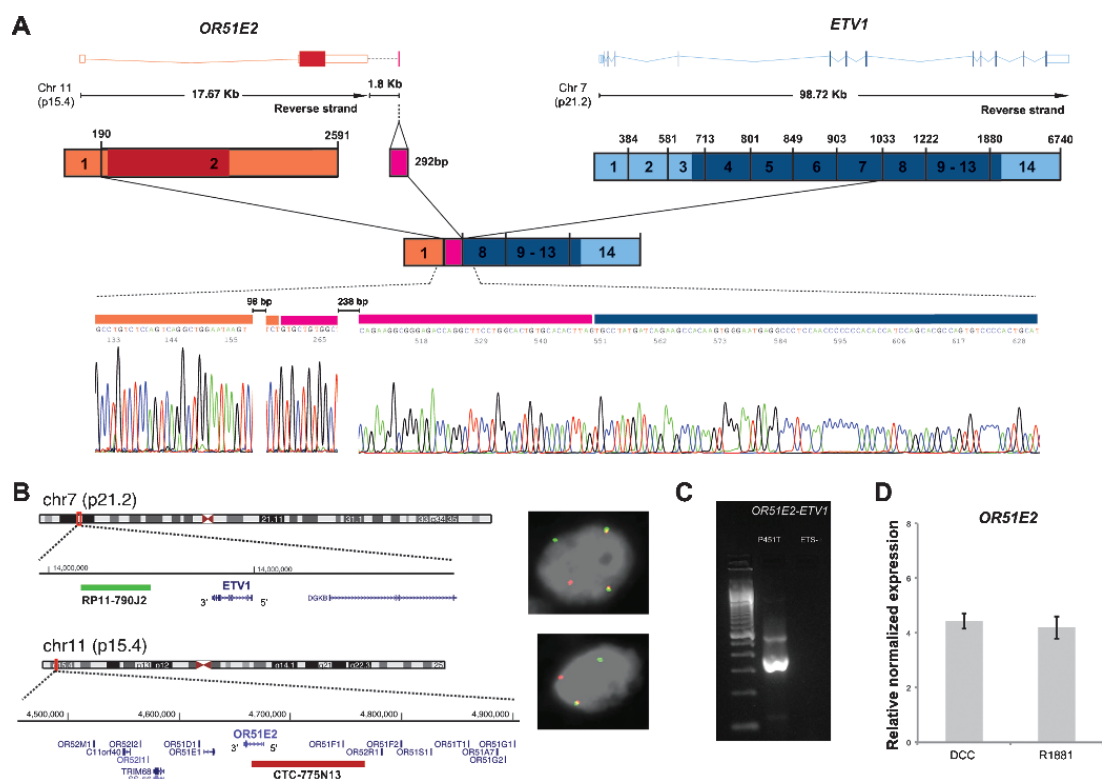


Figure 2. Characterization of the gene fusion involving *OR51E2* and *ETV1* in the clinical prostate cancer P451T with *ETV1* outlier expression and with an *ETV1* genomic rearrangement previously demonstrated by FISH. (A) Fusion transcript of the untranslated exon 1 of *OR51E2* and a downstream sequence of 292 bp of *OR51E2* with exon 8 of *ETV1* detected by sequencing of the 5'RACE PCR products using a reverse primer on exon 10 of *ETV1*. The upper representation depicts wild-type transcripts of *OR51E2* and *ETV1*, indicating its length and chromosomal localization. Exons are represented by boxes (colored if translated) and introns by horizontal lines. The scheme below represents the wild-type cDNA, where untranslated regions are in lighter shades. (B) Interphase FISH on formalin-fixed, paraffin-embedded tissue of the PCa P451T. Co-localization of probes for *OR51E2* (red) and *ETV1* (green) confirms genomic *OR51E2-ETV1* fusion in the cancer cells. (C) RT-PCR assay for detection of *OR51E2-ETV1* fusion transcript in the PCa P451T and in an ETS-negative PCa using a forward primer in the 292-bp sequence downstream of *OR51E2* and a reverse primer in exon 9 of *ETV1*. (D) Expression of *OR51E2* is relatively unchanged in LNCaP cell lines treated with an androgen analog (R1881) versus cells grown in dextran-coated charcoal culture medium as a control. The expression data were obtained from the Gene Expression Omnibus (GSE32875).

genes. It has been described by several authors to have a prostate-specific expression pattern and has also been found overexpressed in PCa [14] (Figure W2). The *OR51E2-ETV1* fusion retains the tissue specific regulatory activity present in exon 1 of *OR51E2*, one of the two described *OR51E2* promoters [15]. A recent study of the gene expression profile of benign and malignant prostate tissues from three radical prostatectomies and from three prostate biopsies of surgically castrated patients showed higher *OR51E2* expression in benign/cancer tissues compared to castrated prostate tissues [16], suggesting androgen regulation. However, it has been shown that the two described *OR51E2* promoters are regulated by interleukin-6 but not by the synthetic androgen R1881 [15]. Furthermore, we performed *in silico* analysis of public microarray data of R1881-treated LNCaP cells and found no alteration of *OR51E2* expression after androgenic stimulation (Figure 2; microarray data originally published in [10]).

The second novel fusion transcript *UBTF-ETV4* is composed by the untranslated exon 1 and exon 2 of *UBTF* (ENST00000393606)

fused to exon 5 of *ETV4* (Figure 3). Sequencing also revealed a partial deletion of exon 6 and exon 7 of *ETV4* consisting in the loss of 29 and 148 bp, respectively. The presence of the *UBTF-ETV4* fusion transcript was confirmed by RT-PCR. Both genes are located in the same chromosome band, 17q21, and are about 700 kb apart (Figure 3). Break apart probes flanking *ETV4* showed deletion of the probe targeting the 5' *ETV4* region, suggesting a cryptic deletion in chromosome 17 between the two genes as the mechanism of *UBTF-ETV4* fusion. Exon 2 of *UBTF* contains the ATG start codon and is in-frame with exon 5 of *ETV4*, so this gene fusion presumably originates a chimeric protein containing 19 amino acids encoded by exon 2 of *UBTF* and an *ETV4* counterpart lacking the first 67 N-terminal amino acids as well as additional 59 amino acids resulting from the partial deletion on exons 6 and 7 of *ETV4*. Interestingly, a previously reported gene fusion in PCa involving exon 5 of *ETV4* also translates into a chimeric protein [17]. Although relatively rare, there is evidence of other ETS fusion transcripts encoding chimeric proteins, namely,

TMPRSS2 fused with *ERG*, *ETV1*, *ETV4*, and *ETV5*, and the fusion transcripts *FOXP1-ETV1*, *EST14-ETV1*, *HNRPA2B1-ETV1*, and *DDX5-ETV4* [2]. The *UBTF* (upstream binding transcription factor, RNA polymerase I) gene encodes a member of the HMG-box DNA-binding protein family [18,19], which plays a critical role in ribosomal RNA (rRNA) transcription as a key component of the preinitiation complex, mediating the recruitment of RNA polymerase I to ribosomal DNA promoter regions. Synthesis of rRNA by Pol I, which drives ribosome biogenesis, is an important determinant of the cellular growth response [20]. This gene is ubiquitously expressed in different tissues and does not display an increased expression in PCa (Figure W2). Moreover, we here show by *in silico* analysis of public microarray data that the expression of *UBTF* is not altered by androgen stimulus in the LNCaP cell line (Figure 3; microarray data originally published in [10]). Interestingly, it has been shown that the oncoprotein MYC directly influences Pol I transcription of rRNA genes [20] and that it positively regulates *UBTF* expression [21].

Besides *UBTF*, we here describe two additional novel *ETV4* fusion partners by 5'RACE PCR (Table 1). In sample P352T, we found a fusion transcript of the prostate-specific androgen-repressed endogenous retroviral element, *HERVK17*, fused to exon 5 of *ETV4* (Figures 4 and W1). *HERVK17* has previously been described as a fusion partner for *ETV1* [13]. Confirming the earlier findings by RT-PCR, sequencing of the 5'RACE PCR product of sample P236T showed an *SLC45A3-ETV4* fusion transcript, in which exon 1 of *SLC45A3* (ENST00000367145) is fused in-frame with exon 3 of *ETV4* (Figures 4 and W1). *ETV4* rearrangements involving exon 3 have been previously described [5]. Interestingly, this tumor also harbors an *SLC45A3-ERG* fusion in a different area of the same cancerous focus [3], evidencing intratumor genetic heterogeneity in the form of two different ETS genes fused with the same 5' fusion partner, which most likely resulted from collision of initially separated neoplasms/foci. With the findings we here report, *SLC45A3* has now been found fused with all five ETS members known to be involved in

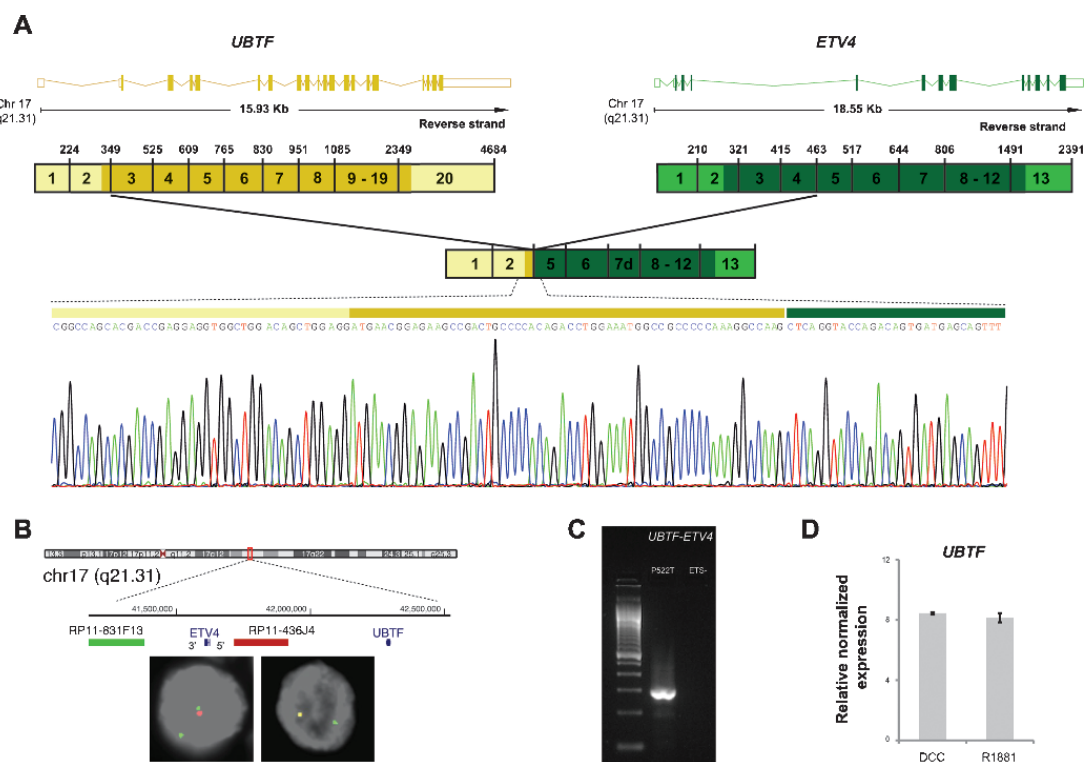


Figure 3. Characterization of the gene fusion involving *UBTF* and *ETV4* in the clinical prostate cancer P522T with *ETV4* outlier expression and with an *ETV4* genomic rearrangement previously demonstrated by FISH. (A) Fusion transcript of the untranslated exon 1 and exon 2 of *UBTF* with exon 5 of *ETV4* detected by sequencing of the 5'RACE PCR products using a reverse primer on exon 10 of *ETV4*. The upper representation depicts wild-type transcripts of *UBTF* and *ETV4* indicating their length and chromosomal localization. Exons are represented by boxes (colored if translated) and introns by horizontal lines. The scheme below represents the wild-type cDNA, where untranslated regions are in lighter shades. (B) Interphase FISH on formalin-fixed, paraffin-embedded tissue of the PCa P522T. Deletion of the probe spanning the chromosome region between *UBTF* and *ETV4* (red) indicates a cryptic deletion at chromosome 17 as the genomic mechanism for *UBTF-ETV4* gene fusion. (C) RT-PCR assay for detection of *UBTF-ETV4* fusion transcript in the PCa P522T and in an ETS-negative PCa using a forward primer in exon 2 of *UBTF* and a reverse primer in exon 6 of *ETV4*. (D) Expression of *UBTF* is relatively unchanged in LNCaP cell lines treated with an androgen analog (R1881) versus cells grown in dextran-coated charcoal culture medium as a control. The expression data were obtained from the Gene Expression Omnibus (GSE32875).

Table 1. Summary of the Experimental Findings in 18 Prostate Tumors with ETV1, ETV4, or ETV5 Rearrangements.

Sample	TLDA Outlier Expression	FISH	Exon Array	RT-PCR (TMPRSS2/ SLC45A3-ETV1)	RT-PCR (TMPRSS2/ SLC45A3-ETV4)	RT-PCR (TMPRSS2/ SLC45A3-ETV5)	FISH (14q13.3-q21.1-ETV1)	5'RACE
P262T	ETV1	ETV1+	D	neg.	—	—	14q13.3-q21.1-ETV1	neg.
P272T	ETV1	ETV1+	W	neg.	—	—	neg.	neg.
P305T	ETV1	ETV1+	W	neg.	—	—	neg.	neg.
P344T	ETV1	ETV1+	W	neg.	—	—	n. a.	neg.
P456T	ETV1	ETV1+	W	neg.	—	—	14q13.3-q21.1-ETV1	neg.
P488T	ETV1	ETV1+	W	neg.	—	—	neg.	neg.
P499T	ETV1	ETV1+	W	neg.	—	—	neg.	neg.
P525T	ETV1	ETV1+	W	neg.	—	—	—	C15orf21-ETV1
P542T	ETV1	ETV1+	D	neg.	—	—	neg.	neg.
P274T	ETV1	ETV1+	D	neg.	—	—	—	EST14-ETV1
P451T	ETV1	ETV1+	D	neg.	—	—	—	OR51E2-ETV1
P271T	ETV1	ETV1+	n. a.	neg.	—	—	neg.	neg.
P498T	ETV1	ETV1+	n. a.	neg.	—	—	neg.	neg.
P500T	ETV1	ETV1+	n. a.	TMPRSS2-ETV1	—	—	—	neg.
P236T	ETV4	ETV4+	D	—	SLC45A3-ETV4	—	—	SLC45A3-ETV4
P352T	ETV4	ETV4+	n. a.	—	neg.	—	—	HERVK17-ETV4
P522T	ETV4	ETV4+	D	—	neg.	—	—	UBTF-ETV4
P262T	ETV5	ETV5+	D	—	—	neg.	—	neg.

TLDA indicates TaqMan low-density array; W, whole gene overexpression; D, differential exon expression; neg., negative; n. a., not analyzable.

prostate carcinogenesis, whereas *TMPRSS2* has been described as 5' fusion partner with four of these (all except *FLII*).

Finally, sequencing of the 5'RACE PCR fragments showed gene fusions that have previously been described in two additional cases. Sample P274T showed the fusion of *EST14* with exon 7 of *ETV1*, as previously described by Hermans and co-workers [13], whereas in sample P525T we found that the untranslated exon 1 of *C15orf21* (ENST00000409454) is fused with exon 3 of *ETV1*, thus giving rise to a full-length ETV1 protein (Figures 4 and W1). The latter is different from the previously described *C15orf21-ETV1* fusion [22], where exon 2 of *C15orf21* was fused with exon 8 of *ETV1*. There is evidence in the literature of fusion transcripts involving ETS genes

that include all their coding exons. Helgeson and co-workers described a prostate tumor with four fusion transcripts of *TMPRSS2-ETV5*, each including all coding exons of *ETV5* [6]. More recently, Gasi and colleagues described a prostate tumor harboring *EST14-ETV1* fusion transcripts including all coding exons of *ETV1* and overexpressing full-length ETV1 [23].

Contrarily to case P236T, in which the initial RT-PCR finding of the *SLC45A3-ETV4* gene fusion was later confirmed by 5'RACE PCR, in one PCa the *TMPRSS2-ETV1* fusion transcript was detected by RT-PCR but not by 5'RACE PCR, which may be due to technical difficulties of the 5'RACE methodology. The 11 cases where no 5' fusion partner was found using both RT-PCR and 5'RACE included 10 samples with evidence of *ETV1* rearrangement and 1 with evidence of *ETV5* rearrangement. The exon-level expression profile of the prostate tumor P261T displayed differential expression of *ETV5* exons 6 to13 compared to exons 1 to 5 (Figure 1); however, no 5' fusion partner was found either by RT-PCR or 5'RACE. Moreover, this sample was tested using a FISH approach with probes flanking *ETV5* combined with two additional probes targeting the 5' region of the two known *ETV5* fusion partners (*TMPRSS2* and *SLC45A3*), but no evidence of involvement of these genes was found. Further FISH analysis lead to the identification of two cases with 14q13.3-q21.1-*ETV1* rearrangement. Regarding the eight *ETV1* cases where no 5' fusion partner was found, exon-level expression data were available in six cases, of which one displayed differential exon-level expression and the remaining five showed overexpression of full-length *ETV1* transcripts. This is in agreement with a recent study of Gasi et al. [23], where, of five prostate tumors with overexpression of full-length *ETV1* transcripts and genomic rearrangement of *ETV1* determined by FISH, only in one case the 5'RACE was efficient in identifying a fusion transcript, which turned out to be *EST14-ETV1*.

Recent studies have brought new insights on the mechanisms underlying chromosomal rearrangements in human cancer. Reports describing androgen receptor–driven chromatin looping [24] and ligand-bound androgen receptor recruitment of enzymes capable of inducing double-strand breaks [25] suggest that androgen receptor may play an important role in gene fusion formation in PCa.

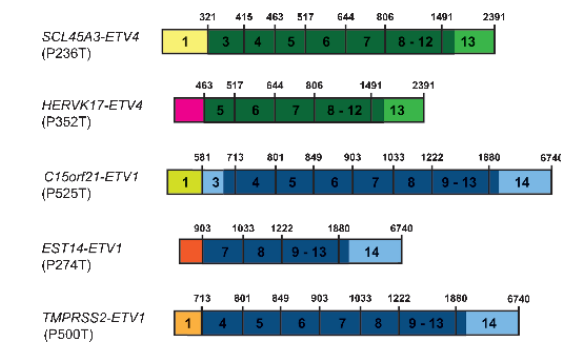


Figure 4. Schematic representation of gene fusions involving known ETS 5'fusion partners detected in PCas using 5'RACE, including the two novel ETV4 fusion partners. ETV1 exons are represented in blue and ETV4 exons in green, and noncoding regions are in lighter shades. From top to bottom: Exon 1 of *SLC45A3* fused with exon 3 of *ETV4* (prostate cancer P236T); transcript of the endogenous retroviral element *HERVK17* fused with exon 4 of *ETV4* (prostate cancer P352T); exon 1 of *C15orf21* fused with exon 3 of *ETV1* (prostate cancer P525T); exon 1 of *EST14* fused with exon 7 of *ETV1* (prostate cancer P274T); exon 1 of *TMPRSS2* fused with exon 4 of *ETV1* (prostate cancer P500T).

Another critical aspect of chromosome rearrangements is the spatial proximity of the partners involved. Growing evidence indicates that the formation of tumor translocations is partially affected by higher order genomic organization in nuclear territory [26]. In light of this, future work may show that the new fusion partners here described occupy the same nuclear domain.

In summary, this study contributes significantly to characterize the pattern of fusion genes in PCa, as we report two novel 5' fusion partners (*OR51E2-ETV1* and *UBTF-ETV4*) of ETS rearrangements, as well as two novel gene fusion combinations involving previously described genes (*SLC45A3-ETV4* and *HERVK17-ETV4*). Our findings suggests that not only may there be more 5' partner genes yet to be identified in PCa but also that all the genes described as 5' fusion partners with one ETS gene can most likely be rearranged with any of the other ETS genes known to be involved in PCa.

References

- [1] Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, et al. (2005). Recurrent fusion of *TMPRSS2* and ETS transcription factor genes in prostate cancer. *Science* **310**, 644–648.
- [2] Clark JP and Cooper CS (2009). ETS gene fusions in prostate cancer. *Nat Rev Urol* **6**, 429–439.
- [3] Paulo P, Barros-Silva JD, Ribeiro FR, Ramalho-Carvalho J, Jeronimo C, Henrique R, Lind GE, Skotheim RI, Lothe RA, and Teixeira MR (2012). FLI1 is a novel ETS transcription factor involved in gene fusions in prostate cancer. *Genes Chromosomes Cancer* **51**, 240–249.
- [4] Cerveira N, Ribeiro FR, Peixoto A, Costa V, Henrique R, Jeronimo C, and Teixeira MR (2006). *TMPRSS2-ERG* gene fusion causing *ERG* overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions. *Neoplasia* **8**, 826–832.
- [5] Tomlins SA, Mehra R, Rhodes DR, Smith LR, Roulston D, Helgeson BE, Cao X, Wei JT, Rubin MA, Shah RB, et al. (2006). *TMPRSS2:ETV4* gene fusions define a third molecular subtype of prostate cancer. *Cancer Res* **66**, 3396–3400.
- [6] Helgeson BE, Tomlins SA, Shah N, Laxman B, Cao Q, Prensner JR, Cao X, Singla N, Montie JE, Varambally S, et al. (2008). Characterization of *TMPRSS2:ETV5* and *SLC45A3:ETV5* gene fusions in prostate cancer. *Cancer Res* **68**, 73–80.
- [7] Maher CA, Palanisamy N, Brenner JC, Cao X, Kalyana-Sundaram S, Luo S, Khrebukova I, Barrette TR, Grasso C, Yu J, et al. (2009). Chimeric transcript discovery by paired-end transcriptome sequencing. *Proc Natl Acad Sci USA* **106**, 12353–12358.
- [8] Pflueger D, Rickman DS, Sboner A, Perner S, LaFargue CJ, Svensson MA, Moss BJ, Kitabayashi N, Pan Y, de la Taille A, et al. (2009). N-myc downstream regulated gene 1 (*NDRG1*) is fused to *ERG* in prostate cancer. *Neoplasia* **11**, 804–811.
- [9] Barros-Silva JD, Ribeiro FR, Rodrigues A, Cruz R, Martins AT, Jeronimo C, Henrique R, and Teixeira MR (2011). Relative 8q gain predicts disease-specific survival irrespective of the *TMPRSS2-ERG* fusion status in diagnostic biopsies of prostate cancer. *Genes Chromosomes Cancer* **50**, 662–671.
- [10] Rajan P, Dalglish C, Carling PJ, Buist T, Zhang C, Grellscheid SN, Armstrong K, Stockley J, Simillion C, Gaughan L, et al. (2011). Identification of novel androgen-regulated pathways and mRNA isoforms through genome-wide exon-specific profiling of the LNCaP transcriptome. *PLoS One* **6**, e29088.
- [11] Paulo P, Ribeiro FR, Santos J, Mesquita D, Almeida M, Barros-Silva JD, Ikonen H, Henrique R, Jeronimo C, Sveen A, et al. (2012). Molecular subtyping of primary prostate cancer reveals specific and shared target genes of different ETS rearrangements. *Neoplasia* **14**, 600–611.
- [12] Attard G, Clark J, Ambroisine L, Mills IG, Fisher G, Flohr P, Reid A, Edwards S, Kovacs G, Berney D, et al. (2008). Heterogeneity and clinical significance of *ETV1* translocations in human prostate cancer. *Br J Cancer* **99**, 314–320.
- [13] Hermans KG, van der Korput HA, van Marion R, van de Wijngaert DJ, Ziel-van der Made A, Dits NF, Boormans JL, van der Kwast TH, van Dekken H, Bangma CH, et al. (2008). Truncated *ETV1*, fused to novel tissue-specific genes, and full-length *ETV1* in prostate cancer. *Cancer Res* **68**, 7541–7549.
- [14] Xu LL, Stackhouse BG, Florence K, Zhang W, Shanmugam N, Sesterhenn IA, Zou Z, Srikantan V, Augustus M, Roschke V, et al. (2000). *PSGR*, a novel prostate-specific gene with homology to a G protein-coupled receptor, is overexpressed in prostate cancer. *Cancer Res* **60**, 6568–6572.
- [15] Weng J, Ma W, Mitchell D, Zhang J, and Liu M (2005). Regulation of human prostate-specific G-protein coupled receptor, *PSGR*, by two distinct promoters and growth factors. *J Cell Biochem* **96**, 1034–1048.
- [16] Vaarala MH, Hirvikoski P, Kauppila S, and Paavonen TK (2012). Identification of androgen-regulated genes in human prostate. *Mol Med Rep* **6**, 466–472.
- [17] Han B, Mehra R, Dhanasekaran SM, Yu J, Menon A, Lonigro RJ, Wang X, Gong Y, Wang L, Shankar S, et al. (2008). A fluorescence *in situ* hybridization screen for E26 transformation-specific aberrations: identification of *DDX5-ETV4* fusion protein in prostate cancer. *Cancer Res* **68**, 7629–7637.
- [18] Jones KA, Black DM, Griffiths BL, and Solomon E (1995). Localization of the human RNA polymerase I transcription factor gene (*UBTF*) to the *D17S183* locus on chromosome 17q21 and construction of a long-range restriction map of the region. *Genomics* **30**, 602–604.
- [19] Matera AG, Wu W, Imai H, O'Keefe CL, and Chan EK (1997). Molecular cloning of the RNA polymerase I transcription factor hUBF/NOR-90 (*UBTF*) gene and localization to 17q21.3 by fluorescence *in situ* hybridization and radiation hybrid mapping. *Genomics* **41**, 135–138.
- [20] Grandori C, Gomez-Roman N, Felton-Edkins ZA, Ngouenet C, Galloway DA, Eisenman RN, and White RJ (2005). c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. *Nat Cell Biol* **7**, 311–318.
- [21] Poortinga G, Hannan KM, Snelling H, Walkley CR, Jenkins A, Sharkey K, Wall M, Brandenburger Y, Palatsides M, Pearson RB, et al. (2004). *MAD1* and c-MYC regulate UBF and rDNA transcription during granulocyte differentiation. *EMBO J* **23**, 3325–3335.
- [22] Tomlins SA, Laxman B, Dhanasekaran SM, Helgeson BE, Cao X, Morris DS, Menon A, Jing X, Cao Q, Han B, et al. (2007). Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature* **448**, 595–599.
- [23] Gasi D, van der Korput HA, Douben HC, de Klein A, de Ridder CM, van Weerden WM, and Trapman J (2011). Overexpression of full-length *ETV1* transcripts in clinical prostate cancer due to gene translocation. *PLoS One* **6**, e16332.
- [24] Lin C, Yang L, Tanasa B, Hutt K, Ju BG, Ohgi K, Zhang J, Rose DW, Fu XD, Glass CK, et al. (2009). Nuclear receptor-induced chromosomal proximity and DNA breaks underlie specific translocations in cancer. *Cell* **139**, 1069–1083.
- [25] Haffner MC, Aryee MJ, Toubaji A, Esopi DM, Albadine R, Gurel B, Isaacs WB, Bova GS, Liu W, Xu J, et al. (2010). Androgen-induced TOP2B-mediated double-strand breaks and prostate cancer gene rearrangements. *Nat Genet* **42**, 668–675.
- [26] Engreitz JM, Agarwala V, and Mirny LA (2012). Three-dimensional genome architecture influences partner selection for chromosomal translocations in human disease. *PLoS One* **7**, e44196.

Supplementary Reference

- [1] Kilpinen S, Autio R, Ojala K, Iljin K, Bucher E, Sara H, Pisto T, Saarela M, Skotheim RI, Björkman M, et al. (2008). Systematic bioinformatic analysis of expression levels of 17,330 human genes across 9,783 samples from 175 types of healthy and pathological tissues. *Genome Biol* **9**, R139.

Table W1. Oligonucleotide Primer Sequences for 5'RACE PCR.

Primer	Oligonucleotide Sequence (5'→3')	Ensembl Transcript	Exon
ETV1_R_GSP	GTTCTGCTGGGATGAGCCAGGAAGC	ENST00000430479	10
ETV1_F_IC	TCCCTCCATCGCAGTCCATACCAGA	ENST00000430479	8
ETV1_R_NGSP	AAGCCTTGTGGTGGGAAGGGGATGT	ENST00000430479	9
ETV4_R_GSP	CAGGGACAACGCAGACATCATCTGG	ENST00000319349	10
ETV4_F_IC	CATGGCGAGCAGTGCCTTTACTCCA	ENST00000319349	6
ETV4_R_NGSP	GGCGAAGTCCGTCTGTTCCTGTTTGA	ENST00000319349	8
ETV5_R_GSP	CTCTCAGGCACAACACAAGTGTCGTCAA	ENST00000306376	10
ETV5_F_IC	CCCTTCCCTCCTCAGCCAGGAGTTC	ENST00000306376	8
ETV5_R_NGSP	TGGCTGCTGGAGAAATAACCCCTCT	ENST00000306376	9

Table W2. Oligonucleotide Primers Used for RT-PCR.

Primer	Oligonucleotide Sequence (5'→3')	Ensembl Transcript	Exon
C15orf21_S	TCTTTTGGTTGGTGATTTTGATGA	ENST00000409454	1
SLC45A3_S	GGAACCAGCCTGCACGC	ENST00000367145	1
UBTF_S	TTGACCCCGGGTTGCC	ENST00000393606	2
TMPRSS2	TAGGCGCGAGCTAAGCAGGAG	ENST00000332149	1
OR51E2_S	ACCACCACCAGGAATTGGC		
EST14_S	GAAGAGAAGAGAATTCACAGAGTGAAAA		
HERVK17_S	TTTCCACACTCTCATTTCCGGA		
ETV1_AS1	TCGTTACAATTTCTCCACGCT	ENST00000430479	4
ETV1_AS2	GCCTAAAGCCTTGTGGTGGG	ENST00000430479	9
ETV4_AS	GGAGTAAAGGCACTGCTCGC	ENST00000319349	6

Table W3. BAC Probes Used to Assess Genomic Rearrangements of ETS Transcription Factors and Novel 5' Fusion Partners.

Gene Symbol	5' Probe (Red)	3' Probe (Green)
<i>ETV1</i>	RP11-643C13	RP11-790J2
<i>ETV4</i>	RP11-436J4	RP11-831F13
<i>OR51E2</i>	CTC-775N13	NA
<i>Chr14 (q13.3-21.1)</i>	RP11-945C4 RP11-666J24	NA

NA indicates not applicable.

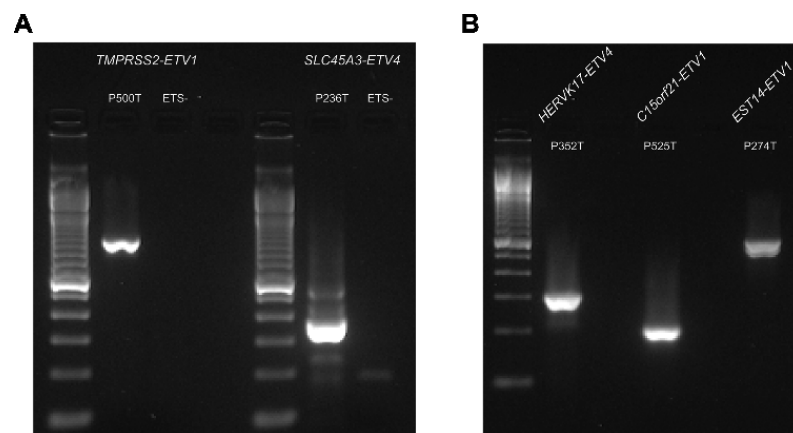


Figure W1. RT-PCR screening of gene fusions involving known 5' fusion partners. Detection of *TMPRSS2-ETV1* and *SLC45A3-ETV4* fusion transcripts in PCas P500T and P236T, respectively (A). RT-PCR validation of 5'RACE findings of gene fusions involving known 5' partners (B).

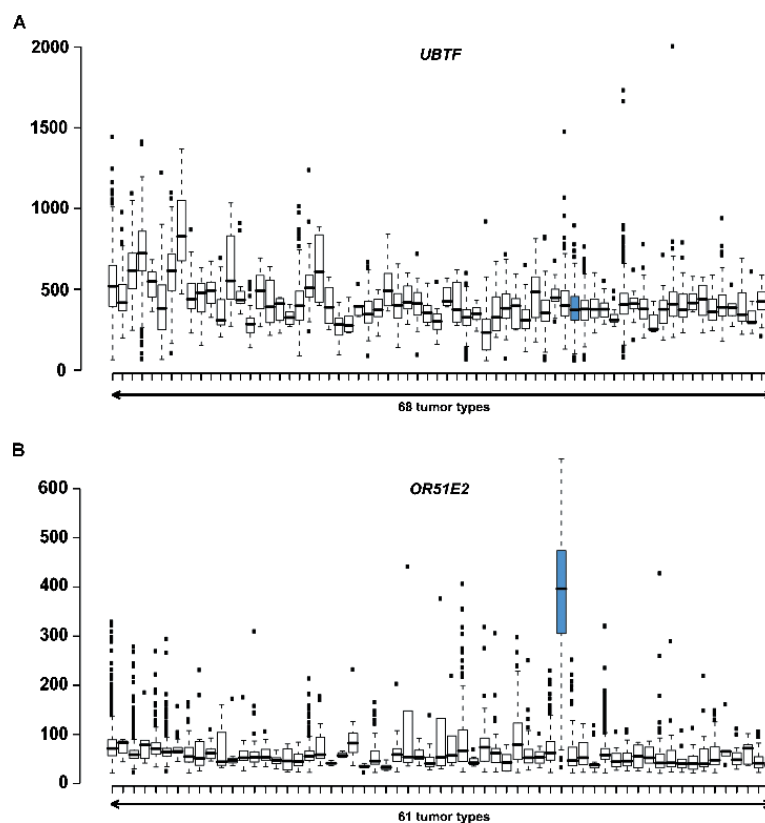


Figure W2. Expression of *UBTF* and *OR51E2* in different tumor types. The plots representing *UBTF* (a) and *OR51E2* expression (b), respectively, were obtained from the publicly available GeneSapiens database [1]. The blue box represents PCas.

Chapter 5

Original publication # III

Relative 8q Gain Predicts Disease-Specific Survival Irrespective of the *TMPRSS2-ERG* Fusion Status in Diagnostic Biopsies of Prostate Cancer

João D. Barros-Silva^{1,4}, Franclim R. Ribeiro^{1,4}, Ângelo Rodrigues², Ricardo Cruz³, Ana T. Martins^{2,5}, Carmen Jerónimo^{1,5,6}, Rui Henrique^{2,5,6}, and Manuel R. Teixeira^{1,4,6*}

¹*Department of Genetics, Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal;*

²*Department of Pathology, Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal;*

³*Department of Urology, Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal;*

⁴*Cancer Genetics Group, Research Center of the Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072, Porto, Portugal;*

⁵*Cancer Epigenetics Group, Research Center of the Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072, Porto, Portugal;*

⁶*Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Largo Prof. Abel Salazar, 2, 4099-003 Porto, Portugal.*

Relative 8q Gain Predicts Disease-Specific Survival Irrespective of the *TMPRSS2-ERG* Fusion Status in Diagnostic Biopsies of Prostate Cancer

João D. Barros-Silva,^{1,2} Francim R. Ribeiro,^{1,2} Ângelo Rodrigues,³ Ricardo Cruz,⁴ Ana T. Martins,^{3,5} Carmen Jerónimo,^{1,5,6} Rui Henrique,^{3,5,6} and Manuel R. Teixeira^{1,4,6*}

¹Department of Genetics, Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal

²Cancer Genetics Group, Research Center of the Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072, Porto, Portugal

³Department of Pathology, Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal

⁴Department of Urology, Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal

⁵Cancer Epigenetics Group, Research Center of the Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072, Porto, Portugal

⁶Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Largo Prof. Abel Salazar, 2, 4099-003 Porto, Portugal

Screening tools have greatly improved prostate cancer (PCa) detection, but the disease course is heterogeneous, and standard clinicopathological parameters do not fully discriminate aggressive from indolent tumors. To evaluate the prognostic value of the *TMPRSS2-ERG* fusion gene combined with chromosome arm 8q relative gain in diagnostic biopsies of PCa, we studied a consecutive series of 200 diagnostic needle biopsies from patients with 10-year disease-specific survival data. *TMPRSS2-ERG* fusion gene status and relative 8q gain were assessed by fluorescent in situ hybridization in whole formalin fixed paraffin-embedded biopsies. The *TMPRSS2-ERG* fusion gene was detected in 43.5% of PCa and was associated with lower Gleason score ($P = 0.045$), whereas relative 8q gain was present in 48% of PCa and was associated in high-Gleason score ($P < 0.001$). *ERG* rearrangement alone was not associated with clinical outcome, whereas relative 8q gain predicted worse disease-specific survival in PCa patients both with and without the *TMPRSS2-ERG* fusion gene ($P < 0.001$), independently of Gleason score, clinical stage, and treatment modality. We conclude that relative 8q gain in diagnostic needle biopsies is a poor prognostic factor independent of the *TMPRSS2-ERG* fusion gene status and of standard clinicopathological parameters. © 2011 Wiley-Liss, Inc.

INTRODUCTION

Prostate cancer (PCa) is the malignant disease with the highest incidence rate and the second in estimated cancer deaths in Western countries (Jemal et al., 2009). The clinical outcome of these patients is variable, with 35% of the patients treated by radical prostatectomy (RP) experiencing biochemical recurrence (BR) (Amling et al., 2000; Han et al., 2001; Hull et al., 2002; Roehl et al., 2004; Sardana et al., 2008). Among those, however, only a subset ranging from 8.3 to 18.5% will eventually perish from metastatic spread of the disease (Ward et al., 2003; Freedland et al., 2005; Porter et al., 2006). Although widely used for screening and monitoring PCa, prostate-specific antigen (PSA) is less efficient to predict disease outcome.

The ETS transcription factor family of genes is involved in recurrent genetic rearrangements in PCa, most commonly the *ERG* gene. Frequently, there is a fusion of *TMPRSS2* and *ERG*, with the

androgen-regulated elements of the former causing overexpression of the latter (Tomlins et al., 2005). We and others have subsequently confirmed the presence of this chimeric gene in about 50% of PCa patients (Cerveira et al., 2006; Hermans et al., 2006; Perner et al., 2006; Soller et al., 2006; Yoshimoto et al., 2006). The mechanism behind this rearrangement is either an interstitial deletion in 21q22.2–3, where *TMPRSS2* and *ERG* are located 3 Mbp apart or an insertion

Additional Supporting Information may be found in the online version of this article.

Supported by: Fundação para a Ciência e a Tecnologia (FCT), Grant number: PTDC/SAU/OBD/70543/2006; JDBS, Grant number: SFRH/BD/46574/2008; FRR, Grant number: SFRH/BPD/26492/2006.

*Correspondence to: Manuel R. Teixeira, Department of Genetics, Portuguese Oncology Institute, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal.

E-mail: manuel.teixeira@ipporto.min-saude.pt

Received 16 February 2011; Accepted 5 April 2011

DOI 10.1002/gcc.20888

Published online 16 May 2011 in

Wiley Online Library (wileyonlinelibrary.com).

of the sequences between the two fusion partners into another chromosome, although a translocation mechanism is theoretically also possible (Teixeira, 2008). Other ETS family genes, namely *ETV1*, *ETV4*, and *ETV5*, have been found fused with *TMPRSS2* or with other 5' partners (Prensner and Chinnaiyan, 2009), although at much lower frequencies.

The data available thus far support the role of ETS fusions as early events in prostate carcinogenesis. Our group has for the first time detected this fusion oncogene in 21% of high-grade prostatic intraepithelial neoplasia (HGPIN) lesions, thereby indicating that HGPIN is a precursor of PCa (Cerveira et al., 2006). Recent studies with *TMPRSS2-ERG* transgenic mice have shown that *ERG* overexpression alone (Klezovitch et al., 2008) or in combination with PI3-kinase pathway activation (King et al., 2009) induces PIN lesions in these models, although the chimeric gene does not seem to be sufficient to cause progression to PCa. To understand the natural history of PCa, it is therefore crucial to identify the subsequent genetic events that underlie progression from a precursor lesion to a potentially lethal disease. Using comparative genomic hybridization (CGH), we have previously shown that the *TMPRSS2-ERG* fusion precedes chromosome copy number alterations in prostate carcinogenesis (Paris et al., 2003, 2004; van Dekken et al., 2004; Cerveira et al., 2006; Ribeiro et al., 2006). Although the ETS fusion gene is arguably the initial genetic event in a large subset of PCa, secondary chromosome copy number changes may be more relevant to understand PCa progression.

Although some studies have assessed the prognostic value of the *TMPRSS2-ERG* oncogene in PCa, most studies used BR as end point and its association with clinical outcome remains controversial (Perner et al., 2006; Attard et al., 2008; Furusato et al., 2008; Saramäki et al., 2008; Gopalan et al., 2009). Yoshimoto et al. (2008) showed that the combined presence of the *TMPRSS2-ERG* fusion gene with *PTEN* loss predicts earlier BR, thus indicating that particular combinations of ETS gene fusions and chromosome copy number changes may be better predictors of clinical outcome. Given our previous findings on the prognostic value of 8q gain in PCa patients (Ribeiro et al., 2006, 2007), we aimed to evaluate the relative clinical relevance of the *TMPRSS2-ERG* fusion and of relative 8q gain in a series of diagnostic needle biopsies from 200 PCa patients with long disease-specific survival data.

PATIENTS AND METHODS

Biopsy Specimens and Clinical Data

Archival paraffin-embedded biopsy specimens from 200 patients diagnosed with PCa from 1997 to 2001 at the Portuguese Oncology Institute, Porto were reevaluated by an expert pathologist to assess the presence and grade of each tumor (Supporting Information Table 1). A representative sample from each patient was selected for interphase fluorescent in situ hybridization (FISH) analyses. Tumor content was evaluated for each PCa biopsy and categorized into four groups: 46 (23%) biopsies had <25% tumor content, 54 (27%) had 25-50%, 57 (28.5%) had 50-75%, and 43 (21.5%) had >75%. Of the 200 biopsies, 12% had Gleason score (GS) lower than 7, whereas 40.5% were classified as GS = 7 and 47.5% as GS >7. Fifty-four of the 200 patients were previously reported regarding testing of 8q gain alone (Ribeiro et al., 2007).

Relevant clinical data at diagnosis were obtained from medical records. Patient age at diagnosis ranged from 52 to 87 (mean 72, median 73), and median PSA value was 36.3 ng/ml (range, 2.38-12,500). At diagnosis, 34.5% of the patients had the disease classified as clinical stage (CS) II, 32% as CS III, and 31% as CS IV. In five patients it was not possible to collect CS data. Regarding treatment, 9% of patients were submitted to RP, 57.5% to hormone therapy (HT), and 3.5% to radiation therapy (RT). Some patients were submitted to more than one treatment: 11% to RT+HT (in this order), 5% to HT and chemotherapy (QT), and 8.5% to HT+RT. A group of patients, including four submitted to surgical castration and HT, two to active surveillance, one to RP + HT + QT, and two to HT+QT+RT was categorized as "other treatment" (4.5%). Treatment data were not available for two patients. Concerning follow-up data, patients were grouped in one of the following categories: alive, no evidence of disease; alive with disease; dead, no evidence of disease; dead with disease; and dead from disease. The median follow-up time was 67.7 months (range, 0.6-155.8).

FISH

From each of the 200 paraffin-embedded biopsy samples, 4-μm-thick sections were cut onto SuperFrost PlusAdhesion slides (Menzel-Glaser, Braunschweig, Germany). Sample processing and

hybridization were performed as described previously (Ribeiro et al., 2006, 2007). Slides were counterstained with DAPI (Vector Laboratories, Burlingame, California, USA) and fluorescent images corresponding to DAPI, SpectrumGreen, SpectrumOrange, and SpectrumAqua were sequentially captured with a Cohu 4900 CCD camera, with the use of an automated filter wheel coupled to a Zeiss Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) and a Cyto-Vision system version 3.9 (Applied Imaging, Santa Clara, California, USA).

To detect *TMPRSS2-ERG* fusions, a break-apart probe strategy flanking *ERG* was used (Perner et al., 2006). Bacterial artificial chromosome (BAC) clones targeting the 5'(RP11-137J13) and 3'(RP11-24A11) regions of *ERG* were selected using the UCSC Human Genome Browser and obtained from the BACPAC Resources Center (Oakland, California, USA). BAC DNA was extracted, amplified, labeled, and prepared for hybridization as previously reported (Vieira et al., 2010). Adequate mapping and probe specificity of all BAC clones was confirmed by hybridization onto normal human metaphases.

Relative 8q24 gain was assessed as previously described (Ribeiro et al., 2006, 2007). A ratio between *MYC* and CEP18 signals within each nucleus of a representative cancer cell population was computed for each sample. Whenever several representative populations existed for a given tumor, the highest ratio was used. Cases were categorized as having relative 8q gain when *MYC*/CEP18 ≥ 1.5 (Ribeiro et al., 2007). Additionally, cases with *MYC*/CEP18 ≥ 2 ratio were deemed 8q-amplified. *ERG* copy number (assessed by the number of 5'*ERG* or 3'*ERG* signals, whichever higher) was also tested as ploidy control (as an alternative to CEP18) in the matching tumor areas with *MYC* copy number increase to compute the *MYC*/*ERG* ratio.

For scoring purposes, only morphologically intact, nonoverlapping nuclei were considered. An abnormal signal pattern was considered representative when present in a minimum of 50 cancer cells.

Statistical Analyses

The χ^2 -square test was used for comparison of categorical data and the Student's *t* test was used for parametric data when comparing two means. For non-parametric data, the Kruskal-Wallis test was used. Disease-specific survival was calculated through Kaplan-Meier plots representing patients

with versus patients without a given genetic alteration. All events other than death from disease were censored. Differences between groups were compared using the log-rank test. A Cox regression hazard model comprising clinicopathological (GS, CS, PSA, % tumor content) as well as genetic data (*TMPRSS2-ERG* rearrangement, 8q gain) was used to assess their independent prognostic value. All statistical analyses were conducted using SPSS (Chicago, Illinois, USA).

RESULTS

ERG Gene Changes

TMPRSS2-ERG fusions were found in 87 (43.5%) patients (Fig. 1; Supporting Information Table 1). A split of the 3' and 5' probes flanking *ERG* (Fig. 1B) was observed in 45 (22.5%) patients (mechanism of insertion), and deletion of the 5'*ERG* probe (Fig. 1C) was observed in 42 (21%), of which 14 (7%) displayed two or more copies of the 5'*ERG* deletion. Additionally, 48 (24%) cases displayed increased *ERG* copy number (Fig. 1D), and *ERG* loss was observed in 4 (2%) cases.

Chromosome Arm 8q Changes

Overall, tumor cell populations with *MYC* copy number increase were found in 132 PCa biopsies. Of these, 36 PCa had a *MYC*/CEP18 < 1.5 ratio (Fig. 1F) and were therefore considered negative for relative 8q gain. Of the 96 PCa biopsies with relative 8q gain (*MYC*/CEP18 ≥ 1.5 ; Fig. 1G), 69 had *MYC* amplification (*MYC*/CEP18 ≥ 2 ; Fig. 1H). Of the PCa biopsies with *MYC* copy number increase, 33 presented a *MYC*/*ERG* < 1.5 ratio and 99 had a *MYC*/*ERG* ≥ 1.5 ratio. The 8q status using each of the two ploidy controls was in agreement in 79.5% of PCa biopsies, whereas 9.5% of *MYC*/CEP18 ≥ 1.5 cases were *MYC*/*ERG* < 1.5 and 11% of *MYC*/CEP18 < 1.5 were *MYC*/*ERG* ≥ 1.5 .

Ten cases harbored putative *MYC* structural rearrangements. In brief, a split of 3' and 5'*MYC* flanking probes was found in five cases, 3'*MYC* deletion was found in two cases, 5'*MYC* deletion was also found in two cases, and 5'*MYC* was amplified in one case (Supporting Information Table 1).

Relationship Between *TMPRSS2-ERG* and 8q Gain

Relative 8q gain (*MYC*/CEP18 ≥ 1.5) was present in 59.4% of *TMPRSS2-ERG* fusion positive

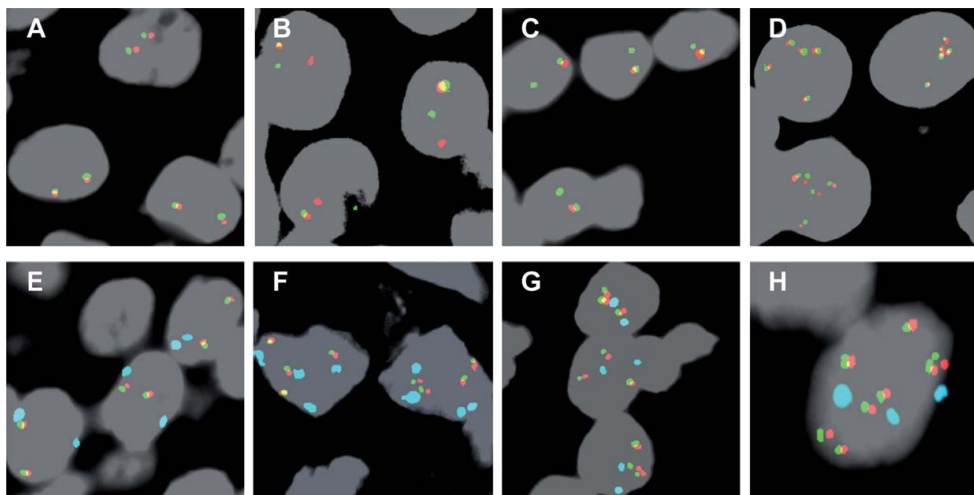


Figure 1. FISH analysis of prostate cancer biopsies. Bacterial artificial chromosome clones targeting 5'*ERG* and 3'*ERG*, labeled red and green, respectively, were used for detection of the *TMPRSS2-ERG* fusion (A–D). Cells displaying no rearrangement (A), rearrangement through insertion (B), rearrangement through deletion (C), and *ERG* copy num-

ber increase (D) are shown. Dual-color probes (orange and green) flanking *MYC* and a chromosome 18 centromeric probe (aqua) were used to detect relative 8q gain (E–H). Cells displaying *MYC/CEP18* ratios of 1 (E and F), 1.5 (G), and 3 (H) are shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and in 40.6% of fusion negative PCa biopsies ($P = 0.066$). Of the fusion positive, 51.3% of PCa biopsies with deletion and 48.7% of PCa biopsies with insertion displayed *MYC/CEP18* ≥ 1.5 ratio ($P = 0.873$). Considering *ERG* as ploidy control instead of CEP18, relative 8q gain (*MYC/ERG* ≥ 1.5) was present in 45.5% of PCa biopsies with and 54.5% without the *TMPRSS2-ERG* fusion ($P = 0.366$). Of the fusion positive, 48.9% of PCa biopsies with deletion and 51.1% with insertion displayed *MYC/ERG* ≥ 1.5 ratio ($P = 0.881$).

Clinicopathological Associations of Genetic Changes

TMPRSS2-ERG rearrangements were associated with lower PSA levels at diagnosis ($P = 0.017$), with lower age at diagnosis ($P = 0.002$), and were less frequent in high GS biopsies (34% in GS >7 vs. 50.6% in GS = 7 and 54.2% in GS <7 , $P = 0.045$). No association was found between *TMPRSS2-ERG* status and clinical stage, percentage of tumor content, or treatment.

Patients with *MYC/CEP18* ≥ 1.5 had higher PSA levels at diagnosis ($P < 0.001$), and no association with age at diagnosis was found. Relative 8q gain was less frequent in low GS biopsies (12.5% in GS <7 vs. 49.4% in GS = 7 and 55.3% in GS >7 ; $P = 0.001$), in patients of lower CS (30.4% in CS II vs. 56.3% in CS III and 61.3% in

CS IV, $P = 0.003$), and in biopsies with lower percentage of tumor content (17.4% in $<25\%$ vs. 55.6% in 25–50%, 57.9% in 50–75%, and 58.1% in $>75\%$; $P < 0.001$). Relative 8q gain was less frequent ($P = 0.008$) in patients treated with RP (11.1%) or RT (14.3%) than in patients whose treatment included HT (up to 57.4%). All clinicopathological associations are summarized in Table 1.

Disease-Specific Survival and Multivariate Analyses

The *TMPRSS2-ERG* fusion gene was not associated with overall disease-specific survival ($P = 0.535$; Fig. 2A) irrespective of treatment subgroup. Additionally, rearrangement type (insertion or deletion) showed no association with clinical outcome (Supporting Information Fig. 1; $P = 0.505$). On the other hand, patients with 8q gain (*MYC/CEP18* ≥ 1.5) had significantly lower 10-year disease-specific survival than those without (47% vs. 75%, $P = 1.5 \times 10^{-5}$; Fig. 2B). Using *ERG* as ploidy control, patients with relative 8q gain (*MYC/ERG* ≥ 1.5) also displayed lower 10-year disease-specific survival than those without (49% vs. 74%, $P = 7.1 \times 10^{-5}$, Fig. 2D). Because this difference is slightly smaller than that with the *MYC/CEP18* ratio, we kept it as our main definition of 8q gain.

TABLE I. Association of Clinical Parameters with FISH Results for the *TMPRSS2-ERG* Fusion Gene and Relative 8q Gain

Clinical parameters	<i>TMPRSS2-ERG</i>			8q gain		
	–	+	<i>P</i>	–	+	<i>P</i>
Age ($\pm$ std)	73.33 $\pm$ 6.51	70.26 $\pm$ 6.96	0.002	71.67 $\pm$ 6.88	72.34 $\pm$ 7.07	0.491
PSA (median)	50	26.3	0.017	21.75	63.26	<0.001
Gleason score						
GS < 7	11 (45.8%)	13 (54.2%)	0.056	21 (87.5%)	3 (12.5%)	0.001
GS = 7	40 (49.4%)	41 (50.6%)		41 (50.6%)	40 (49.4%)	
GS > 7	62 (65.3%)	33 (34.7%)		42 (44.7%)	52 (55.3%)	
Clinical stage						
II	38 (55.1%)	31 (44.9%)	0.250	48 (69.6%)	21 (30.4%)	0.001
III	32 (50%)	32 (50%)		28 (43.8%)	36 (56.3%)	
IV	40 (64.5%)	22 (35.5%)		24 (38.7%)	38 (61.3%)	
Tumor content						
<25%	30 (65.2%)	16 (34.8%)	0.267	38 (82.6%)	8 (17.4%)	<0.001
$\geq$ 25–50%	25 (46.3%)	29 (53.7%)		24 (44.4%)	30 (55.6%)	
$\geq$ 50–75%	34 (59.6%)	23 (40.4%)		24 (42.1%)	33 (57.9%)	
$\geq$ 75%	24 (55.8%)	19 (44.2%)		18 (41.9%)	25 (58.1%)	
Treatment						
RP ^a	7 (38.9%)	11 (61.1%)	0.243	16 (88.9%)	2 (11.1%)	0.005
HT	70 (60.9%)	45 (39.1%)		49 (42.6%)	66 (57.4%)	
RT	5 (71.4%)	2 (28.6%)		6 (85.7%)	1 (14.3%)	
RT + HT	9 (40.9%)	13 (59.1%)		14 (63.6%)	8 (36.4%)	
HT + QT	4 (40%)	6 (60%)		5 (50%)	5 (50%)	
HT + RT	11 (64.7%)	6 (35.3%)		9 (52.9%)	8 (47.1%)	
Other	6 (66.7%)	3 (33.3%)		4 (54.4%)	5 (55.6%)	

^aSix prostate cancer patients were additionally treated with HT and one with RT. RP, radical prostatectomy; HT, hormone therapy; RT, radiation therapy; QT, chemotherapy.

The presence of the two genetic alterations predicted low-disease-specific survival (Fig. 2C), but it was similar to that of patients with relative 8q gain lacking *TMPRSS2-ERG* ($P = 0.926$). The prognostic value was related with relative 8q gain and not to plain 8q copy number increase (Supporting Information Fig. 2; $P = 0.03$).

In patients with GS > 7, relative 8q gain was associated with poor disease-specific survival ($P = 0.015$; Fig. 3A). In patients with GS = 7 and *MYC/CEP18* ≥ 1.5 , there was a trend for shorter cancer-specific survival ($P = 0.078$; Fig. 3B), whereas 8q amplification was associated with a statistically significant worse disease-specific survival ($P = 0.028$; Fig. 3C). Of GS < 7 patients with 8q gain ($n = 3$) one died from disease, but the small number of patients did not allow evaluation of prognostic value in this subgroup. Relative 8q gain predicted worse disease-specific survival in CS = II ($P = 0.021$) and in CS = IV ($P = 0.013$) patients (Fig. 4A–C). Finally, in patients undergoing HT only, those with *MYC/CEP18* ≥ 1.5 had worse disease-specific survival ($P = 0.013$; Fig. 4D), as was also the case in patients treated with RP alone ($P = 0.008$) and with RT alone ($P = 0.046$).

A Cox proportional hazard regression model including clinical parameters and the genetic alterations herein studied are summarized in Table 2. Briefly, relative 8q gain presented increased risk of death from disease (HR = 4.00, 95% C.I.: 2.19–7.30, $P < 0.001$) independently from clinical stage (HR = 10.05, 95% C.I.: 3.55–28.48, $P < 0.001$). The *TMPRSS2-ERG* status showed no independent prognostic value (HR = 1.32, 95% C.I.: 0.76–2.29, $P = 0.327$).

DISCUSSION

Needle biopsies are essential to establish the diagnosis of PCa. However, grading and staging in prostate biopsies are not as accurate as data obtained in prostatectomy specimens, with overgrading and undergrading being described in up to 12.8 and 41.2%, respectively (Isariyawongse et al., 2008). Ancillary methods to obtain refined prognostic information in diagnostic needle biopsies before any treatment decision would therefore be a major contribution to predict the clinical heterogeneity that characterizes PCa.

ERG rearrangements were found in 43.5% of the diagnostic biopsies, which is in agreement

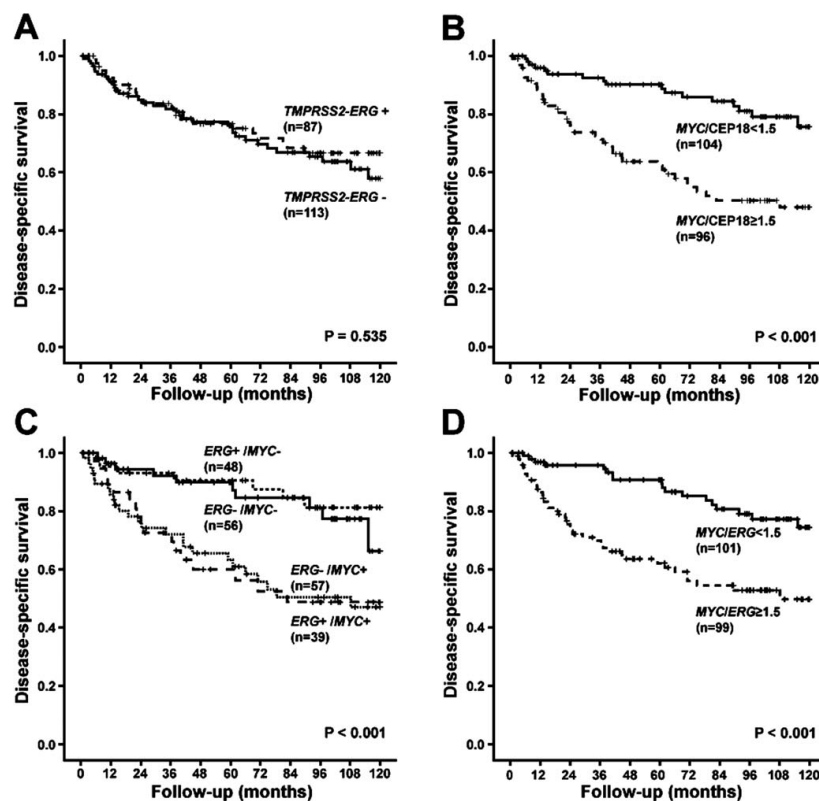


Figure 2. Kaplan-Meier curves illustrating overall disease-specific survival between groups of prostate cancer patients defined by the presence (+) or absence (−) of *TMPRSS2-ERG* rearrangements (A), presence (*MYC/CEP18* ≥ 1.5) or absence (*MYC/CEP18* < 1.5) of rela-

tive 8q gain using CEP18 as ploidy control (B), combination of the fusion gene and relative 8q gain (C), and presence (*MYC/ERG* ≥ 1.5) or absence (*MYC/ERG* < 1.5) of relative 8q gain using *ERG* (chromosome 21) as ploidy control (D).

with the rearrangement frequency of 46% reported in the only published study on prostate biopsies using FISH to detect the *TMPRSS2-ERG* fusion (Mosquera et al., 2009). We found no association of *ERG* rearrangements with cancer-specific survival. The prognostic value of the *TMPRSS2-ERG* fusion gene is controversial in the literature when using the same end point described here (Demichelis et al., 2007; Fitzgerald et al., 2008; Rubio-Briones et al., 2010), but also among authors using BR as end point (Perner et al., 2006; Lapointe et al., 2007; Mehra et al., 2007; Nam et al., 2007; Saramäki et al., 2008; Yoshimoto et al., 2008). The last large FISH-based report to evaluate the clinical relevance of the *TMPRSS2-ERG* fusion in patients treated with RP found no association of the fusion gene with clinical outcome (Gopalan et al., 2009). Furthermore, a subgroup with copy num-

ber increase and rearrangement by deletion, previously described by Attard et al. (2008) and Yoshimoto et al. (2008), tended to be clinically more aggressive. However, assessment of whole DNA ploidy by flow cytometry and of an independent chromosome (#9) by FISH led the authors to conclude that the aggressive phenotype is associated with aneuploidy and not with the duplication of *TMPRSS2-ERG* itself, therefore indicating that other genomic changes may be better prognostic markers.

One of the best candidate genetic prognostic markers thus far described is 8q gain, as we have previously shown using genome-wide analysis with CGH (Ribeiro et al., 2006) and confirmed by FISH (Ribeiro et al., 2007) with locus-specific probes. Among patients with 8q gain, the computed *MYC/CEP18* ratio discriminated patients with generalized aneuploidy from those with

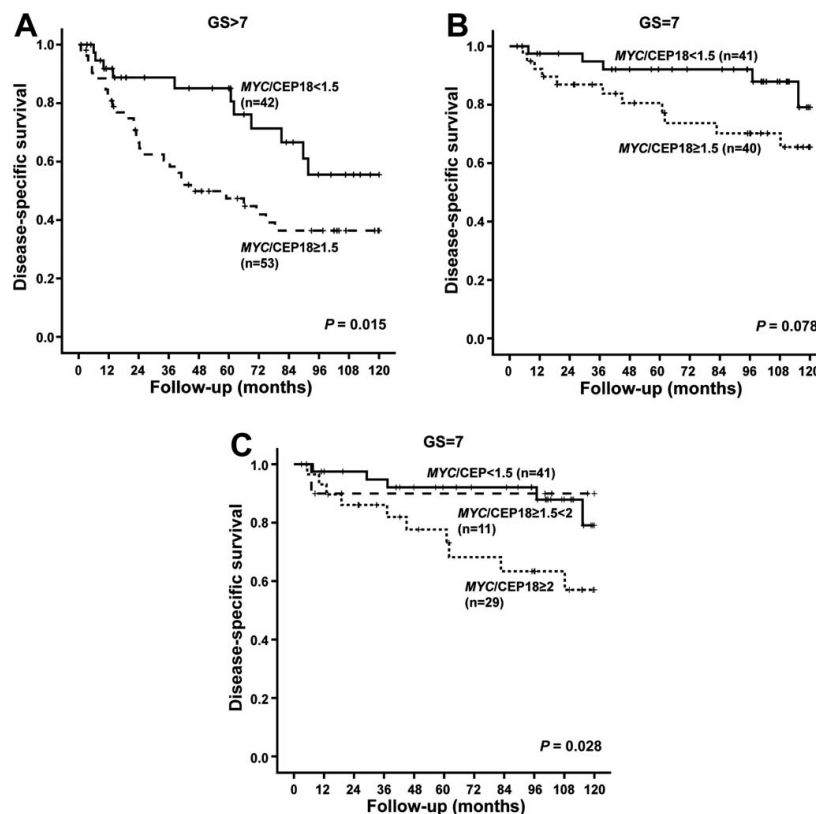


Figure 3. Kaplan-Meier curves illustrating disease-specific survival according to the presence ($MYC/CEP18 \geq 1.5$) or absence ($MYC/CEP18 < 1.5$) of relative 8q gains in PCa patients with Gleason score greater than 7 (A) and Gleason score 7 (B). Kaplan-Meier curve illus-

trating disease-specific survival in patients with Gleason score 7 grouped as no 8q gain ($MYC/CEP18 < 1.5$), relative 8q gain but no amplification ($MYC/CEP18 \geq 1.5 < 2$), and 8q amplification ($MYC/CEP18 \geq 2$) (C).

relative 8q gain, and we here show that the latter displayed worse disease-specific survival. In the present study, relative 8q gain was found in 48% of PCa patients, which is within the range of 12.8 to 54% reported in previous studies (Miyoshi et al., 2000; Saramäki et al., 2001; Qian et al., 2002; Tsuchiya et al., 2002). We conclusively demonstrate that patients with relative 8q gain have significantly lower 10-year disease-specific survival than those without this genetic aberration and that this prognostic value is independent of the standard clinical variables GS and CS. These data confirm our previous reports (Ribeiro et al., 2006, 2007) and reports by other authors (Saramäki et al., 2001) stressing the importance of relative 8q gain as an independent predictor of poor survival in PCa patients. Interestingly, we show for the first time that 8q gain predicts death by

disease irrespective of *TMPRSS2-ERG* status, indicating that, whereas ETS fusion genes are the primary genetic events in a large subset of prostate carcinomas, secondary genetic features like relative 8q gain may be the most clinically relevant prognostic factors in this disease.

In contrast to *PTEN* deletion, which tends to be associated with *TMPRSS2-ERG* (Reid et al., 2010) in the same tumors, we here show that 8q gain is not associated with the fusion gene, indicating that 8q gain is a prognostically relevant, secondary genetic alteration in PCa independent of the genetic pathway in which ETS fusions are the primary events. Furthermore, we show that the use of *ERG* copy number as ploidy control also allows discrimination of PCa with poor prognosis associated with relative 8q gain. Although originating similar classifications in most cases,

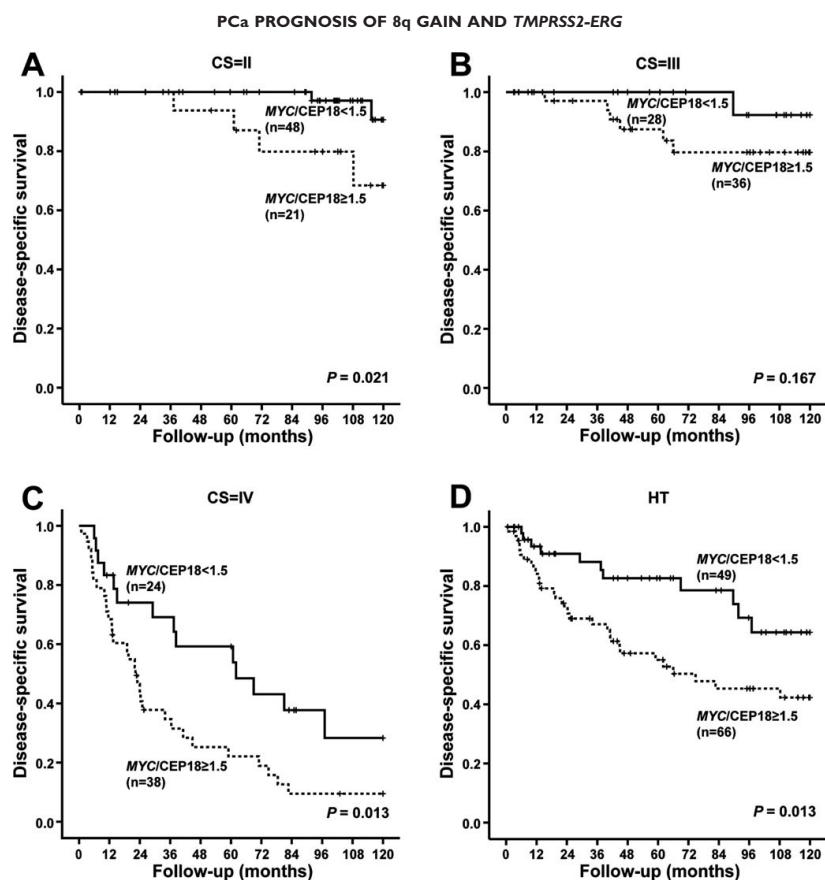


Figure 4. Kaplan–Meier curves illustrating disease-specific survival according to the presence (*MYC/CEP18* ≥ 1.5) or absence (*MYC/CEP18* < 1.5) of relative 8q gain in patients with clinical stage II (A), III (B), or IV (C) as well as in the subgroup of prostate cancer patients treated only with hormone therapy (HT) (D).

TABLE 2. Cox Regression Analysis of Clinical and Genetic Variables

	HR	95% CI	P value
PSA ≥ 30 (ng/ml)	1.97	0.89–4.37	0.095
Tumor content (≥50%)	0.63	0.36–1.13	0.119
CS III versus CS II	0.71	0.22–2.32	0.567
CS IV versus CS II	10.05	3.55–28.48	<0.001
GS = 7 versus GS < 7	2.14	0.26–17.92	0.482
GS > 7 versus GS < 7	4.208	0.50–35.58	0.187
<i>TMPRSS2-ERG</i> +	1.32	0.76–2.29	0.327
<i>MYC/CEP18</i> ≥ 1.5	4.00	2.19–7.30	<0.001

HR, hazard ratio; CI, confidence interval.

the use of the two methods for determining relative 8q gain allows discrimination of groups of PCa patients with poor, favorable, and intermediate disease-specific survival, the latter representing the patients with different risk group by each

method (Supporting Information Fig. 3). This emphasizes our conclusion that good ploidy controls are essential to identify the poor prognostic PCa associated with relative 8q gain. The most relevant target genes of 8q gain remain unknown, but *EIF3S3*, *TCEB1*, *EIF3S3*, *BOP1*, and *RAD21* have been described as being amplified and over-expressed in PCa (Nupponen et al., 1999; Sarimäki et al., 2001; Jalava et al., 2009). Given our findings of structural rearrangements and some reported data (Gurel et al., 2008), *MYC* itself might be relevant in prostate carcinogenesis.

The 200 patients we present here received different treatment modalities, and so it is important to evaluate if the prognostic value is influenced by the therapy. Considering patients treated only with HT, relative 8q gain identified a subset of patients with worse disease-specific survival.

Despite the reduced frequency of 8q gain in the fewer patients treated solely with RP or RT, also these presented lower disease-specific survival when their tumors displayed *MYC*/CEP18 ≥ 1.5 . Altogether, these data indicate that 8q gain has prognostic value independently from treatment modality. On the other hand, the *TMPRSS2-ERG* gene fusion was not associated with disease-specific survival in HT treated patients, which is agreement with previous reports (Boormans et al., 2010; Leinonen et al., 2010) nor with any other treatment subset. Recently, El Gammal et al. (2010) found association of 8q gain with BR in patients treated by RP, but multivariate analysis showed no independent predictive effect. As previously discussed (Narod et al., 2008), we consider that the prognostic value of a given PCa genetic biomarker must be evaluated with disease-specific survival instead of recurrence-free survival, as there is evidence in the literature that only a minority of patients with BR will succumb to PCa (Ward et al., 2003; Porter et al., 2006).

CONCLUSION

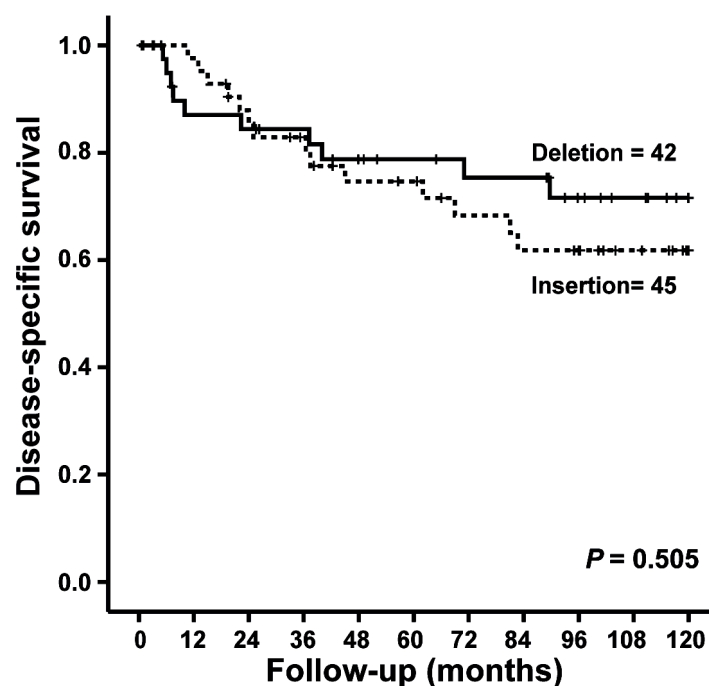
We conclude that relative 8q gain in diagnostic needle biopsies predicts poor disease-specific survival independently of the presence of the *TMPRSS2-ERG* fusion gene, standard clinicopathologic variables, and treatment modalities. Because this genetic marker can be evaluated in a prostate needle biopsy before the delineation of the therapeutic plan, it has the potential to improve the clinical management of PCa patients.

REFERENCES

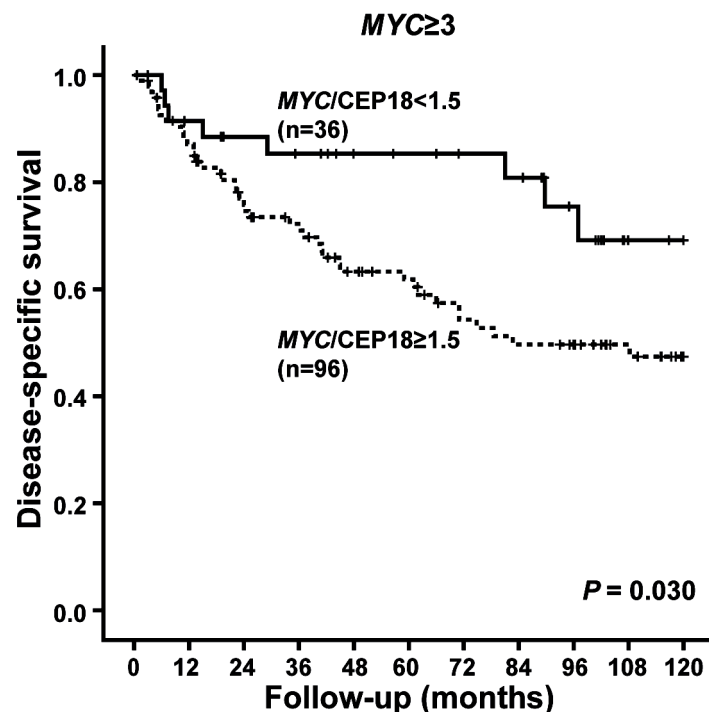
- Ameling CL, Blute ML, Bergstralh EJ, Seay TM, Slezak J, Zineke H. 2000. Long-term hazard of progression after radical prostatectomy for clinically localized prostate cancer: Continued risk of biochemical failure after 5 years. *J Urol* 164:101–105.
- Attard G, Clark J, Ambrosino L, Fisher G, Kovacs G, Flohr P, Berney D, Foster CS, Fletcher A, Gerald WL, Moller H, Reuter V, De Bono JS, Scardino P, Cuzick J, Cooper CS. 2008. Duplication of the fusion of *TMPRSS2* to *ERG* sequences identifies fatal human prostate cancer. *Oncogene* 27:253–263.
- Boormans JL, Hermans KG, Made AC, van Leenders GJ, Wildhagen MF, Collette L, Schroder FH, Trapman J, Verhagen PC. 2010. Expression of the androgen-regulated fusion gene *TMPRSS2-ERG* does not predict response to endocrine treatment in hormone-naïve, node-positive prostate cancer. *Eur Urol* 57:830–835.
- Cerveira N, Ribeiro F, Peixoto A, Costa V, Henrique R, Jerónimo C, Teixeira M. 2006. *TMPRSS2-ERG* gene fusion causing *ERG* overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions. *Neoplasia* 8:826–832.
- Demicheli F, Fall K, Perner S, Andrén O, Schmidt F, Setlur SR, Hoshida Y, Mosquera J-M, Pawitan Y, Lee C, Adami H-O, Mucci LA, Kantoff PW, Andersson S-O, Chinnaiyan AM, Johansson J-E, Rubin MA. 2007. *TMPRSS2:ERG* gene fusion associated with lethal prostate cancer in a watchful waiting cohort. *Oncogene* 26:4596–4599.
- El Gammal AT, Bruchmann M, Zustin J, Isbarn H, Hellwinkel OJC, Köllermann J, Sauter G, Simon R, Wilczak W, Schwarz J, Bokemeyer C, Brümmendorf TH, Izbicki JR, Yekebas E, Fisch M, Huland H, Graefen M, Schlomm T. 2010. Chromosome 8p deletions and 8q gains are associated with tumor progression and poor prognosis in prostate cancer. *Clin Cancer Res* 16:56–64.
- Fitzgerald LM, Agalliu I, Johnson K, Miller MA, Kwon EM, Hurtado-Coll A, Fazli L, Rajput AB, Gleave ME, Cox ME, Ostrander EA, Stanford JL, Huntsman DG. 2008. Association of *TMPRSS2-ERG* gene fusion with clinical characteristics and outcomes: Results from a population-based study of prostate cancer. *BMC Cancer* 8:230.
- Freedland SJ, Humphreys EB, Mangold LA, Eisenberger M, Dorey FJ, Walsh PC, Partin AW. 2005. Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy. *J Am Med Assoc* 294:433–439.
- Furusato B, Gao C-L, Ravindranath L, Chen Y, Cullen J, McLeod DG, Dobi A, Srivastava S, Petrovics G, Sesterhenn IA. 2008. Mapping of *TMPRSS2-ERG* fusions in the context of multifocal prostate cancer. *Mod Pathol* 21:67–75.
- Gopalan A, Leversha MA, Satagopan JM, Zhou Q, Al-Ahmadie HA, Fine SW, Eastham JA, Scardino PT, Scher HI, Tickoo SK, Reuter VE, Gerald WL. 2009. *TMPRSS2-ERG* gene fusion is not associated with outcome in patients treated by prostatectomy. *Cancer Res* 69:1400–1406.
- Gurel B, Iwata T, Koh C M, Jenkins RB, Lan F, Van Dang C, Hicks JL, Morgan J, Cornish TC, Sutcliffe S, Isaacs WB, Luo J, De Marzo AM. 2008. Nuclear *MYC* protein overexpression is an early alteration in human prostate carcinogenesis. *Mod Pathol* 21:1156–1167.
- Han M, Partin AW, Pound CR, Epstein JI, Walsh PC. 2001. Long-term biochemical disease-free and cancer-specific survival following anatomic radical retropubic prostatectomy. The 15-year Johns Hopkins experience. *Urol Clin North Am* 28:555–565.
- Hermans KG, Van Marion R, Van Dekken H, Jenster G, Van Weerden WM, Trapman J. 2006. *TMPRSS2:ERG* fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. *Cancer Res* 66:10658–10663.
- Hull GW, Rabbani F, Abbas F, Wheeler TM, Kattan MW, Scardino PT. 2002. Cancer control with radical prostatectomy alone in 1,000 consecutive patients. *J Urol* 167:528–534.
- Isariyawongse BK, Sun L, Banez LL, Robertson C, Polascik TJ, Maloney K, Donatucci C, Albala D, Mouraviev V, Madden JF, Moul JW. 2008. Significant discrepancies between diagnostic and pathologic Gleason sums in prostate cancer: The predictive role of age and prostate-specific antigen. *Urology* 72:882–886.
- Jalava SE, Porkka KP, Rauhala HE, Isotalo J, Tammela TL, Visakorpi T. 2009. *TCEB1* promotes invasion of prostate cancer cells. *Int J Cancer* 124:95–102.
- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. 2009. Cancer statistics, 2009. *CA Cancer J Clin* 59:225–249.
- King JC, Xu J, Wongvipat J, Hieronymus H, Carver BS, Leung DH, Taylor BS, Sander C, Cardiff RD, Couto SS, Gerald WL, Sawyers CL. 2009. Cooperativity of *TMPRSS2-ERG* with *PI3-kinase* pathway activation in prostate oncogenesis. *Nat Genet* 41:524–526.
- Klezovitch O, Risk M, Coleman I, Lucas JM, Null M, True LD, Nelson PS, Vasioukhin V. 2008. A causal role for *ERG* in neoplastic transformation of prostate epithelium. *Proc Natl Acad Sci USA* 105:2105–2110.
- Lapointe J, Kim YH, Miller MA, Li C, Kaygusuz G, Van De Rijn M, Huntsman DG, Brooks JD, Pollack JR. 2007. A variant *TMPRSS2* isoform and *ERG* fusion product in prostate cancer with implications for molecular diagnosis. *Mod Pathol* 20:467–473.
- Leinonen KA, Tolonen TT, Bracken H, Stenman UH, Tammela TL, Saramäki OR, Visakorpi T. 2010. Association of *SPINK1* expression and *TMPRSS2:ERG* fusion with prognosis in endocrine-treated prostate cancer. *Clin Cancer Res* 16:2845–2851.
- Mehra R, Tomlins SA, Shen R, Nadeem O, Wang L, Wei JT, Pienta KJ, Ghosh D, Rubin MA, Chinnaiyan AM, Shah RB. 2007. Comprehensive assessment of *TMPRSS2* and *ETS* family gene aberrations in clinically localized prostate cancer. *Mod Pathol* 20:538–544.
- Miyoshi Y, Uemura H, Fujinami K, Mikata K, Harada M, Kitamura H, Koizumi Y, Kubota Y. 2000. Fluorescence in situ hybridization evaluation of *c-myc* and androgen receptor gene amplification and chromosomal anomalies in prostate cancer in Japanese patients. *Prostate* 43:225–232.

- Mosquera J-M, Mehra R, Regan MM, Perner S, Genega EM, Bueti G, Shah RB, Gaston S, Tomlins SA, Wei JT, Kearney MC, Johnson LA, Tang JM, Chinnaiyan AM, Rubin MA, Sanda MG. 2009. Prevalence of TMPRSS2-ERG fusion prostate cancer among men undergoing prostate biopsy in the United States. *Clin Cancer Res* 15:4706–4711.
- Nam RK, Sugar L, Yang W, Srivastava S, Klotz LH, Yang L-Y, Stanimirovic A, Encioiu E, Neill M, Loblaw DA, Trachtenberg J, Narod SA, Seth A. 2007. Expression of the TMPRSS2:ERG fusion gene predicts cancer recurrence after surgery for localised prostate cancer. *Br J Cancer* 97:1690–1695.
- Narod SA, Seth A, Nam R. 2008. Fusion in the ETS gene family and prostate cancer. *Br J Cancer* 99:847–851.
- Nupponen NN, Porkka K, Kakkola L, Tanner M, Persson K, Borg A, Isola J, Visakorpi T. 1999. Amplification and overexpression of p40 subunit of eukaryotic translation initiation factor 3 in breast and prostate cancer. *Am J Pathol* 154:1777–1783.
- Paris PL, Albertson DG, Alers JC, Andaya A, Carroll P, Fridlyand J, Jain AN, Kamkar S, Kowbel D, Krijtenburg PJ, Pinkel D, Schroder FH, Vissers KJ, Watson VJ, Wildhagen MF, Collins C, Van Dekken H. 2003. High-resolution analysis of paraffin-embedded and formalin-fixed prostate tumors using comparative genomic hybridization to genomic microarrays. *Am J Pathol* 162:763–770.
- Paris PL, Andaya A, Fridlyand J, Jain AN, Weinberg V, Kowbel D, Brebner JH, Simko J, Watson JE, Volik S, Albertson DG, Pinkel D, Alers JC, van der Kwast TH, Vissers KJ, Schroder FH, Wildhagen MF, Febbo PG, Chinnaiyan AM, Pienta KJ, Carroll PR, Rubin MA, Collins C, van Dekken H. 2004. Whole genome scanning identifies genotypes associated with recurrence and metastasis in prostate tumors. *Hum Mol Genet* 13:1303–1313.
- Perner S, Demicheli F, Beroukhi R, Schmidt FH, Mosquera JM, Setlur S, Tchinda J, Tomlins SA, Hofer MD, Pienta KG, Kuefer R, Vessella R, Sun XW, Meyerson M, Lee C, Sellers WR, Chinnaiyan AM, Rubin MA. 2006. TMPRSS2:ERG fusion-associated deletions provide insight into the heterogeneity of prostate cancer. *Cancer Res* 66:8337–8341.
- Porter CR, Kodama K, Gibbons RP, Correa R, Jr., Chun FK, Perrotte P, Karakiewicz PI. 2006. 25-year prostate cancer control and survival outcomes: A 40-year radical prostatectomy single institution series. *J Urol* 176:569–574.
- Prensner JR, Chinnaiyan AM. 2009. Oncogenic gene fusions in epithelial carcinomas. *Curr Opin Genet Dev* 19:82–91.
- Qian J, Hirasawa K, Bostwick DG, Bergstralh EJ, Slezak JM, Anderl KL, Borell TJ, Lieber MM, Jenkins RB. 2002. Loss of p53 and c-myc overrepresentation in stage T₂₋₃N₁₋₃M₀ prostate cancer are potential markers for cancer progression. *Mod Pathol* 15:35–44.
- Reid AH, Attard G, Ambrosini L, Fisher G, Kovacs G, Brewer D, Clark J, Flohr P, Edwards S, Berney DM, Foster CS, Fletcher A, Gerald WL, Moller H, Reuter VE, Scardino PT, Cuzick J, de Bono JS, Cooper CS. 2010. Molecular characterisation of ERG, ETV1 and PTEN gene loci identifies patients at low and high risk of death from prostate cancer. *Br J Cancer* 102:678–684.
- Ribeiro FR, Henrique R, Martins AT, Jerónimo C, Teixeira MR. 2007. Relative copy number gain of MYC in diagnostic needle biopsies is an independent prognostic factor for prostate cancer patients. *Eur Urol* 52:116–125.
- Ribeiro FR, Jerónimo C, Henrique R, Fonseca D, Oliveira J, Lothe RA, Teixeira MR. 2006. 8q gain is an independent predictor of poor survival in diagnostic needle biopsies from prostate cancer suspects. *Clin Cancer Res* 12:3961–3970.
- Rochl KA, Han M, Ramos CG, Antenor JA, Catalona WJ. 2004. Cancer progression and survival rates following anatomical radical retropubic prostatectomy in 3,478 consecutive patients: Long-term results. *J Urol* 172:910–914.
- Rubio-Briones J, Fernandez-Serra A, Calatrava A, Garcia-Casado Z, Rubio L, Bonillo MA, Iborra I, Solsona E, Lopez-Guerrero JA. 2010. Clinical implications of TMPRSS2-ERG gene fusion expression in patients with prostate cancer treated with radical prostatectomy. *J Urol* 183:2054–2061.
- Saramäki O, Willi N, Bratt O, Gasser TC, Koivisto P, Nupponen NN, Bubendorf L, Visakorpi T. 2001. Amplification of EIF3S3 gene is associated with advanced stage in prostate cancer. *Am J Pathol* 159:2089–2094.
- Saramäki OR, Harjula AE, Martikainen PM, Vessella RL, Tammele TLJ, Visakorpi T. 2008. TMPRSS2:ERG fusion identifies a subgroup of prostate cancers with a favorable prognosis. *Clin Cancer Res* 14:3395–3400.
- Sardana G, Dowell B, Diamandis EP. 2008. Emerging biomarkers for the diagnosis and prognosis of prostate cancer. *Clin Chem* 54:1951–1960.
- Soller MJ, Isaksson M, Elfving P, Soller W, Lundgren R, Panagopoulos I. 2006. Confirmation of the high frequency of the TMPRSS2/ERG fusion gene in prostate cancer. *Genes Chromosomes Cancer* 45:717–719.
- Teixeira MR. 2008. Chromosome mechanisms giving rise to the TMPRSS2-ERG fusion oncogene in prostate cancer and HGPIN lesions. *Am J Surg Pathol* 32:642–644.
- Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun X-W, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM. 2005. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 310:644–648.
- Tsuchiya N, Slezak JM, Lieber MM, Bergstralh EJ, Jenkins RB. 2002. Clinical significance of alterations of chromosome 8 detected by fluorescence in situ hybridization analysis in pathologic organ-confined prostate cancer. *Genes Chromosomes Cancer* 34:363–371.
- van Dekken H, Paris PL, Albertson DG, Alers JC, Andaya A, Kowbel D, van der Kwast TH, Pinkel D, Schroder FH, Vissers KJ, Wildhagen MF, Collins C. 2004. Evaluation of genetic patterns in different tumor areas of intermediate-grade prostatic adenocarcinomas by high-resolution genomic array analysis. *Genes Chromosomes Cancer* 39:249–256.
- Vieira J, Henrique R, Ribeiro FR, Barros-Silva JD, Peixoto A, Santos C, Pinheiro M, Costa VL, Soares MJ, Oliveira J, Jerónimo C, Teixeira MR. 2010. Feasibility of differential diagnosis of kidney tumors by comparative genomic hybridization of fine needle aspiration biopsies. *Genes Chromosomes Cancer* 49:935–947.
- Ward JF, Blute ML, Slezak J, Bergstralh EJ, Zincke H. 2003. The long-term clinical impact of biochemical recurrence of prostate cancer 5 or more years after radical prostatectomy. *J Urol* 170:1872–1876.
- Yoshimoto M, Joshua AM, Chilton-Macneill S, Bayani J, Selvarajah S, Evans AJ, Zielenska M, Squire JA. 2006. Three-Color FISH analysis of TMPRSS2/ERG fusions in prostate cancer indicates that genomic microdeletion of chromosome 21 is associated with rearrangement. *Neoplasia* 8:465–469.
- Yoshimoto M, Joshua AM, Cunha IW, Coudry RA, Fonseca FP, Ludkovski O, Zielenska M, Soares FA, Squire JA. 2008. Absence of TMPRSS2:ERG fusions and PTEN losses in prostate cancer is associated with a favorable outcome. *Mod Pathol* 21:1451–1460.

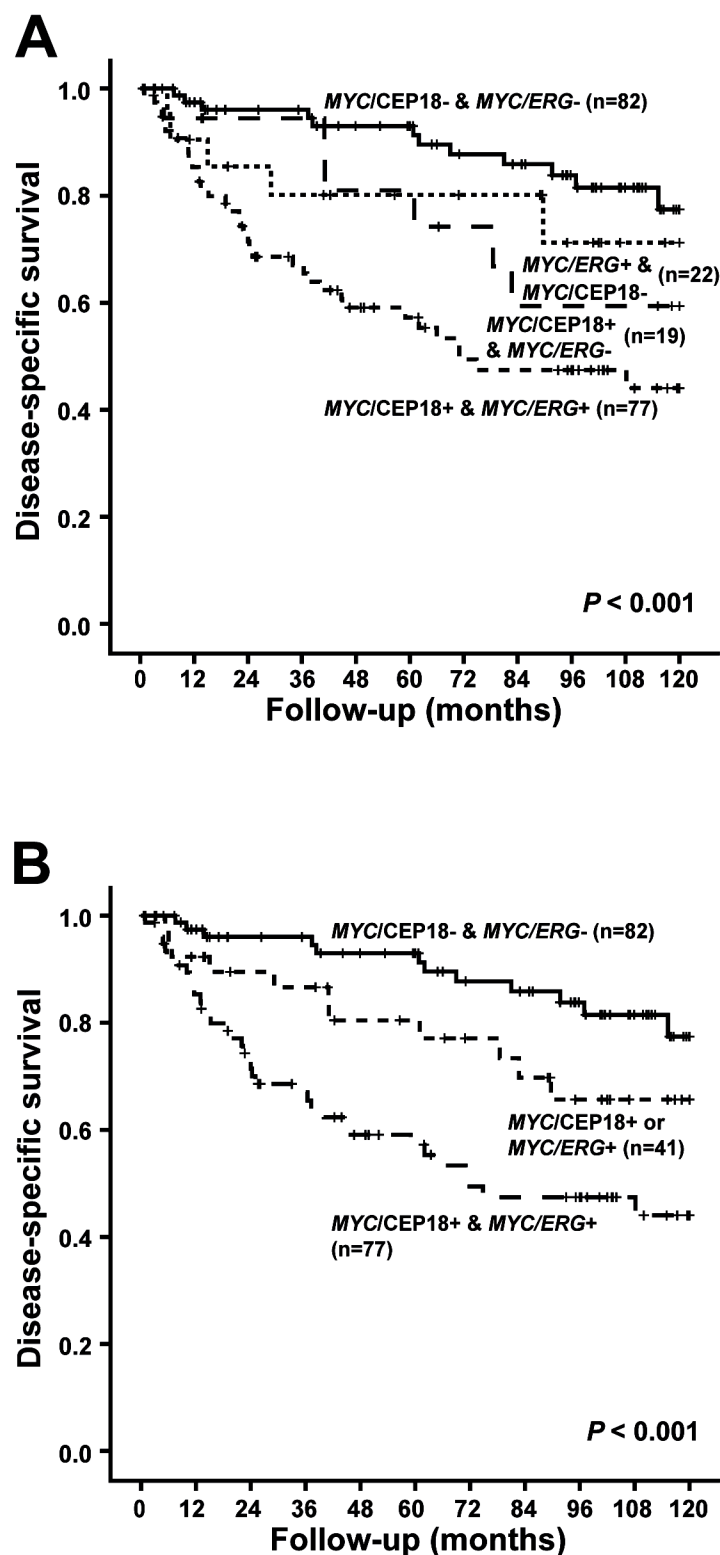
Supporting information



Supplementary Figure 1. Kaplan-Meier curve illustrating disease-specific survival in prostate carcinoma patients displaying *TMPRSS2-ERG* rearrangement through deletion and insertion.



Supplementary Figure 2. Kaplan-Meier curve illustrating disease-specific survival in prostate carcinoma patients with increased *MYC* copy number according to *MYC*/CEP18 ratio.



Supplementary Figure 3. Kaplan-Meier curves illustrating disease-specific survival in prostate carcinoma patients grouped according to the combination of relative 8q gain status computed using CEP18 and ERG copy number, considering *MYC*/CEP18+ & *MYC*/ERG- and *MYC*/CEP18- & *MYC*/ERG+ patients separately (A) or in the same group (B).

Chapter 6

General Discussion

GENERAL DISCUSSION

Chromosomal alterations such as structural rearrangements and copy number aberrations are an important feature of human cancer. The seminal discovery in this field was made in chronic myelogenous leukemia (CML), where classical cytogenetic techniques revealed a small marker chromosome, called the Philadelphia chromosome (Nowell and Hungerford, 1960), latter shown to arise from a reciprocal translocation between the long arms of chromosomes 9 and 22, $t(9;22)(q34;q11)$. This led to the discovery of the recombination of *BCR* (from 22q11) and *ABL1* (from 9q34) genes to a novel *BCR-ABL1* fusion gene that produces a new chimeric protein with abnormal tyrosine kinase properties, identified as the driving force in CML (Daley et al., 1990; Lugo et al., 1990). By establishing a causal link between a specific chromosomal lesion and a specific neoplasia, *BCR-ABL1* also pioneered cancer therapy: the tyrosine kinase inhibitor imatinib (Gleevec) was introduced as the first widely used therapy targeting a fusion gene (Druker et al., 2001). Similar discoveries led to the characterization of numerous gene fusions in other hematological malignancies (including Burkitt's lymphoma, T-cell lymphomas, and acute promyelocytic leukemia) and in soft tissue tumors. Ewing's sarcoma, included in the latter, is characterized by harboring tumor-specific gene fusions involving members of the TET (TLS/EWS/TAF15) family of RNA-binding proteins with ETS transcription factors (reviewed by (Sankar and Lessnick, 2011), being the *EWSR1-FLI1* fusion the most frequent (present in 85% of cases). Although this type of gene rearrangement has long been recognized as a fundamental pathogenic mechanism in hematological malignancies and soft-tissue tumors, it has until recently only rarely been described in the common carcinomas (reviewed by (Prensner and Chinnaiyan, 2009), which are responsible for about 80% of the human cancer burden (Look, 1997; Mitelman et al., 2004; Mitelman et al., 2005). The finding of gene fusions involving ETS family genes in prostate carcinomas constituted the first time this type of genomic aberration was commonly found (more than 50% of all cases) in a highly prevalent epithelial tumor.

The advent of molecular cytogenetics, specifically comparative genomic hybridization technologies, enabled great advances in the knowledge of prostate cancer (Kallioniemi et al., 1994; Kraus et al., 2003; Teixeira et al., 2004; Ribeiro et al., 2006). This methodology detects genomic imbalances in tumor cells pinpointing specific DNA gains and/or losses within chromosomes, providing a

whole-genome analysis in a single experiment. Among the most commonly observed deletions (8p, 16q, 5q, 13q and 6q) and gains (8q and 7q) in prostate tumors, gain at 8q has been shown to have prognostic value (Ribeiro et al., 2006; Ribeiro et al., 2007).

The work presented in this thesis aimed to clarify relevant questions that arose subsequently to the discovery of ETS fusion genes in prostate carcinomas. The results obtained will be integrated in two major discussion topics, one more focused on the molecular biology of ETS rearrangements and the other concerning its clinical relevance combined with other chromosomal aberrations, namely 8q gains.

1. Characterization of ETS rearrangements in prostate cancer

An effective and timely search for genomic rearrangements knowing at least one of the genes involved can be performed by molecular cytogenetic or molecular genetic approaches. FISH analysis in whole-tumor sections provides unequivocal evidence of genomic rearrangements in tumor cells, allowing also estimation of its frequency and distinguishing its presence in different tumor foci. However, the analysis of whole-tumor sections of a large series can be a very time-consuming and expensive task. A PCR-based approach, on the other hand, provides high-throughput data of specific transcripts in a timely fashion. One should, however, not neglect careful dissection of the tumor area to be effective. In order to perform a comprehensive characterization of a cohort of 200 consecutive prostate carcinomas regarding ETS rearrangement status, we combined the strategies mentioned above taking advantage of the strong points and minimizing the drawbacks of each method.

1.1. Identification of known ETS fusion genes

As an initial screening, we opted for the assessment of expression levels of a panel of selected ETS genes (**Paper I**), a strategy supported on the evidence that rearranged ETS genes display abnormally high expression levels (Tomlins et al., 2005). In fact, the discovery of the first ETS rearrangements in prostate carcinomas was based on outlier expression analysis (Tomlins et al., 2005). Thus, we initially performed the high-throughput real-time PCR-based Taqman Low Density Array (TLDA) to assess the expression levels of ETS genes known to be

involved in rearrangements in prostate carcinomas (*ERG*, *ETV1*, *ETV4*, and *ETV5*) and/or in other tumors (*FLI1*, *FEV* and *ETV6*). Two other ETS genes were included in the panel, namely *ELK4* for being reported as overexpressed in a subset of PCa (Rickman et al., 2009), and *ETS2* for its localization in 21q22.2, between *TMPRSS2* and *ERG*. Additionally, a probe targeting the most common *TMPRSS2-ERG* fusion transcript variant (type III) was included. This last probe was particularly useful for the typing of *ERG* rearrangements, particularly for defining the 5' partner of *ERG*. As expected, given the predicted high frequency of *ERG* rearrangements, the distribution of *ERG* expression did not reveal cases with extreme outlier expression. Thus, tumors were sorted by the threshold cycle (Ct *ERG*) for *ERG* expression, revealing a bimodal distribution, and FISH was used to clarify the rearrangement status in samples with intermediate *ERG* expression and in sample with high *ERG* expression but low *TMPRSS2-ERG* type III expression. Due to its specificity, FISH was the gold standard for defining samples as positive or negative for *ERG* rearrangement, either with *TMPRSS2* or other 5' fusion partner. Of the 200 PCa, *TMPRSS2-ERG* and *SLC45A3-ERG* were found in 99 and 5 tumors, respectively. Given that one patient harbored both gene fusions (see below), *ERG* rearrangements were found in 51.5% of patients, which is in keeping with previous studies of patients from western countries (Clark and Cooper, 2009). The frequency of *ERG* rearrangement found in an independent cohort of 200 diagnostic biopsies with PCa (**Paper III**) was somewhat lower (43.5%), although the difference is not statistically significant ($P = 0.109$). Given that there are no differences in patient ethnicity, two major reasons may explain this difference: first, tumor heterogeneity may be underlying these differences due to lower tumor content sampled in biopsy specimens when compared to radical prostatectomy specimens; second, the difference may be originated by the case selection criteria, as one series is made of consecutive diagnostic biopsies and the other a consecutive series of prostatectomy specimens.

Regarding the other ETS genes known to be involved in PCa, analysis of the TLDA data revealed extreme outlier expression of *ETV1* in 16 cases, of *ETV4* in four cases and *ETV5* in one case. Using a FISH break-apart probe strategy with BAC probes flanking the overexpressed ETS gene, we demonstrated genomic rearrangement in 14 of the 16 cases with *ETV1* overexpression, which represents 7% of all PCa. Genomic rearrangement was also confirmed in three of the four PCa with *ETV4* overexpression and in the single PCa showing *ETV5* overexpression, representing a frequency of 1.5% and 0.5%, respectively. For the

remaining three tumors (two with *ETV1* overexpression and one with *ETV4* overexpression) we did not find evidence of genomic rearrangement by FISH. Other authors have reported similar observations before (Hermans et al., 2006; Tu et al., 2007; Hermans et al., 2008). Altered regulatory mechanisms can be driving the aberrant ETS expression in these tumors, but it is also possible that genomic rearrangements involve a small part of a prostate-specific gene or a strong promoter, as described by Helgeson and co-workers, which may be below the resolution level of the FISH strategy used (Helgeson et al., 2008).

Further approaches to identify the respective 5' fusion partners involved in the rearrangements with *ETV1*, *ETV4* and *ETV5* are discussed below.

1.2. Identification of novel ETS genes involved in rearrangements

As previously stated, apart from the ETS genes known to be involved in PCa, the expression of *ETV6*, *ELK4*, *ETS2*, *FEV* and *FLI1* was also assessed in the cohort of 200 clinically localized PCa. Interestingly, one sample displayed extreme outlier expression of *FLI1* and further FISH break-apart assays showed genomic rearrangement of this ETS gene (**Paper I**). Given that *FLI1* is a member of the same ETS subfamily as *ERG*, the most common *ERG* fusion partners were tested by FISH analysis, and *SLC45A3* was found to be the 5' fusion partner. The involvement of *FLI1* in gene fusions is well known in Ewing sarcoma, where the *EWSR1-FLI1* gene fusion is found in 85% of the cases (Sankar and Lessnick, 2011). The resulting chimeric protein in Ewing sarcoma is a potent transcriptional activator due to the transactivation domain of *EWSR1* and the DNA-binding domain of *FLI1* (Welford et al., 2001; Uren et al., 2004; Uren and Toretzky, 2005). On the other hand, our finding strongly indicates that the *SLC45A3-FLI1* fusion in prostate cancer results in *FLI1* overexpression, as exon-level quantitative RT-PCR confirmed 5'/3' differential *FLI1* exon expression. Qualitative RT-PCR followed by sequencing demonstrated the presence of *SLC45A3-FLI1* fusion gene, in which the untranslated *SLC45A3* exon 1 is fused with *FLI1* exon 3. The here described novel *SLC45A3-FLI1* fusion gene is therefore predicted to result in overexpression of full length *FLI1* and not a chimeric protein. In most of the ETS fusions so far described in PCa rearrangements, the genomic sequences of the 5' partner gene corresponds to untranslated regions (Clark and Cooper, 2009), being the promoter region of the 5' partner gene the most likely driver of ETS overexpression. Considering that *FLI1* and *ERG* display 98% sequence identity in

the DNA binding domain (Laudet et al., 1999; Hollenhorst et al., 2011), it is expected that, in the same cellular and tissue context, these two transcription factors share a high proportion of target genes, thus playing similar roles in prostate carcinogenesis. Moreover, in Ewing sarcoma it has been described that *EWSR1-FLI1* and *EWSR1-ERG* chimeric transcription factors have similar target genes (Ginsberg et al., 1999; Im et al., 2000). This interesting finding is also a rewarding validation of the strategy followed to identify novel ETS fusion genes.

Putative rearrangements with other ETS genes would most likely be of low frequency. The TLDA-based approach made possible a fast and reliable candidate ETS gene expression assessment, making it feasible to include a large number of patients in this study. In fact, *FLI1* rearrangement was found in one out of 200 patients. Han and co-workers (2008) have previously searched for novel ETS genes involved in rearrangements. These authors used a comprehensive fluorescence in situ hybridization split probe strategy interrogating all 27 ETS family members using a tissue microarray composed by 110 clinically localized prostate cancer patients (Han et al., 2008). Apart from *ERG* (43%), *ETV1* (5%), and *ETV4* (5%), no other ETS gene showed evidence of rearrangement. Although FISH is a reliable method for detection of genomic rearrangements, the use of TMA as initial screening has some drawbacks. Due to the documented heterogeneity of PCa, the number of cores included per sample may be insufficient to detect alterations that may be confined to limited tumor foci (Clark et al., 2008). Another aspect is the relatively low efficiency of performing FISH on TMAs due to the number of non-analyzable tumors associated with technical limitations (FitzGerald et al., 2008).

1.3. Identification of novel fusion partners in ETS rearrangements

In order to look for novel 5' fusion partners in ETS rearrangements we focused on the subset of 18 tumors with *ETV1*, *ETV4* or *ETV5* outlier overexpression and evidence of rearrangement by FISH. Exon-level expression data available for 14 of the 18 samples confirmed ETS overexpression compared to samples with no rearrangement. Six tumors displayed differential exon expression pinpointing the breakpoint while the remaining displayed whole-gene overexpression. Initial RT-PCR screening identified the fusion partners in two samples, one with the *TMPRSS2-ETV1* fusion and other with the *SLC45A3-ETV4* fusion. The latter had never been described as an *ETV4* partner. Notwithstanding

these findings, we searched for potential fusion partners using 5'RACE in all samples. Sequencing of the 5'RACE PCR products unveiled *OR51E2* and *UBTF* as novel 5' fusion partners of *ETV1* and *ETV4*, respectively (**Paper II**). These two genes have dissimilar characteristics. The olfactory receptor, family 51, subfamily E, member 2 (*OR51E2*) displays a highly prostate-specific expression and is overexpressed in prostate tumors (Xu et al., 2000; Weng et al., 2005b; Xu et al., 2006). Our observations showed that *OR51E2* differs from other known prostate-specific fusion partners because it is neither induced (like *TMPRSS2*) nor repressed (like *C15orf21*) by androgens. Most likely resulting from a translocation involving the chromosome sub-bands 11p15.4 and 7p21.2, the fusion transcript combines a truncated *ETV1* transcript with the untranslated exon 1 of *OR51E2*, which contains a promoter region not responsive to the synthetic androgen R1881 (Weng et al., 2005a).

The upstream binding transcription factor (*UBTF*) encodes a member of the HMG-box DNA-binding protein family (Jones et al., 1995; Matera et al., 1997) and displays a ubiquitous expression pattern. The *in silico* analysis performed showed that this gene is not androgen-regulated and is not overexpressed in PCa. FISH analysis provided evidence of a deletion between *UBTF* and *ETV4*, which are about 700kb apart in the long arm of chromosome 17, being the likely mechanism underlying the rearrangement. The *UBTF-ETV4* fusion transcript found encodes a putative chimeric protein containing 19 amino acids from *UBTF* exon 2 and a N-truncated *ETV4* transcript. As described above, this 5' fusion partner does not display the *TMPRSS2*-like characteristics (i.e. androgen-induced, prostate-specific). Instead, similarly to *DDX5* (Tomlins et al., 2007; Han et al., 2008) and *HNRPA2B1* (Tomlins et al., 2007), *UBTF* has an androgen-irresponsive ubiquitously expression pattern. Interestingly, the proteins that these genes encode display either RNA-binding (*DDX5*, *HNRPA2B1*) or DNA-binding properties (*UBTF*) and all these ETS-fusion transcripts originate chimerical proteins.

The identification of two novel gene fusion combinations involving previously described genes (*SLC45A3-ETV4* and *HERVK17-ETV4*) contributes to further understanding of ETS rearrangements in PCa. Up until now, *TMPRSS2* and *SLC45A3* were the only 5' partners found fused with different ETS genes, whereas the remaining were specific to a given ETS gene. Our findings suggest that not only there may be more 5' partner genes yet to be identified in PCa, but also that all the genes described as 5' fusion partners with one ETS gene can most likely be rearranged with any of the other ETS genes known to be involved in PCa.

The 11 samples where no fusion partner was found using RT-PCR and 5'RACE included 10 tumors with evidence of *ETV1* rearrangement and one with evidence of *ETV5* rearrangement. Considering the latter, despite the strong evidence provided by exon-level expression indicating differential *ETV5* exon expression of exons 1 to 5 compared to exons 6 to 13, neither 5'RACE nor FISH testing of the known fusion partners allowed the identification of the putative 5' fusion partner. Regarding the samples with positive break-apart FISH assays for *ETV1*, we also sought potential fusion partners with specific FISH assays. With this approach we were able to identify two cases with the 14q13.3-q21.1-*ETV1* gene fusion. Of the remaining 8 tumors with no fusion partner found, exon-level expression data was available in six cases, of which one displayed 5'/3' differential expression and five showed overexpression of full-length *ETV1* transcripts. Similar results have been previously described (Gasi et al., 2011), pinpointing that whole *ETV1* overexpression might also have a role in prostate carcinogenesis.

1.4. Intratumor heterogeneity of ETS gene fusions

The thorough characterization of the ETS rearrangements present in the 200 prostatectomy specimens (**Paper I** and **Paper II**) revealed an interesting feature in some tumors, providing further insights on prostate cancer clonal heterogeneity. We observed three tumors displaying overexpression of different ETS genes, of which two co-overexpressed *ERG* and *ETV4* and one *ERG* and *FLI1*. We further identified the gene fusions underlying the ETS co-overexpression, confirming that, in fact, each of these cases harbored two distinct gene fusions in the same index tumor. In case P236T we found *SLC45A3-ERG* and *SLC45A3-ETV4*, whereas cases P352T and P453T harbored *TMPRSS2-ERG* and additionally *HERVK17-ETV4* or *SLC45A3-FLI1*, respectively. Moreover, in one of the five randomly selected cases for FISH validation of the *TMPRSS2-ERG* status (having *ERG* and *TMPRSS2-ERG* Ct values less than 27), we observed both *TMPRSS2-ERG* and *SLC45A3-ERG* rearrangements in the index tumor. This shows that the ETS expression-based typing used alone, while allowing the detection of tumor genetic heterogeneity involving different ETS genes, may underestimate intratumor genetic heterogeneity involving the same ETS gene. In none of the tumors with two fusion genes did tumor cells present more than one rearrangement; instead, the different rearrangements were found in distinct

regions of the index tumor. Considering that ETS rearrangements may arise early in PCa carcinogenesis (Cerveira et al., 2006; Perner et al., 2007), our observations suggest that collision of independent clonal populations occurred during tumor growth. Adding to our results, other studies have also reported the presence of PCa with rearrangements involving different ETS genes, namely *ERG* and *ETV1*, either in the same tumor focus (Attard et al., 2009; Svensson et al., 2011) or in different tumor foci (Clark et al., 2008). This data thus demonstrates that, although the original observation that ETS fusions are mutually exclusive (Tomlins et al., 2005) within an individual tumor focus holds true in the majority of cases so far reported, intratumor heterogeneity of ETS rearrangements does exist in prostate cancer and that the strategy used strongly influences the ability to detect it. Svensson and co-workers applied a FISH-based ETS rearrangement assessment approach in 88 PCa patients and found 7 tumors with evidence of more than one rearrangement (Svensson et al., 2011). Adding to the complexity of ETS rearrangements, a recent study using immunohistochemistry observed intrafocal heterogeneity of ERG overexpression in 39% of a total of 178 PCa patients (Minner et al., 2013), showing that there may be tumor sub-clonal populations in ETS positive tumors with no genetic rearrangement or, perhaps more likely, that lost overexpression during tumor progression.

2. Clinical impact of ETS rearrangements and 8q gains

There has been considerable interest in unraveling the potential role of ETS rearrangements as prognostic biomarkers in patients with prostate carcinoma. This interest is supported by the fact that chromosomal rearrangement has been found in some hematological and soft-tissue malignancies to be associated with clinical outcomes. As an example, considering the pediatric acute lymphoblastic leukemia, patients harboring *ETV6-RUNX1* gene fusion have a favorable clinical outcome (Gandemer et al., 2012), whilst *MLL-AF4* fusion is associated with a dismal prognosis especially in patients younger than 6 months (Pui and Campana, 2007). The impact of *ERG* gene rearrangements in PCa has been investigated in a variety of clinical contexts, but no clear consensus has been reached. *ERG* rearrangements have been reported to be associated with favorable outcomes (Winnes et al., 2007; Saramaki et al., 2008), to have no association with clinical outcome (Yoshimoto et al., 2006; Lapointe et al., 2007; Tu et al., 2007; Furusato et al., 2008; Gopalan et al., 2009) or to be associated

with poor prognosis and more aggressive PCa (Perner et al., 2006; Wang et al., 2006; Demichelis et al., 2007; Nam et al., 2007a; Rajput et al., 2007; Attard et al., 2008; Mehra et al., 2008; Yoshimoto et al., 2008). To clarify this topic, we assessed *ERG* rearrangements using a break-apart FISH strategy in a consecutive series of 200 paraffin-embedded formalin-fixed diagnostic needle biopsies from PCa patients with 10-year disease-specific survival data. We additionally looked for 8q relative gains to validate its prognostic value and to test if the combination with *ERG* rearrangements increases discrimination of aggressive disease.

2.1. Impact of *TMPRSS2-ERG* and 8q gain on disease-specific survival

The identification of potentially lethal PCa is arguably the most important challenge for the management of this disease. The prognostic value of *TMPRSS2-ERG* gene fusion is a controversial subject in the literature, both for authors using biochemical recurrence as clinical end point (Perner et al., 2006; Mehra et al., 2007; Nam et al., 2007b; Saramaki et al., 2008; Gopalan et al., 2009) and also for authors using disease-specific survival (Demichelis et al., 2007; FitzGerald et al., 2008). To address this issue, we studied a clinically heterogeneous cohort of 200 diagnostic prostate needle-biopsies with confirmed PCa and a long follow up using FISH for detection of *ERG* rearrangements and 8q gains. This allowed the characterization of a wider spectrum of prostate carcinomas with a likely enrichment of more aggressive tumors, as compared to cohorts including solely radical prostatectomy specimens. Furthermore, we used disease-specific survival as clinical end point instead of biochemical recurrence, based on the evidence that only a minority of patients with biochemical recurrence will succumb to PCa (Ward et al., 2003; Porter et al., 2006).

We observed no statistically significant differences in disease-specific survival between patients with and without *ERG* rearrangements (**Paper III**). Additionally, we also found no differences when comparing the two described genomic mechanisms underlying fusion of *TMPRSS2* and *ERG* (i.e. insertion and deletion). Fitzgerald and co-workers also found no significant association between *TMPRSS2-ERG* gene fusion (detected in 35.5% of 214 PCa) and disease-specific mortality (FitzGerald et al., 2008). Studying a cohort of men with localized PCa followed by expectant therapy, Demichelis and co-workers observed a statistically significant association of the *TMPRSS2-ERG* fusion (detected in 15% of 111 PCa) with metastases or disease-specific death. However, when adjusting

for Gleason score the cumulative incidence ratio was not statistically significant (Demichelis et al., 2007). Attard and co-workers described that tumors with two or more copies of the *ERG* rearrangement by deletion was associated with worst clinical outcome (Attard et al., 2008). However, Gopalan and co-workers, based on assessment of whole DNA ploidy by flow cytometry and of an independent chromosome (#9) by FISH, concluded that the aggressive phenotype is more likely to be associated with aneuploidy than with the duplication of *TMPRSS2-ERG* itself (Gopalan et al., 2009).

Recent immunohistochemical (IHC) approaches using *ERG*-specific antibodies have shown that *ERG* protein overexpression is a sensitive and specific surrogate for the presence of *TMPRSS2-ERG* gene fusion detected by FISH or qRT-PCR (Park et al., 2010; Falzarano et al., 2011; van Leenders et al., 2011; Hoogland et al., 2012; Rosen et al., 2012). *ERG* immunohistochemistry is thus a rapid, cost-effective methodology to infer the presence of *ERG* rearrangements. Using this approach, Hoogland and co-workers studied 437 patients treated with radical prostatectomy and did not find any statistically significant prognostic association of *ERG* overexpression with subsequent biochemical or clinical recurrence, overall death or disease-specific death (Hoogland et al., 2012). This lack of prognostic significance in surgically treated patients was confirmed by recent meta-analysis using biochemical recurrence and disease-specific mortality as endpoints (Pettersson et al., 2012).

Relative 8q gains were present in 48% of PCa patients in our series of 200 diagnostic biopsies (**Paper III**), fitting in the range of 12% to 54% reported in previous studies (Miyoshi et al., 2000; Saramaki et al., 2001; Qian et al., 2002; Tsuchiya et al., 2002). Contrarily to *ERG* rearrangements, we demonstrated that patients with relative 8q gain have worse disease-specific survival and that this prognostic value is independent from clinical variables with known prognostic relevance, namely Gleason score and tumor staging. We additionally showed the importance of the ratio (*MYC*/*CEP18*) computed to discriminate patients with generalized aneuploidy from those with relative 8q gains. When we restricted the analysis including only patients with increased *MYC* copy number (three or more gene copies), the adverse clinical outcome was confined to the subset of patients displaying a *MYC*/*CEP18* ratio ≥ 1.5 .

PTEN deletions are also common genomic aberrations in prostate cancer and this copy number change has been shown to occur more frequently in *TMPRSS2-ERG* positive tumors (Carver et al., 2009). Although they can

occasionally be found in HGPIN lesions, *PTEN* deletions are more frequent in androgen-independent metastatic prostate cancer, being thus more likely to have a relevant role in late progression stages (Han et al., 2009). Moreover, it has been reported that *ERG* rearrangements and *PTEN* deletions combined are predictors of early recurrence (Yoshimoto et al., 2008). Regarding the possibility of a combined prognostic value of *ERG* rearrangements and 8q gains, we observed that the latter predicts death by disease irrespective of *ERG* fusion status. Since we found no association between these two genetic events, it is likely that they occur independently in prostatic carcinogenesis and that relative 8q gain is not associated with *PTEN* deletions.

2.2. Association of ETS fusion genes and 8q gains with clinico-pathological parameters

Besides disease-specific survival, we also looked for potential associations of *ERG* rearrangements and 8q gains with clinico-pathological parameters such as histological grade (measured by the Gleason score) and clinical stage, and also with the different treatment modalities. We observed that tumors with *ERG* rearrangements were associated with lower PSA levels, with Gleason score equal or lower than 7, and were not associated with clinical stage (**Paper III**). Similarly, our study of clinically localized PCa also revealed an association of the *TMPRSS2-ERG* fusion gene with Gleason score lower of equal to 7 and with more advanced pathological stage (pT3) (**Paper I**). This type of tumor staging cannot be determined in biopsy specimens, so it is not possible to compare the data of the two series. As in studies assessing the prognostic value of *TMPRSS2-ERG*, published data obtained using either FISH, qRT-PCR or IHC to assess putative associations of *TMPRSS2-ERG* with clinico-pathological parameters have reached contradictory conclusions. A recent publication sums up the available data in the literature, which includes more than 10,000 PCa patients (reviewed by Xu et al., 2013). Considering pre-operative PSA levels, in 64.7% of the patients studied the presence of the gene fusion was associated with lower PSA levels, while in 29.8% of the patients no association was found. Conversely, 5.5% of PCa patients displayed an association of the gene fusion with higher PSA levels. Regarding associations of the gene fusion with Gleason score, in 47.3% of the patients no association was found. On the other hand, in 39.6% of the patients the presence of the gene fusion was associated with lower Gleason scores. Finally, *TMPRSS2-*

ERG was associated with higher Gleason score in 13.1% of the patients. The associations of the gene fusion with pathological stage were only of two sorts. In 54% of the patients no association of the gene fusion with pathological stage was found, whereas in 46% of the patients studied there was an association with more advanced pathological stage.

Regarding treatment options, *ERG* rearrangements were not associated with disease-specific survival in patients treated with hormone therapy, in keeping with previous studies (Boormans et al., 2010; Leinonen et al., 2010). Pre-clinical studies have indicated that the *TMPRSS2-ERG* gene fusion, leading to *ERG* overexpression, may be a biomarker of DNA double strand break (DSB) repair capacity with potential implications for sensitivity to radiotherapy (Swanson et al., 2011) or DNA damaging modifying agents, such as PARP inhibitors (Brenner et al., 2011). In our study, the RT treated patients harboring *ERG* rearrangements did not display diverging disease-specific survival compared to those without the rearrangement. This observation may lack statistical power due to the reduced number of patients treated with RT in this study. However, our results were corroborated by a recent study questioning the hypothesis of whether *TMPRSS2-ERG* is a surrogate marker for DNA repair status and thus for sensitivity to radiotherapy. The authors did not observe any clinical outcome differences between the two groups of intermediate-risk radiotherapy treated PCa patients, defined by the presence or absence of the fusion gene (Dal Pra et al., 2013). These clinical results suggest that the presence of a *TMPRSS2-ERG* fusion is not consistently predictive of resistance to radiotherapy. In none of the remaining treatment groups (RP treated patients and patients treated with chemotherapy) was a prognostic or predictive value of *ERG* rearrangements evidenced. However, each of these subgroups had less than 20 patients, which is insufficient to draw any reliable conclusion.

Contrarily to what was observed for *ERG* gene fusions, relative 8q gains displayed features of aggressive tumors, being associated with higher PSA levels, lower degree of differentiation, and more advanced clinical stages. Nevertheless, it is noteworthy that Cox regression analysis demonstrated that the prognostic value of relative 8q gains is independent from the clinico-pathological parameters mentioned above. Regarding treatment modalities, relative 8q gains predicted worse clinical outcome among HT treated patients. Relative 8q gains also indicated poorer disease-specific survival among patients treated solely with RP

or RT, but these particular conclusions lack statistical power due to the reduced number of patients in each treatment subset.

Chapter 7

Conclusions

CONCLUSIONS

The aims of this thesis were addressed in the included peer-reviewed publications. The main conclusions drawn are summarized in the following topics:

- The assessment of ETS expression using high-throughput technologies combined with selective genomic validation through FISH experiments is a valid strategy to study the known ETS rearrangements and to search for novel gene fusions;
- Genomic rearrangements of the ETS genes *ERG*, *ETV1*, *ETV4* and *ETV5* were found in 51%, 7%, 1% and 0.5%, respectively, of PCa patients;
- We have for the first time described the ETS gene *FLI1* rearranged in PCa, having as 5' fusion partner the *SLC45A3* gene. This finding makes *FLI1* the fifth ETS transcription factor known to be involved in PCa gene fusions;
- We unveiled two novel 5' fusion partners, namely *OR51E2* and *UBTF*, rearranged with ETS transcription factors. Two novel gene fusion combinations involving previously described genes (*SLC45A3*-*ETV4* and *HERVK17*-*ETV4*) were also found. These findings suggests that not only there may be more 5' partner genes yet to be identified in PCa, but also that all the genes described as 5' fusion partners with one ETS gene can most likely be rearranged with any of the other ETS genes known to be involved in PCa;
- Relative 8q gain detected in diagnostic needle biopsies predicts poor disease-specific survival independently of the presence of the *TMPRSS2-ERG* fusion gene, standard clinicopathologic variables, and treatment modalities. Given that this genetic marker can be evaluated before the delineation of the therapeutic plan, it has the potential to improve the clinical management of PCa patients.

Chapter 8

Future Perspectives

FUTURE PERSPECTIVES

As it is common in scientific projects, these are originated by a set of questions that after being addressed give rise to a new set of questions that will fuel further studies. The following topics represent, from our point of view, relevant topics for future studies:

1. Impact of ETS rearrangements diversity in clinical outcome

We studied the clinical impact of the rearrangements involving *ERG*, which are by far the most common. The impact of rearrangements specifically involving *ETV1*, the second most commonly rearranged ETS, remains poorly documented, not to mention the rarer *ETV4*, *ETV5* and *FLI1*. The frequency of these alternative ETS rearrangements is low, constituting a significant hurdle to successfully clarify this issue. We have recent data showing that different ETS rearrangements share some downstream targets with *ERG* but also have specific target genes, so a prognostic value different from that of *ERG* rearrangements cannot at present be excluded.

Another interesting question is related to the nature of the regulation of the fusion partner. For instance, the androgen-repressed non-coding RNA *C15orf21* is among the most common 5' partners of *ETV1*. Additionally, as found in the work presented in this thesis there are also fusion partners insensitive to androgen stimuli. Since the most common ETS fusion partners are androgen dependent, is there any difference in clinical behavior of tumors with ETS rearrangements involving 5' fusion partners that are not?

2. Characterization of non-ETS gene fusions in PCa

The assessment of chromosome 8q copy number has incidentally revealed structural rearrangements possibly involving the gene *MYC*. The design of the probes used does not allow a precise identification of the gene involved due to the 200Mb span between the break-apart probes. To clarify this issue, the selection of BAC clones closely mapping the 3' and 5' genomic regions flanking the candidate gene would need to be employed. Interestingly, it has been recently reported a case of a PCa patient harboring a genomic fusion of *MYC* with *C15orf21*, a known *ETV1* fusion partner. We thus consider that a break-apart BAC

probe strategy in the cohort of the 200 patients treated with radical prostatectomy may reveal novel rearrangement types.

3. Validation of the prognostic value of relative 8q gain in a clinically localized PCa cohort

Our results clearly demonstrate an association of relative 8q gain with aggressive disease independently from known prognostic markers, namely tumor staging and grading. Hence, this genomic aberration is a potential genetic marker that can be evaluated in prostate needle-biopsy prior to the delineation of the therapeutic plan. The relatively small number of patients treated with radical prostatectomy included in this thesis requires a validation study in a broader cohort of PCa patients with clinically localized disease.

4. Targets of relative 8q copy number gain

The findings presented in this thesis strongly associate relative 8q gains with aggressive prostate carcinomas. Assuming that there is one or more genes in 8q that are operative in this subset of PCa patients with aggressive disease, it would be very interesting to identify genes differentially expressed between tumors with and without relative 8q gains.

References

Abate-Shen, C., Shen, M.M., and Gelmann, E. (2008). Integrating differentiation and cancer: the Nkx3.1 homeobox gene in prostate organogenesis and carcinogenesis. *Differentiation; research in biological diversity* 76, 717-727.

Abdellaoui, A., Iyengar, S., and Freeman, S. (2011). Imaging in prostate cancer. *Future oncology* 7, 679-691.

Adolfsson, J., Tribukait, B., and Levitt, S. (2007). The 20-Yr outcome in patients with well- or moderately differentiated clinically localized prostate cancer diagnosed in the pre-PSA era: the prognostic value of tumour ploidy and comorbidity. *European urology* 52, 1028-1035.

Alers, J.C., Rochat, J., Krijtenburg, P.J., Hop, W.C., Kranse, R., Rosenberg, C., Tanke, H.J., Schroder, F.H., and van Dekken, H. (2000). Identification of genetic markers for prostatic cancer progression. *Laboratory investigation; a journal of technical methods and pathology* 80, 931-942.

Andreoiu, M., and Cheng, L. (2010). Multifocal prostate cancer: biologic, prognostic, and therapeutic implications. *Human pathology* 41, 781-793.

Attard, G., Clark, J., Ambrosine, L., Fisher, G., Kovacs, G., Flohr, P., Berney, D., Foster, C.S., Fletcher, A., Gerald, W.L., *et al.* (2008a). Duplication of the fusion of TMPRSS2 to ERG sequences identifies fatal human prostate cancer. *Oncogene* 27, 253-263.

Attard, G., Clark, J., Ambrosine, L., Mills, I.G., Fisher, G., Flohr, P., Reid, A., Edwards, S., Kovacs, G., Berney, D., *et al.* (2008b). Heterogeneity and clinical significance of ETV1 translocations in human prostate cancer. *British journal of cancer* 99, 314-320.

Attard, G., Jameson, C., Moreira, J., Flohr, P., Parker, C., Dearnaley, D., Cooper, C.S., and de Bono, J.S. (2009). Hormone-sensitive prostate cancer: a case of ETS gene fusion heterogeneity. *Journal of clinical pathology* 62, 373-376.

Aus, G., Abbou, C.C., Bolla, M., Heidenreich, A., Schmid, H.P., van Poppel, H., Wolff, J., Zattoni, F., and European Association of, U. (2005). EAU guidelines on prostate cancer. *European urology* 48, 546-551.

Ayala, G., Thompson, T., Yang, G., Frolov, A., Li, R., Scardino, P., Otori, M., Wheeler, T., and Harper, W. (2004). High levels of phosphorylated form of Akt-1 in prostate cancer and non-neoplastic prostate tissues are strong predictors of

biochemical recurrence. *Clinical cancer research : an official journal of the American Association for Cancer Research* 10, 6572-6578.

Bachmann, I.M., Halvorsen, O.J., Collett, K., Stefansson, I.M., Straume, O., Haukaas, S.A., Salvesen, H.B., Otte, A.P., and Akslen, L.A. (2006). EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 24, 268-273.

Balk, S.P., Ko, Y.J., and Bubley, G.J. (2003). Biology of prostate-specific antigen. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 21, 383-391.

Bangma, C.H., Bul, M., and Roobol, M. (2012). The Prostate cancer Research International: Active Surveillance study. *Current opinion in urology* 22, 216-221.

Bangma, C.H., and Roobol, M.J. (2012). Defining and predicting indolent and low risk prostate cancer. *Critical reviews in oncology/hematology* 83, 235-241.

Barbieri, C.E., Baca, S.C., Lawrence, M.S., Demichelis, F., Blattner, M., Theurillat, J.P., White, T.A., Stojanov, P., Van Allen, E., Stransky, N., *et al.* (2012). Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. *Nature genetics* 44, 685-689.

Barbieri, C.E., Bangma, C.H., Bjartell, A., Catto, J.W., Culig, Z., Gronberg, H., Luo, J., Visakorpi, T., and Rubin, M.A. (2013). The mutational landscape of prostate cancer. *European urology* 64, 567-576.

Barski, A., and Zhao, K. (2009). Genomic location analysis by ChIP-Seq. *Journal of cellular biochemistry* 107, 11-18.

Beahrs, O.H., and Henson, D.E. (1992). Fourth edition of the manual for staging of cancer. *Cancer* 69, 2869-2869.

Berney, D.M., and Warren, A.Y. (2013). Premalignant Lesions of the Prostate. In *Prostate Cancer: A Comprehensive Perspective*, A. Tewari, ed. (London: Springer-Verlag), pp. 215-233.

Bertram, J., Peacock, J.W., Tan, C., Mui, A.L., Chung, S.W., Gleave, M.E., Dedhar, S., Cox, M.E., and Ong, C.J. (2006). Inhibition of the phosphatidylinositol 3'-kinase pathway promotes autocrine Fas-induced death of phosphatase and tensin homologue-deficient prostate cancer cells. *Cancer research* 66, 4781-4788.

Bethel, C.R., Faith, D., Li, X., Guan, B., Hicks, J.L., Lan, F., Jenkins, R.B., Bieberich, C.J., and De Marzo, A.M. (2006). Decreased NKX3.1 protein expression in focal prostatic atrophy, prostatic intraepithelial neoplasia, and adenocarcinoma: association with gleason score and chromosome 8p deletion. *Cancer research* 66, 10683-10690.

Bill-Axelson, A., Holmberg, L., Ruutu, M., Garmo, H., Stark, J.R., Busch, C., Nordling, S., Haggman, M., Andersson, S.O., Bratell, S., *et al.* (2011). Radical prostatectomy versus watchful waiting in early prostate cancer. *The New England journal of medicine* 364, 1708-1717.

Bill-Axelson, A., Holmberg, L., Ruutu, M., Haggman, M., Andersson, S.O., Bratell, S., Spangberg, A., Busch, C., Nordling, S., Garmo, H., *et al.* (2005). Radical prostatectomy versus watchful waiting in early prostate cancer. *The New England journal of medicine* 352, 1977-1984.

Boormans, J.L., Hermans, K.G., Made, A.C., van Leenders, G.J., Wildhagen, M.F., Collette, L., Schroder, F.H., Trapman, J., and Verhagen, P.C. (2010). Expression of the androgen-regulated fusion gene TMPRSS2-ERG does not predict response to endocrine treatment in hormone-naïve, node-positive prostate cancer. *European urology* 57, 830-835.

Borboroglu, P.G., Sur, R.L., Roberts, J.L., and Amling, C.L. (2001). Repeat biopsy strategy in patients with atypical small acinar proliferation or high grade prostatic intraepithelial neoplasia on initial prostate needle biopsy. *The Journal of urology* 166, 866-870.

Bostwick, D.G. (1996). Progression of prostatic intraepithelial neoplasia to early invasive adenocarcinoma. *European urology* 30, 145-152.

Bostwick, D.G., and Cheng, L. (2012). Precursors of prostate cancer. *Histopathology* 60, 4-27.

Bostwick, D.G., Shan, A., Qian, J., Darson, M., Maihle, N.J., Jenkins, R.B., and Cheng, L. (1998). Independent origin of multiple foci of prostatic intraepithelial neoplasia: comparison with matched foci of prostate carcinoma. *Cancer* 83, 1995-2002.

Boyd, L.K., Mao, X., and Lu, Y.J. (2012). The complexity of prostate cancer: genomic alterations and heterogeneity. *Nature reviews Urology* 9, 652-664.

Bozeman, C.B., Carver, B.S., Caldito, G., Venable, D.D., and Eastham, J.A. (2005). Prostate cancer in patients with an abnormal digital rectal examination and serum prostate-specific antigen less than 4.0 ng/mL. *Urology* 66, 803-807.

- Brawer, M.K. (2000). Prostate-specific antigen. *Seminars in surgical oncology* 18, 3-9.
- Brenner, J.C., Ateeq, B., Li, Y., Yocum, A.K., Cao, Q., Asangani, I.A., Patel, S., Wang, X., Liang, H., Yu, J., *et al.* (2011). Mechanistic rationale for inhibition of poly(ADP-ribose) polymerase in ETS gene fusion-positive prostate cancer. *Cancer cell* 19, 664-678.
- Breslow, N., Chan, C.W., Dhom, G., Drury, R.A., Franks, L.M., Gellei, B., Lee, Y.S., Lundberg, S., Sparke, B., Sternby, N.H., *et al.* (1977). Latent carcinoma of prostate at autopsy in seven areas. The International Agency for Research on Cancer, Lyons, France. *International journal of cancer Journal international du cancer* 20, 680-688.
- Bushman, W. (2009). Etiology, epidemiology, and natural history of benign prostatic hyperplasia. *The Urologic clinics of North America* 36, 403-415, v.
- Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J.G., Jen, J., Isaacs, W.B., Bova, G.S., and Sidransky, D. (1997). Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer research* 57, 4997-5000.
- Campbell, T., Blasko, J., Crawford, E.D., Forman, J., Hanks, G., Kuban, D., Montie, J., Moul, J., Pollack, A., Raghavan, D., *et al.* (2001). Clinical staging of prostate cancer: reproducibility and clarification of issues. *International journal of cancer Journal international du cancer* 96, 198-209.
- Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 298, 1039-1043.
- Carter, B.S., Beaty, T.H., Steinberg, G.D., Childs, B., and Walsh, P.C. (1992). Mendelian inheritance of familial prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America* 89, 3367-3371.
- Carver, B.S., Tran, J., Gopalan, A., Chen, Z., Shaikh, S., Carracedo, A., Alimonti, A., Nardella, C., Varmeh, S., Scardino, P.T., *et al.* (2009). Aberrant ERG expression cooperates with loss of PTEN to promote cancer progression in the prostate. *Nature genetics* 41, 619-624.
- Catalona, W.J., Richie, J.P., deKernion, J.B., Ahmann, F.R., Ratliff, T.L., Dalkin, B.L., Kavoussi, L.R., MacFarlane, M.T., and Southwick, P.C. (1994). Comparison of prostate specific antigen concentration versus prostate specific antigen density in the early detection of prostate cancer: receiver operating characteristic curves. *The Journal of urology* 152, 2031-2036.

Center, M.M., Jemal, A., Lortet-Tieulent, J., Ward, E., Ferlay, J., Brawley, O., and Bray, F. (2012). International variation in prostate cancer incidence and mortality rates. *European urology* 61, 1079-1092.

Cerveira, N., Ribeiro, F.R., Peixoto, A., Costa, V., Henrique, R., Jeronimo, C., and Teixeira, M.R. (2006). TMPRSS2-ERG gene fusion causing ERG overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions. *Neoplasia* 8, 826-832.

Cheng, L., Montironi, R., Bostwick, D.G., Lopez-Beltran, A., and Berney, D.M. (2012). Staging of prostate cancer. *Histopathology* 60, 87-117.

Cheville, J.C., Reznicek, M.J., and Bostwick, D.G. (1997). The focus of "atypical glands, suspicious for malignancy" in prostatic needle biopsy specimens: incidence, histologic features, and clinical follow-up of cases diagnosed in a community practice. *American journal of clinical pathology* 108, 633-640.

Chi, K.N., Gleave, M.E., Fazli, L., Goldenberg, S.L., So, A., Kollmannsberger, C., Murray, N., Tinker, A., and Pollak, M. (2012). A phase II pharmacodynamic study of preoperative figitumumab in patients with localized prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 18, 3407-3413.

Chi, K.N., Hotte, S.J., Yu, E.Y., Tu, D., Eigl, B.J., Tannock, I., Saad, F., North, S., Powers, J., Gleave, M.E., *et al.* (2010). Randomized phase II study of docetaxel and prednisone with or without OGX-011 in patients with metastatic castration-resistant prostate cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 28, 4247-4254.

Chin, S.P., Dickinson, J.L., and Holloway, A.F. (2011). Epigenetic regulation of prostate cancer. *Clinical epigenetics* 2, 151-169.

Chodak, G.W., Thisted, R.A., Gerber, G.S., Johansson, J.E., Adolfsson, J., Jones, G.W., Chisholm, G.D., Moskovitz, B., Livne, P.M., and Warner, J. (1994). Results of conservative management of clinically localized prostate cancer. *The New England journal of medicine* 330, 242-248.

Clark, J., Attard, G., Jhavar, S., Flohr, P., Reid, A., De-Bono, J., Eeles, R., Scardino, P., Cuzick, J., Fisher, G., *et al.* (2008). Complex patterns of ETS gene alteration arise during cancer development in the human prostate. *Oncogene* 27, 1993-2003.

Clark, J.P., and Cooper, C.S. (2009). ETS gene fusions in prostate cancer. *Nature reviews Urology* 6, 429-439.

- Cohen, R.J., Shannon, B.A., Phillips, M., Moorin, R.E., Wheeler, T.M., and Garrett, K.L. (2008). Central zone carcinoma of the prostate gland: a distinct tumor type with poor prognostic features. *The Journal of urology* 179, 1762-1767; discussion 1767.
- Coolen, M.W., Stirzaker, C., Song, J.Z., Statham, A.L., Kassir, Z., Moreno, C.S., Young, A.N., Varma, V., Speed, T.P., Cowley, M., *et al.* (2010). Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity. *Nature cell biology* 12, 235-246.
- Coppola, V., De Maria, R., and Bonci, D. (2010). MicroRNAs and prostate cancer. *Endocrine-related cancer* 17, F1-17.
- Corcoran, N.M., and Gleave, M.E. (2012). Targeted therapy in prostate cancer. *Histopathology* 60, 216-231.
- Dal Pra, A., Lalonde, E., Sykes, J., Warde, F., Ishkanian, A., Meng, A., Maloff, C., Srigley, J., Joshua, A.M., Petrovics, G., *et al.* (2013). TMPRSS2-ERG status is not prognostic following prostate cancer radiotherapy: implications for fusion status and DSB repair. *Clinical cancer research : an official journal of the American Association for Cancer Research* 19, 5202-5209.
- Daley, G.Q., Van Etten, R.A., and Baltimore, D. (1990). Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science* 247, 824-830.
- de Bono, J.S., Logothetis, C.J., Molina, A., Fizazi, K., North, S., Chu, L., Chi, K.N., Jones, R.J., Goodman, O.B., Jr., Saad, F., *et al.* (2011). Abiraterone and increased survival in metastatic prostate cancer. *The New England journal of medicine* 364, 1995-2005.
- De Bortoli, M., Castellino, R.C., Lu, X.Y., Deyo, J., Sturla, L.M., Adesina, A.M., Perlaky, L., Pomeroy, S.L., Lau, C.C., Man, T.K., *et al.* (2006). Medulloblastoma outcome is adversely associated with overexpression of EEF1D, RPL30, and RPS20 on the long arm of chromosome 8. *BMC cancer* 6, 223.
- Dehm, S.M., and Tindall, D.J. (2006). Ligand-independent androgen receptor activity is activation function-2-independent and resistant to antiandrogens in androgen refractory prostate cancer cells. *The Journal of biological chemistry* 281, 27882-27893.
- Demichelis, F., Fall, K., Perner, S., Andren, O., Schmidt, F., Setlur, S.R., Hoshida, Y., Mosquera, J.M., Pawitan, Y., Lee, C., *et al.* (2007). TMPRSS2:ERG gene

fusion associated with lethal prostate cancer in a watchful waiting cohort. *Oncogene* 26, 4596-4599.

Demichelis, F., Setlur, S.R., Beroukhi, R., Perner, S., Korbel, J.O., Lafargue, C.J., Pflueger, D., Pina, C., Hofer, M.D., Sboner, A., *et al.* (2009). Distinct genomic aberrations associated with ERG rearranged prostate cancer. *Genes, chromosomes & cancer* 48, 366-380.

Donaldson, L.W., Petersen, J.M., Graves, B.J., and McIntosh, L.P. (1994). Secondary structure of the ETS domain places murine Ets-1 in the superfamily of winged helix-turn-helix DNA-binding proteins. *Biochemistry* 33, 13509-13516.

Dong, J.T. (2006). Prevalent mutations in prostate cancer. *Journal of cellular biochemistry* 97, 433-447.

Druker, B.J., Sawyers, C.L., Kantarjian, H., Resta, D.J., Reese, S.F., Ford, J.M., Capdeville, R., and Talpaz, M. (2001). Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *The New England journal of medicine* 344, 1038-1042.

Edge, S., and Compton, C. (2010). The American Joint Committee on Cancer: the 7th Edition of the AJCC Cancer Staging Manual and the Future of TNM. *Ann Surg Oncol* 17, 1471-1474.

Edwards, S.M., and Eeles, R.A. (2004). Unravelling the genetics of prostate cancer. *American journal of medical genetics Part C, Seminars in medical genetics* 129C, 65-73.

Egger, S.E., Scardino, P.T., Walsh, P.C., Han, M., Partin, A.W., Trock, B.J., Feng, Z., Wood, D.P., Eastham, J.A., Yossepowitch, O., *et al.* (2011). Predicting 15-year prostate cancer specific mortality after radical prostatectomy. *The Journal of urology* 185, 869-875.

Emmert-Buck, M.R., Vocke, C.D., Pozzatti, R.O., Duray, P.H., Jennings, S.B., Florence, C.D., Zhuang, Z., Bostwick, D.G., Liotta, L.A., and Linehan, W.M. (1995). Allelic loss on chromosome 8p12-21 in microdissected prostatic intraepithelial neoplasia. *Cancer research* 55, 2959-2962.

Epstein, J.I. (2009). Precursor lesions to prostatic adenocarcinoma. *Virchows Archiv : an international journal of pathology* 454, 1-16.

Epstein, J.I., Allsbrook, W.C., Jr., Amin, M.B., Egevad, L.L., and Committee, I.G. (2005). The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. *The American journal of surgical pathology* 29, 1228-1242.

- Falzarano, S.M., Zhou, M., Carver, P., Tsuzuki, T., Simmerman, K., He, H., and Magi-Galluzzi, C. (2011). ERG gene rearrangement status in prostate cancer detected by immunohistochemistry. *Virchows Archiv : an international journal of pathology* 459, 441-447.
- Farnham, P.J. (2009). Insights from genomic profiling of transcription factors. *Nature reviews Genetics* 10, 605-616.
- Feldman, B.J., and Feldman, D. (2001). The development of androgen-independent prostate cancer. *Nature reviews Cancer* 1, 34-45.
- Ferlay, J., Parkin, D.M., and Steliarova-Foucher, E. (2010a). Estimates of cancer incidence and mortality in Europe in 2008. *European journal of cancer* 46, 765-781.
- Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C., and Parkin, D.M. (2010b). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International journal of cancer Journal international du cancer* 127, 2893-2917.
- FitzGerald, L.M., Agalliu, I., Johnson, K., Miller, M.A., Kwon, E.M., Hurtado-Coll, A., Fazli, L., Rajput, A.B., Gleave, M.E., Cox, M.E., *et al.* (2008). Association of TMPRSS2-ERG gene fusion with clinical characteristics and outcomes: results from a population-based study of prostate cancer. *BMC cancer* 8, 230.
- Fizazi, K., Scher, H.I., Molina, A., Logothetis, C.J., Chi, K.N., Jones, R.J., Staffurth, J.N., North, S., Vogelzang, N.J., Saad, F., *et al.* (2012). Abiraterone acetate for treatment of metastatic castration-resistant prostate cancer: final overall survival analysis of the COU-AA-301 randomised, double-blind, placebo-controlled phase 3 study. *The lancet oncology* 13, 983-992.
- Fu, W., Bubendorf, L., Willi, N., Moch, H., Mihatsch, M.J., Sauter, G., and Gasser, T.C. (2000). Genetic changes in clinically organ-confined prostate cancer by comparative genomic hybridization. *Urology* 56, 880-885.
- Furusato, B., Gao, C.L., Ravindranath, L., Chen, Y., Cullen, J., McLeod, D.G., Dobi, A., Srivastava, S., Petrovics, G., and Sesterhenn, I.A. (2008). Mapping of TMPRSS2-ERG fusions in the context of multi-focal prostate cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 21, 67-75.
- Gandemer, V., Chevret, S., Petit, A., Vermylen, C., Leblanc, T., Michel, G., Schmitt, C., Lejars, O., Schneider, P., Demeocq, F., *et al.* (2012). Excellent prognosis of late relapses of ETV6/RUNX1-positive childhood acute lymphoblastic leukemia: lessons from the FRALLE 93 protocol. *Haematologica* 97, 1743-1750.

Gann, P.H., Hennekens, C.H., and Stampfer, M.J. (1995). A prospective evaluation of plasma prostate-specific antigen for detection of prostatic cancer. *JAMA : the journal of the American Medical Association* 273, 289-294.

Gao, H., Ouyang, X., Banach-Petrosky, W.A., Shen, M.M., and Abate-Shen, C. (2006). Emergence of androgen independence at early stages of prostate cancer progression in Nkx3.1; Pten mice. *Cancer research* 66, 7929-7933.

Gasi, D., van der Korput, H.A., Douben, H.C., de Klein, A., de Ridder, C.M., van Weerden, W.M., and Trapman, J. (2011). Overexpression of full-length ETV1 transcripts in clinical prostate cancer due to gene translocation. *PloS one* 6, e16332.

Gelmann, E.P. (2003). Searching for the gatekeeper oncogene of prostate cancer. *Critical reviews in oncology/hematology* 46 Suppl, S11-20.

Ghadimi, B.M., Grade, M., Liersch, T., Langer, C., Siemer, A., Fuzesi, L., and Becker, H. (2003). Gain of chromosome 8q23-24 is a predictive marker for lymph node positivity in colorectal cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 9, 1808-1814.

Ginsberg, J.P., de Alava, E., Ladanyi, M., Wexler, L.H., Kovar, H., Paulussen, M., Zoubek, A., Dockhorn-Dworniczak, B., Juergens, H., Wunder, J.S., *et al.* (1999). EWS-FLI1 and EWS-ERG gene fusions are associated with similar clinical phenotypes in Ewing's sarcoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 17, 1809-1814.

Gleason, D.F. (1966). Classification of prostatic carcinomas. *Cancer chemotherapy reports Part 1* 50, 125-128.

Glinsky, G.V., Glinskii, A.B., Stephenson, A.J., Hoffman, R.M., and Gerald, W.L. (2004). Gene expression profiling predicts clinical outcome of prostate cancer. *The Journal of clinical investigation* 113, 913-923.

Gopalan, A., Leversha, M.A., Satagopan, J.M., Zhou, Q., Al-Ahmadie, H.A., Fine, S.W., Eastham, J.A., Scardino, P.T., Scher, H.I., Tickoo, S.K., *et al.* (2009). TMPRSS2-ERG gene fusion is not associated with outcome in patients treated by prostatectomy. *Cancer research* 69, 1400-1406.

Gosselaar, C., Roobol, M.J., and Schroder, F.H. (2005). Prevalence and characteristics of screen-detected prostate carcinomas at low prostate-specific antigen levels: aggressive or insignificant? *BJU international* 95, 231-237.

Grasso, C.S., Wu, Y.M., Robinson, D.R., Cao, X., Dhanasekaran, S.M., Khan, A.P., Quist, M.J., Jing, X., Lonigro, R.J., Brenner, J.C., *et al.* (2012). The mutational landscape of lethal castration-resistant prostate cancer. *Nature* 487, 239-243.

Greene, K.L., Albertsen, P.C., Babaian, R.J., Carter, H.B., Gann, P.H., Han, M., Kuban, D.A., Sartor, A.O., Stanford, J.L., Zietman, A., *et al.* (2013). Prostate specific antigen best practice statement: 2009 update. *The Journal of urology* 189, S2-S11.

Grignon, D.J., and Sakr, W.A. (1996). Pathologic staging of prostate carcinoma. What are the issues? *Cancer* 78, 337-340.

Gurel, B., Iwata, T., Koh, C.M., Yegnasubramanian, S., Nelson, W.G., and De Marzo, A.M. (2008). Molecular alterations in prostate cancer as diagnostic, prognostic, and therapeutic targets. *Advances in anatomic pathology* 15, 319-331.

Haas, G.P., Delongchamps, N., Brawley, O.W., Wang, C.Y., and de la Roza, G. (2008). The worldwide epidemiology of prostate cancer: perspectives from autopsy studies. *The Canadian journal of urology* 15, 3866-3871.

Haggman, M.J., Wojno, K.J., Pearsall, C.P., and Macoska, J.A. (1997). Allelic loss of 8p sequences in prostatic intraepithelial neoplasia and carcinoma. *Urology* 50, 643-647.

Hamberg, P., Verhagen, P.C., and de Wit, R. (2008). When to start cytotoxic therapy in asymptomatic patients with hormone refractory prostate cancer? *European journal of cancer* 44, 1193-1197.

Han, B., Mehra, R., Dhanasekaran, S.M., Yu, J., Menon, A., Lonigro, R.J., Wang, X., Gong, Y., Wang, L., Shankar, S., *et al.* (2008). A fluorescence in situ hybridization screen for E26 transformation-specific aberrations: identification of DDX5-ETV4 fusion protein in prostate cancer. *Cancer research* 68, 7629-7637.

Han, B., Mehra, R., Lonigro, R.J., Wang, L., Suleman, K., Menon, A., Palanisamy, N., Tomlins, S.A., Chinnaiyan, A.M., and Shah, R.B. (2009). Fluorescence in situ hybridization study shows association of PTEN deletion with ERG rearrangement during prostate cancer progression. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 22, 1083-1093.

Heidenreich, A., Aus, G., Bolla, M., Joniau, S., Matveev, V.B., Schmid, H.P., Zattoni, F., and European Association of, U. (2008). EAU guidelines on prostate cancer. *European urology* 53, 68-80.

Heidenreich, A., Bellmunt, J., Bolla, M., Joniau, S., Mason, M., Matveev, V., Mottet, N., Schmid, H.P., van der Kwast, T., Wiegel, T., *et al.* (2011). EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. *European urology* 59, 61-71.

Heidenreich, A., and Pfister, D. (2013). Pelvic Lymphadenectomy in Clinically Localised Prostate Cancer: Counting Lymph Nodes or Dissecting Primary Landing Zones of the Prostate? *European urology*.

Helgeson, B.E., Tomlins, S.A., Shah, N., Laxman, B., Cao, Q., Prensner, J.R., Cao, X., Singla, N., Montie, J.E., Varambally, S., *et al.* (2008). Characterization of TMPRSS2:ETV5 and SLC45A3:ETV5 gene fusions in prostate cancer. *Cancer research* 68, 73-80.

Hemminki, K. (2012). Familial risk and familial survival in prostate cancer. *World journal of urology* 30, 143-148.

Hermans, K.G., van der Korput, H.A., van Marion, R., van de Wijngaart, D.J., Ziel-van der Made, A., Dits, N.F., Boormans, J.L., van der Kwast, T.H., van Dekken, H., Bangma, C.H., *et al.* (2008). Truncated ETV1, fused to novel tissue-specific genes, and full-length ETV1 in prostate cancer. *Cancer research* 68, 7541-7549.

Hermans, K.G., van Marion, R., van Dekken, H., Jenster, G., van Weerden, W.M., and Trapman, J. (2006). TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. *Cancer research* 66, 10658-10663.

Hollenhorst, P.C., McIntosh, L.P., and Graves, B.J. (2011). Genomic and biochemical insights into the specificity of ETS transcription factors. *Annual review of biochemistry* 80, 437-471.

Hollenhorst, P.C., Shah, A.A., Hopkins, C., and Graves, B.J. (2007). Genome-wide analyses reveal properties of redundant and specific promoter occupancy within the ETS gene family. *Genes & development* 21, 1882-1894.

Hoogland, A.M., Jenster, G., van Weerden, W.M., Trapman, J., van der Kwast, T., Roobol, M.J., Schroder, F.H., Wildhagen, M.F., and van Leenders, G.J. (2012). ERG immunohistochemistry is not predictive for PSA recurrence, local recurrence or overall survival after radical prostatectomy for prostate cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 25, 471-479.

Huggins, C., and Hodges, C.V. (1972). Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *CA: a cancer journal for clinicians* 22, 232-240.

- Humphrey, P.A. (2004). Gleason grading and prognostic factors in carcinoma of the prostate. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 17, 292-306.
- Im, Y.H., Kim, H.T., Lee, C., Poulin, D., Welford, S., Sorensen, P.H., Denny, C.T., and Kim, S.J. (2000). EWS-FLI1, EWS-ERG, and EWS-ETV1 oncoproteins of Ewing tumor family all suppress transcription of transforming growth factor beta type II receptor gene. *Cancer research* 60, 1536-1540.
- Isaacs, J.T., and Coffey, D.S. (1989). Etiology and disease process of benign prostatic hyperplasia. *The Prostate Supplement* 2, 33-50.
- Jani, A.B., and Hellman, S. (2003). Early prostate cancer: clinical decision-making. *Lancet* 361, 1045-1053.
- Jani, A.B., Roeske, J.C., and Rash, C. (2003). Intensity-modulated radiation therapy for prostate cancer. *Clinical prostate cancer* 2, 98-105.
- Jansson, K.F., Akre, O., Garmo, H., Bill-Axelsson, A., Adolfsson, J., Stattin, P., and Bratt, O. (2012). Concordance of tumor differentiation among brothers with prostate cancer. *European urology* 62, 656-661.
- Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., and Forman, D. (2011). Global cancer statistics. *CA: a cancer journal for clinicians* 61, 69-90.
- Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C., and Thun, M.J. (2006). Cancer statistics, 2006. *CA: a cancer journal for clinicians* 56, 106-130.
- Jeronimo, C., Bastian, P.J., Bjartell, A., Carbone, G.M., Catto, J.W., Clark, S.J., Henrique, R., Nelson, W.G., and Shariat, S.F. (2011). Epigenetics in prostate cancer: biologic and clinical relevance. *European urology* 60, 753-766.
- Jeronimo, C., Usadel, H., Henrique, R., Oliveira, J., Lopes, C., Nelson, W.G., and Sidransky, D. (2001). Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. *Journal of the National Cancer Institute* 93, 1747-1752.
- Jewett, H.J. (1975). The present status of radical prostatectomy for stages A and B prostatic cancer. *The Urologic clinics of North America* 2, 105-124.
- Johansson, J.E., Adami, H.O., Andersson, S.O., Bergstrom, R., Krusemo, U.B., and Kraaz, W. (1989). Natural history of localised prostatic cancer. A population-based study in 223 untreated patients. *Lancet* 1, 799-803.
- Johansson, J.E., Andren, O., Andersson, S.O., Dickman, P.W., Holmberg, L., Magnuson, A., and Adami, H.O. (2004). Natural history of early, localized prostate cancer. *JAMA : the journal of the American Medical Association* 291, 2713-2719.

Jones, K.A., Black, D.M., Griffiths, B.L., and Solomon, E. (1995). Localization of the human RNA polymerase I transcription factor gene (UBTF) to the D17S183 locus on chromosome 17q21 and construction of a long-range restriction map of the region. *Genomics* 30, 602-604.

Jonsson, E., Sigbjarnarson, H.P., Tomasson, J., Benediktsdottir, K.R., Tryggvadottir, L., Hrafnkelsson, J., Olafsdottir, E.J., Tulinius, H., and Jonasson, J.G. (2006). Adenocarcinoma of the prostate in Iceland: a population-based study of stage, Gleason grade, treatment and long-term survival in males diagnosed between 1983 and 1987. *Scandinavian journal of urology and nephrology* 40, 265-271.

July, L.V., Akbari, M., Zellweger, T., Jones, E.C., Goldenberg, S.L., and Gleave, M.E. (2002). Clusterin expression is significantly enhanced in prostate cancer cells following androgen withdrawal therapy. *The Prostate* 50, 179-188.

Kallioniemi, O.P., Kallioniemi, A., Piper, J., Isola, J., Waldman, F.M., Gray, J.W., and Pinkel, D. (1994). Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. *Genes, chromosomes & cancer* 10, 231-243.

Kheirandish, P., and Chinegwundoh, F. (2011). Ethnic differences in prostate cancer. *British journal of cancer* 105, 481-485.

Kim, M.J., Bhatia-Gaur, R., Banach-Petrosky, W.A., Desai, N., Wang, Y., Hayward, S.W., Cunha, G.R., Cardiff, R.D., Shen, M.M., and Abate-Shen, C. (2002). Nkx3.1 mutant mice recapitulate early stages of prostate carcinogenesis. *Cancer research* 62, 2999-3004.

Kim, T.M., Xi, R., Luquette, L.J., Park, R.W., Johnson, M.D., and Park, P.J. (2013). Functional genomic analysis of chromosomal aberrations in a compendium of 8000 cancer genomes. *Genome research* 23, 217-227.

Klatte, T., Kroeger, N., Rampersaud, E.N., Birkhauser, F.D., Logan, J.E., Sonn, G., Riss, J., Rao, P.N., Kabbinar, F.F., Belldegrun, A.S., *et al.* (2012). Gain of chromosome 8q is associated with metastases and poor survival of patients with clear cell renal cell carcinoma. *Cancer* 118, 5777-5782.

Koivisto, P., Kononen, J., Palmberg, C., Tammela, T., Hyytinen, E., Isola, J., Trapman, J., Cleutjens, K., Noordzij, A., Visakorpi, T., *et al.* (1997). Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer research* 57, 314-319.

Koontz, B.F., and Lee, W.R. (2013). Radiation therapy for prostate cancer. *Surg Oncol Clin N Am* 22, 483-494.

- Kraus, J., Pantel, K., Pinkel, D., Albertson, D.G., and Speicher, M.R. (2003). High-resolution genomic profiling of occult micrometastatic tumor cells. *Genes, chromosomes & cancer* 36, 159-166.
- Kristal, A.R., Arnold, K.B., Neuhouser, M.L., Goodman, P., Platz, E.A., Albanes, D., and Thompson, I.M. (2010). Diet, supplement use, and prostate cancer risk: results from the prostate cancer prevention trial. *American journal of epidemiology* 172, 566-577.
- Kronz, J.D., Allan, C.H., Shaikh, A.A., and Epstein, J.I. (2001). Predicting cancer following a diagnosis of high-grade prostatic intraepithelial neoplasia on needle biopsy: data on men with more than one follow-up biopsy. *The American journal of surgical pathology* 25, 1079-1085.
- Kwak, M.K., Johnson, D.T., Zhu, C., Lee, S.H., Ye, D.W., Luong, R., and Sun, Z. (2013). Conditional deletion of the Pten gene in the mouse prostate induces prostatic intraepithelial neoplasms at early ages but a slow progression to prostate tumors. *PloS one* 8, e53476.
- Lapointe, J., Kim, Y.H., Miller, M.A., Li, C., Kaygusuz, G., van de Rijn, M., Huntsman, D.G., Brooks, J.D., and Pollack, J.R. (2007a). A variant TMPRSS2 isoform and ERG fusion product in prostate cancer with implications for molecular diagnosis. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 20, 467-473.
- Lapointe, J., Li, C., Giacomini, C.P., Salari, K., Huang, S., Wang, P., Ferrari, M., Hernandez-Boussard, T., Brooks, J.D., and Pollack, J.R. (2007b). Genomic profiling reveals alternative genetic pathways of prostate tumorigenesis. *Cancer research* 67, 8504-8510.
- Lapointe, J., Li, C., Higgins, J.P., van de Rijn, M., Bair, E., Montgomery, K., Ferrari, M., Egevad, L., Rayford, W., Bergerheim, U., *et al.* (2004). Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America* 101, 811-816.
- Laudet, V., Hanni, C., Stehelin, D., and Duterque-Coquillaud, M. (1999). Molecular phylogeny of the ETS gene family. *Oncogene* 18, 1351-1359.
- Lee, J.S. (2007). GSTP1 promoter hypermethylation is an early event in breast carcinogenesis. *Virchows Archiv : an international journal of pathology* 450, 637-642.
- Leinonen, K.A., Tolonen, T.T., Bracken, H., Stenman, U.H., Tammela, T.L., Saramaki, O.R., and Visakorpi, T. (2010). Association of SPINK1 expression and TMPRSS2:ERG fusion with prognosis in endocrine-treated prostate cancer. *Clinical*

cancer research : an official journal of the American Association for Cancer Research 16, 2845-2851.

Lin, H.K., Hu, Y.C., Lee, D.K., and Chang, C. (2004). Regulation of androgen receptor signaling by PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor through distinct mechanisms in prostate cancer cells. *Molecular endocrinology* 18, 2409-2423.

Lonigro, R.J., Grasso, C.S., Robinson, D.R., Jing, X., Wu, Y.M., Cao, X., Quist, M.J., Tomlins, S.A., Pienta, K.J., and Chinnaiyan, A.M. (2011). Detection of somatic copy number alterations in cancer using targeted exome capture sequencing. *Neoplasia* 13, 1019-1025.

Look, A.T. (1997). Oncogenic transcription factors in the human acute leukemias. *Science* 278, 1059-1064.

Lu-Yao, G.L., Albertsen, P.C., Moore, D.F., Shih, W., Lin, Y., DiPaola, R.S., Barry, M.J., Zietman, A., O'Leary, M., Walker-Corkery, E., *et al.* (2009). Outcomes of localized prostate cancer following conservative management. *JAMA : the journal of the American Medical Association* 302, 1202-1209.

Lubahn, D.B., Joseph, D.R., Sullivan, P.M., Willard, H.F., French, F.S., and Wilson, E.M. (1988). Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science* 240, 327-330.

Lugo, T.G., Pendergast, A.M., Muller, A.J., and Witte, O.N. (1990). Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science* 247, 1079-1082.

Macintosh, C.A., Stower, M., Reid, N., and Maitland, N.J. (1998). Precise microdissection of human prostate cancers reveals genotypic heterogeneity. *Cancer research* 58, 23-28.

Magee, J.A., Abdulkadir, S.A., and Milbrandt, J. (2003). Haploinsufficiency at the Nkx3.1 locus. A paradigm for stochastic, dosage-sensitive gene regulation during tumor initiation. *Cancer cell* 3, 273-283.

Marandola, P., Bonghi, A., Jallous, H., Bombardelli, E., Morazzoni, P., Gerardini, M., Tiscione, D., and Albergati, F. (2004). Molecular biology and the staging of prostate cancer. *Annals of the New York Academy of Sciences* 1028, 294-312.

Marcelli, M., Ittmann, M., Mariani, S., Sutherland, R., Nigam, R., Murthy, L., Zhao, Y., DiConcini, D., Puxeddu, E., Esen, A., *et al.* (2000). Androgen receptor mutations in prostate cancer. *Cancer research* 60, 944-949.

Matera, A.G., Wu, W., Imai, H., O'Keefe, C.L., and Chan, E.K. (1997). Molecular cloning of the RNA polymerase I transcription factor hUBF/NOR-90 (UBTF) gene and localization to 17q21.3 by fluorescence in situ hybridization and radiation hybrid mapping. *Genomics* 47, 135-138.

McNeal, J.E. (1981). The zonal anatomy of the prostate. *The Prostate* 2, 35-49.

Mehra, R., Tomlins, S.A., Shen, R., Nadeem, O., Wang, L., Wei, J.T., Pienta, K.J., Ghosh, D., Rubin, M.A., Chinnaiyan, A.M., *et al.* (2007). Comprehensive assessment of TMPRSS2 and ETS family gene aberrations in clinically localized prostate cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 20, 538-544.

Mehra, R., Tomlins, S.A., Yu, J., Cao, X., Wang, L., Menon, A., Rubin, M.A., Pienta, K.J., Shah, R.B., and Chinnaiyan, A.M. (2008). Characterization of TMPRSS2-ETS gene aberrations in androgen-independent metastatic prostate cancer. *Cancer research* 68, 3584-3590.

Milecki, P., Kwias, Z., and Martenka, D.J. (2007). Radiotherapy combined with hormonal therapy (RT-HT) in prostate cancer patients with low, intermediate, and high risk of biochemical recurrence: perspective and therapeutic gain analysis. *Neoplasma* 54, 7-15.

Minner, S., Gartner, M., Freudenthaler, F., Bauer, M., Kluth, M., Salomon, G., Heinzer, H., Graefen, M., Bokemeyer, C., Simon, R., *et al.* (2013). Marked heterogeneity of ERG expression in large primary prostate cancers. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 26, 106-116.

Mitelman, F., Johansson, B., and Mertens, F. (2004). Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer. *Nature genetics* 36, 331-334.

Mitelman, F., Mertens, F., and Johansson, B. (2005). Prevalence estimates of recurrent balanced cytogenetic aberrations and gene fusions in unselected patients with neoplastic disorders. *Genes, chromosomes & cancer* 43, 350-366.

Miyake, H., Nelson, C., Rennie, P.S., and Gleave, M.E. (2000). Testosterone-repressed prostate message-2 is an antiapoptotic gene involved in progression to androgen independence in prostate cancer. *Cancer research* 60, 170-176.

Miyoshi, Y., Uemura, H., Fujinami, K., Mikata, K., Harada, M., Kitamura, H., Koizumi, Y., and Kubota, Y. (2000). Fluorescence in situ hybridization evaluation

of c-myc and androgen receptor gene amplification and chromosomal anomalies in prostate cancer in Japanese patients. *The Prostate* 43, 225-232.

Montie, J.E. (1994). Staging of prostate cancer. *Cancer* 74, 1-3.

Nam, R.K., Sugar, L., Wang, Z., Yang, W., Kitching, R., Klotz, L.H., Venkateswaran, V., Narod, S.A., and Seth, A. (2007a). Expression of TMPRSS2:ERG gene fusion in prostate cancer cells is an important prognostic factor for cancer progression. *Cancer biology & therapy* 6, 40-45.

Nam, R.K., Sugar, L., Yang, W., Srivastava, S., Klotz, L.H., Yang, L.Y., Stanimirovic, A., Encioiu, E., Neill, M., Loblaw, D.A., *et al.* (2007b). Expression of the TMPRSS2:ERG fusion gene predicts cancer recurrence after surgery for localised prostate cancer. *British journal of cancer* 97, 1690-1695.

Narod, S.A., Seth, A., and Nam, R. (2008). Fusion in the ETS gene family and prostate cancer. *British journal of cancer* 99, 847-851.

Nowell, P.C. (1976). The clonal evolution of tumor cell populations. *Science* 194, 23-28.

Nowell, P.C., and Hungerford, D.A. (1960). Chromosome studies on normal and leukemic human leukocytes. *Journal of the National Cancer Institute* 25, 85-109.

Ohigashi, T., Mizuno, R., Nakashima, J., Marumo, K., and Murai, M. (2005). Inhibition of Wnt signaling downregulates Akt activity and induces chemosensitivity in PTEN-mutated prostate cancer cells. *The Prostate* 62, 61-68.

Okotie, O.T., Roehl, K.A., Han, M., Loeb, S., Gashti, S.N., and Catalona, W.J. (2007). Characteristics of prostate cancer detected by digital rectal examination only. *Urology* 70, 1117-1120.

Ornstein, D.K., Cinquanta, M., Weiler, S., Duray, P.H., Emmert-Buck, M.R., Vocke, C.D., Linehan, W.M., and Ferretti, J.A. (2001). Expression studies and mutational analysis of the androgen regulated homeobox gene NKX3.1 in benign and malignant prostate epithelium. *The Journal of urology* 165, 1329-1334.

Park, K., Tomlins, S.A., Mudaliar, K.M., Chiu, Y.L., Esgueva, R., Mehra, R., Suleman, K., Varambally, S., Brenner, J.C., MacDonald, T., *et al.* (2010). Antibody-based detection of ERG rearrangement-positive prostate cancer. *Neoplasia* 12, 590-598.

Perner, S., Demichelis, F., Beroukhi, R., Schmidt, F.H., Mosquera, J.M., Setlur, S., Tchinda, J., Tomlins, S.A., Hofer, M.D., Pienta, K.G., *et al.* (2006). TMPRSS2:ERG fusion-associated deletions provide insight into the heterogeneity of prostate cancer. *Cancer research* 66, 8337-8341.

Perner, S., Mosquera, J.M., Demichelis, F., Hofer, M.D., Paris, P.L., Simko, J., Collins, C., Bismar, T.A., Chinnaiyan, A.M., De Marzo, A.M., *et al.* (2007). TMPRSS2-ERG fusion prostate cancer: an early molecular event associated with invasion. *The American journal of surgical pathology* 31, 882-888.

Perner, S., Rupp, N.J., Braun, M., Rubin, M.A., Moch, H., Dietel, M., Wernert, N., Jung, K., Stephan, C., and Kristiansen, G. (2013). Loss of SLC45A3 protein (prostein) expression in prostate cancer is associated with SLC45A3-ERG gene rearrangement and an unfavorable clinical course. *International journal of cancer Journal international du cancer* 132, 807-812.

Petrovics, G., Liu, A., Shaheduzzaman, S., Furusato, B., Sun, C., Chen, Y., Nau, M., Ravindranath, L., Chen, Y., Dobi, A., *et al.* (2005). Frequent overexpression of ETS-related gene-1 (ERG1) in prostate cancer transcriptome. *Oncogene* 24, 3847-3852.

Petrylak, D.P., Tangen, C.M., Hussain, M.H., Lara, P.N., Jr., Jones, J.A., Taplin, M.E., Burch, P.A., Berry, D., Moinpour, C., Kohli, M., *et al.* (2004). Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *The New England journal of medicine* 351, 1513-1520.

Pettersson, A., Graff, R.E., Bauer, S.R., Pitt, M.J., Lis, R.T., Stack, E.C., Martin, N.E., Kunz, L., Penney, K.L., Ligon, A.H., *et al.* (2012). The TMPRSS2:ERG rearrangement, ERG expression, and prostate cancer outcomes: a cohort study and meta-analysis. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 21, 1497-1509.

Porter, C.R., Kodama, K., Gibbons, R.P., Correa, R., Jr., Chun, F.K., Perrotte, P., and Karakiewicz, P.I. (2006). 25-year prostate cancer control and survival outcomes: a 40-year radical prostatectomy single institution series. *The Journal of urology* 176, 569-574.

Postma, R., Schroder, F.H., and van der Kwast, T.H. (2005). Atrophy in prostate needle biopsy cores and its relationship to prostate cancer incidence in screened men. *Urology* 65, 745-749.

Prensner, J.R., and Chinnaiyan, A.M. (2009). Oncogenic gene fusions in epithelial carcinomas. *Current opinion in genetics & development* 19, 82-91.

Pui, C.H., and Campana, D. (2007). Age-related differences in leukemia biology and prognosis: the paradigm of MLL-AF4-positive acute lymphoblastic leukemia. *Leukemia* 21, 593-594.

Qian, J., Hirasawa, K., Bostwick, D.G., Bergstralh, E.J., Slezak, J.M., Anderl, K.L., Borell, T.J., Lieber, M.M., and Jenkins, R.B. (2002). Loss of p53 and c-myc overrepresentation in stage T(2-3)N(1-3)M(0) prostate cancer are potential markers for cancer progression. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 15, 35-44.

Qian, J., Wollan, P., and Bostwick, D.G. (1997). The extent and multicentricity of high-grade prostatic intraepithelial neoplasia in clinically localized prostatic adenocarcinoma. *Human pathology* 28, 143-148.

Rajput, A.B., Miller, M.A., De Luca, A., Boyd, N., Leung, S., Hurtado-Coll, A., Fazli, L., Jones, E.C., Palmer, J.B., Gleave, M.E., *et al.* (2007). Frequency of the TMPRSS2:ERG gene fusion is increased in moderate to poorly differentiated prostate cancers. *Journal of clinical pathology* 60, 1238-1243.

Rehman, Y., and Rosenberg, J.E. (2012). Abiraterone acetate: oral androgen biosynthesis inhibitor for treatment of castration-resistant prostate cancer. *Drug design, development and therapy* 6, 13-18.

Ribeiro, F.R., Diep, C.B., Jeronimo, C., Henrique, R., Lopes, C., Eknaes, M., Lingjaerde, O.C., Lothe, R.A., and Teixeira, M.R. (2006a). Statistical dissection of genetic pathways involved in prostate carcinogenesis. *Genes, chromosomes & cancer* 45, 154-163.

Ribeiro, F.R., Henrique, R., Martins, A.T., Jeronimo, C., and Teixeira, M.R. (2007). Relative copy number gain of MYC in diagnostic needle biopsies is an independent prognostic factor for prostate cancer patients. *European urology* 52, 116-125.

Ribeiro, F.R., Jeronimo, C., Henrique, R., Fonseca, D., Oliveira, J., Lothe, R.A., and Teixeira, M.R. (2006b). 8q gain is an independent predictor of poor survival in diagnostic needle biopsies from prostate cancer suspects. *Clinical cancer research : an official journal of the American Association for Cancer Research* 12, 3961-3970.

Rickman, D.S., Pflueger, D., Moss, B., VanDoren, V.E., Chen, C.X., de la Taille, A., Kuefer, R., Tewari, A.K., Setlur, S.R., Demichelis, F., *et al.* (2009). SLC45A3-ELK4 is a novel and frequent erythroblast transformation-specific fusion transcript in prostate cancer. *Cancer research* 69, 2734-2738.

Rosen, P., Sesterhenn, I.A., Brassell, S.A., McLeod, D.G., Srivastava, S., and Dobi, A. (2012). Clinical potential of the ERG oncoprotein in prostate cancer. *Nature reviews Urology* 9, 131-137.

Rubin, M.A., and Chinnaiyan, A.M. (2006). Bioinformatics approach leads to the discovery of the TMPRSS2:ETS gene fusion in prostate cancer. *Laboratory investigation; a journal of technical methods and pathology* 86, 1099-1102.

Sanchez-Ortiz, R.F., Troncoso, P., Babaian, R.J., Lloreta, J., Johnston, D.A., and Pettaway, C.A. (2006). African-American men with nonpalpable prostate cancer exhibit greater tumor volume than matched white men. *Cancer* 107, 75-82.

Sandblom, G., Dufmats, M., and Varenhorst, E. (2000). Long-term survival in a Swedish population-based cohort of men with prostate cancer. *Urology* 56, 442-447.

Sandhu, J.S., and Te, A.E. (2004). Benign Prostatic Hyperplasia (BPH). In *Encyclopedia of Endocrine Diseases*, L. Martini, ed. (Milano: Academic Press), pp. 341-344.

Sankar, S., and Lessnick, S.L. (2011). Promiscuous partnerships in Ewing's sarcoma. *Cancer genetics* 204, 351-365.

Saramaki, O., Willi, N., Bratt, O., Gasser, T.C., Koivisto, P., Nupponen, N.N., Bubendorf, L., and Visakorpi, T. (2001). Amplification of EIF3S3 gene is associated with advanced stage in prostate cancer. *The American journal of pathology* 159, 2089-2094.

Saramaki, O.R., Harjula, A.E., Martikainen, P.M., Vessella, R.L., Tammela, T.L., and Visakorpi, T. (2008). TMPRSS2:ERG fusion identifies a subgroup of prostate cancers with a favorable prognosis. *Clinical cancer research : an official journal of the American Association for Cancer Research* 14, 3395-3400.

Sarker, D., Reid, A.H., Yap, T.A., and de Bono, J.S. (2009). Targeting the PI3K/AKT pathway for the treatment of prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 15, 4799-4805.

Sato, K., Qian, J., Slezak, J.M., Lieber, M.M., Bostwick, D.G., Bergstralh, E.J., and Jenkins, R.B. (1999). Clinical significance of alterations of chromosome 8 in high-grade, advanced, nonmetastatic prostate carcinoma. *Journal of the National Cancer Institute* 91, 1574-1580.

Scher, H.I., Fizazi, K., Saad, F., Taplin, M.E., Sternberg, C.N., Miller, K., de Wit, R., Mulders, P., Chi, K.N., Shore, N.D., *et al.* (2012). Increased survival with enzalutamide in prostate cancer after chemotherapy. *The New England journal of medicine* 367, 1187-1197.

Schulz, W.A., Burchardt, M., and Cronauer, M.V. (2003). Molecular biology of prostate cancer. *Molecular human reproduction* 9, 437-448.

- Seth, A., and Watson, D.K. (2005). ETS transcription factors and their emerging roles in human cancer. *European journal of cancer* 41, 2462-2478.
- Sharrocks, A.D. (2001). The ETS-domain transcription factor family. *Nature reviews Molecular cell biology* 2, 827-837.
- Sharrocks, A.D., Brown, A.L., Ling, Y., and Yates, P.R. (1997). The ETS-domain transcription factor family. *The international journal of biochemistry & cell biology* 29, 1371-1387.
- Shen, M.M., and Abate-Shen, C. (2010). Molecular genetics of prostate cancer: new prospects for old challenges. *Genes & development* 24, 1967-2000.
- Shevchuk, M.M., and Robinson, B.D. (2013). The Pathology of Prostatic Carcinoma. In *Prostate Cancer: A Comprehensive Perspective*, A. Tewari, ed. (London: Springer-Verlag), pp. 235-262.
- Siegel, R., Naishadham, D., and Jemal, A. (2012). Cancer statistics, 2012. *CA: a cancer journal for clinicians* 62, 10-29.
- Singh, D., Febbo, P.G., Ross, K., Jackson, D.G., Manola, J., Ladd, C., Tamayo, P., Renshaw, A.A., D'Amico, A.V., Richie, J.P., *et al.* (2002). Gene expression correlates of clinical prostate cancer behavior. *Cancer cell* 1, 203-209.
- Sircar, K., Yoshimoto, M., Monzon, F.A., Koumakpayi, I.H., Katz, R.L., Khanna, A., Alvarez, K., Chen, G., Darnel, A.D., Aprikian, A.G., *et al.* (2009). PTEN genomic deletion is associated with p-Akt and AR signalling in poorer outcome, hormone refractory prostate cancer. *The Journal of pathology* 218, 505-513.
- Smith, M.R., Saad, F., Coleman, R., Shore, N., Fizazi, K., Tombal, B., Miller, K., Sieber, P., Karsh, L., Damiao, R., *et al.* (2012). Denosumab and bone-metastasis-free survival in men with castration-resistant prostate cancer: results of a phase 3, randomised, placebo-controlled trial. *Lancet* 379, 39-46.
- Soller, M.J., Isaksson, M., Elfving, P., Soller, W., Lundgren, R., and Panagopoulos, I. (2006). Confirmation of the high frequency of the TMPRSS2/ERG fusion gene in prostate cancer. *Genes, chromosomes & cancer* 45, 717-719.
- Squire, J.A., Park, P.C., Yoshimoto, M., Alami, J., Williams, J.L., Evans, A., and Joshua, A.M. (2011). Prostate cancer as a model system for genetic diversity in tumors. *Advances in cancer research* 112, 183-216.
- Stamey, T.A., Yang, N., Hay, A.R., McNeal, J.E., Freiha, F.S., and Redwine, E. (1987). Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *The New England journal of medicine* 317, 909-916.

Steiner, T., Junker, K., Burkhardt, F., Braunsdorf, A., Janitzky, V., and Schubert, J. (2002). Gain in chromosome 8q correlates with early progression in hormonal treated prostate cancer. *European urology* 41, 167-171.

Sternberg, C.N. (2008). Systemic chemotherapy and new experimental approaches in the treatment of metastatic prostate cancer. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 19 Suppl 7, vii91-95.

Svensson, M.A., LaFargue, C.J., MacDonald, T.Y., Pflueger, D., Kitabayashi, N., Santa-Cruz, A.M., Garsha, K.E., Sathyanarayana, U.G., Riley, J.P., Yun, C.S., *et al.* (2011). Testing mutual exclusivity of ETS rearranged prostate cancer. *Laboratory investigation; a journal of technical methods and pathology* 91, 404-412.

Swalwell, J.I., Vocke, C.D., Yang, Y., Walker, J.R., Grouse, L., Myers, S.H., Gillespie, J.W., Bostwick, D.G., Duray, P.H., Linehan, W.M., *et al.* (2002). Determination of a minimal deletion interval on chromosome band 8p21 in sporadic prostate cancer. *Genes, chromosomes & cancer* 33, 201-205.

Swanson, T.A., Krueger, S.A., Galoforo, S., Thibodeau, B.J., Martinez, A.A., Wilson, G.D., and Marples, B. (2011). TMPRSS2/ERG fusion gene expression alters chemo- and radio-responsiveness in cell culture models of androgen independent prostate cancer. *The Prostate*.

Takahashi, S., Qian, J., Brown, J.A., Alcaraz, A., Bostwick, D.G., Lieber, M.M., and Jenkins, R.B. (1994). Potential markers of prostate cancer aggressiveness detected by fluorescence in situ hybridization in needle biopsies. *Cancer research* 54, 3574-3579.

Tannock, I.F., de Wit, R., Berry, W.R., Horti, J., Pluzanska, A., Chi, K.N., Oudard, S., Theodore, C., James, N.D., Turesson, I., *et al.* (2004). Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *The New England journal of medicine* 351, 1502-1512.

Taplin, M.E., Bubley, G.J., Shuster, T.D., Frantz, M.E., Spooner, A.E., Ogata, G.K., Keer, H.N., and Balk, S.P. (1995). Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *The New England journal of medicine* 332, 1393-1398.

Taylor, B.S., Schultz, N., Hieronymus, H., Gopalan, A., Xiao, Y., Carver, B.S., Arora, V.K., Kaushik, P., Cerami, E., Reva, B., *et al.* (2010). Integrative genomic profiling of human prostate cancer. *Cancer cell* 18, 11-22.

Teixeira, M.R. (2008). Chromosome mechanisms giving rise to the TMPRSS2-ERG fusion oncogene in prostate cancer and HGPIN lesions. *The American journal of surgical pathology* 32, 642-644; author reply 644.

Teixeira, M.R., Ribeiro, F.R., Eknaes, M., Waehre, H., Stenwig, A.E., Giercksky, K.E., Heim, S., and Lothe, R.A. (2004). Genomic analysis of prostate carcinoma specimens obtained via ultrasound-guided needle biopsy may be of use in preoperative decision-making. *Cancer* 101, 1786-1793.

Thompson, I.M., Pauler, D.K., Goodman, P.J., Tangen, C.M., Lucia, M.S., Parnes, H.L., Minasian, L.M., Ford, L.G., Lippman, S.M., Crawford, E.D., *et al.* (2004). Prevalence of prostate cancer among men with a prostate-specific antigen level $< \text{or } = 4.0$ ng per milliliter. *The New England journal of medicine* 350, 2239-2246.

Tomlins, S.A., Laxman, B., Dhanasekaran, S.M., Helgeson, B.E., Cao, X., Morris, D.S., Menon, A., Jing, X., Cao, Q., Han, B., *et al.* (2007). Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature* 448, 595-599.

Tomlins, S.A., Mehra, R., Rhodes, D.R., Smith, L.R., Roulston, D., Helgeson, B.E., Cao, X., Wei, J.T., Rubin, M.A., Shah, R.B., *et al.* (2006). TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. *Cancer research* 66, 3396-3400.

Tomlins, S.A., Rhodes, D.R., Perner, S., Dhanasekaran, S.M., Mehra, R., Sun, X.W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., *et al.* (2005). Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 310, 644-648.

Tran, C., Ouk, S., Clegg, N.J., Chen, Y., Watson, P.A., Arora, V., Wongvipat, J., Smith-Jones, P.M., Yoo, D., Kwon, A., *et al.* (2009). Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* 324, 787-790.

Tsuchiya, N., Slezak, J.M., Lieber, M.M., Bergstralh, E.J., and Jenkins, R.B. (2002). Clinical significance of alterations of chromosome 8 detected by fluorescence in situ hybridization analysis in pathologic organ-confined prostate cancer. *Genes, chromosomes & cancer* 34, 363-371.

Tu, J.J., Rohan, S., Kao, J., Kitabayashi, N., Mathew, S., and Chen, Y.T. (2007). Gene fusions between TMPRSS2 and ETS family genes in prostate cancer: frequency and transcript variant analysis by RT-PCR and FISH on paraffin-

embedded tissues. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 20, 921-928.

Uren, A., Tcherkasskaya, O., and Toretsky, J.A. (2004). Recombinant EWS-FLI1 oncoprotein activates transcription. *Biochemistry* 43, 13579-13589.

Uren, A., and Toretsky, J.A. (2005). Ewing's sarcoma oncoprotein EWS-FLI1: the perfect target without a therapeutic agent. *Future oncology* 1, 521-528.

van Dekken, H., Alers, J.C., Damen, I.A., Vissers, K.J., Krijtenburg, P.J., Hoedemaeker, R.F., Wildhagen, M.F., Hop, W.C., van der Kwast, T.H., Tanke, H.J., *et al.* (2003). Genetic evaluation of localized prostate cancer in a cohort of forty patients: gain of distal 8q discriminates between progressors and nonprogressors. *Laboratory investigation; a journal of technical methods and pathology* 83, 789-796.

van den Bosch, T., van Beek, J.G., Vaarwater, J., Verdijk, R.M., Naus, N.C., Paridaens, D., de Klein, A., and Kilic, E. (2012). Higher percentage of FISH-determined monosomy 3 and 8q amplification in uveal melanoma cells relate to poor patient prognosis. *Investigative ophthalmology & visual science* 53, 2668-2674.

van Leenders, G.J., Boormans, J.L., Vissers, C.J., Hoogland, A.M., Bressers, A.A., Furusato, B., and Trapman, J. (2011). Antibody EPR3864 is specific for ERG genomic fusions in prostate cancer: implications for pathological practice. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 24, 1128-1138.

Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vanderwinden, J.M., *et al.* (2006). The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 439, 871-874.

Vis, A.N., Hoedemaeker, R.F., Roobol, M., Schroder, F.H., and van der Kwast, T.H. (2001). The predictive value for prostate cancer of lesions that raise suspicion of concomitant carcinoma: an evaluation from a randomized, population-based study of screening for prostate cancer. *Cancer* 92, 524-534.

Vocke, C.D., Pozzatti, R.O., Bostwick, D.G., Florence, C.D., Jennings, S.B., Strup, S.E., Duray, P.H., Liotta, L.A., Emmert-Buck, M.R., and Linehan, W.M. (1996). Analysis of 99 microdissected prostate carcinomas reveals a high frequency of allelic loss on chromosome 8p12-21. *Cancer research* 56, 2411-2416.

Voeller, H.J., Augustus, M., Madike, V., Bova, G.S., Carter, K.C., and Gelmann, E.P. (1997). Coding region of NKX3.1, a prostate-specific homeobox

gene on 8p21, is not mutated in human prostate cancers. *Cancer research* 57, 4455-4459.

Vogelstein, B., and Kinzler, K.W. (1993). The multistep nature of cancer. *Trends in genetics : TIG* 9, 138-141.

Walsh, P.C. (1975). Physiologic basis for hormonal therapy in carcinoma of the prostate. *The Urologic clinics of North America* 2, 125-140.

Wang, J., Cai, Y., Ren, C., and Ittmann, M. (2006). Expression of variant TMPRSS2/ERG fusion messenger RNAs is associated with aggressive prostate cancer. *Cancer research* 66, 8347-8351.

Wang, Y., Romigh, T., He, X., Orloff, M.S., Silverman, R.H., Heston, W.D., and Eng, C. (2010). Resveratrol regulates the PTEN/AKT pathway through androgen receptor-dependent and -independent mechanisms in prostate cancer cell lines. *Human molecular genetics* 19, 4319-4329.

Ward, J.F., Blute, M.L., Slezak, J., Bergstralh, E.J., and Zincke, H. (2003). The long-term clinical impact of biochemical recurrence of prostate cancer 5 or more years after radical prostatectomy. *The Journal of urology* 170, 1872-1876.

Wasylyk, B., Hagman, J., and Gutierrez-Hartmann, A. (1998). Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends in biochemical sciences* 23, 213-216.

Weischenfeldt, J., Simon, R., Feuerbach, L., Schlangen, K., Weichenhan, D., Minner, S., Wuttig, D., Warnatz, H.J., Stehr, H., Rausch, T., *et al.* (2013). Integrative genomic analyses reveal an androgen-driven somatic alteration landscape in early-onset prostate cancer. *Cancer cell* 23, 159-170.

Welford, S.M., Hebert, S.P., Deneen, B., Arvand, A., and Denny, C.T. (2001). DNA binding domain-independent pathways are involved in EWS/FLI1-mediated oncogenesis. *The Journal of biological chemistry* 276, 41977-41984.

Weng, J., Ma, W., Mitchell, D., Zhang, J., and Liu, M. (2005a). Regulation of human prostate-specific G-protein coupled receptor, PSGR, by two distinct promoters and growth factors. *Journal of cellular biochemistry* 96, 1034-1048.

Weng, J., Wang, J., Cai, Y., Stafford, L.J., Mitchell, D., Ittmann, M., and Liu, M. (2005b). Increased expression of prostate-specific G-protein-coupled receptor in human prostate intraepithelial neoplasia and prostate cancers. *International journal of cancer Journal international du cancer* 113, 811-818.

Wheat, W., Fitzsimmons, D., Lennox, H., Krautkramer, S.R., Gentile, L.N., McIntosh, L.P., and Hagman, J. (1999). The highly conserved beta-hairpin of the

paired DNA-binding domain is required for assembly of Pax-Ets ternary complexes. *Molecular and cellular biology* 19, 2231-2241.

Whitesell, L., and Lindquist, S.L. (2005). HSP90 and the chaperoning of cancer. *Nature reviews Cancer* 5, 761-772.

Winnes, M., Lissbrant, E., Damber, J.E., and Stenman, G. (2007). Molecular genetic analyses of the TMPRSS2-ERG and TMPRSS2-ETV1 gene fusions in 50 cases of prostate cancer. *Oncology reports* 17, 1033-1036.

Xu, B., Chevarie-Davis, M., Chevalier, S., Scarlata, E., Zeizafoon, N., Dragomir, A., Tanguay, S., Kassouf, W., Aprikian, A., Fadi, B. (2013). The prognostic role of erg-immunopositivity in prostatic acinar adenocarcinoma: a study including 454 cases and review of the literature. *Human Pathology*

Xu, L.L., Stackhouse, B.G., Florence, K., Zhang, W., Shanmugam, N., Sesterhenn, I.A., Zou, Z., Srikantan, V., Augustus, M., Roschke, V., *et al.* (2000). PSGR, a novel prostate-specific gene with homology to a G protein-coupled receptor, is overexpressed in prostate cancer. *Cancer research* 60, 6568-6572.

Xu, L.L., Sun, C., Petrovics, G., Makarem, M., Furusato, B., Zhang, W., Sesterhenn, I.A., McLeod, D.G., Sun, L., Moul, J.W., *et al.* (2006). Quantitative expression profile of PSGR in prostate cancer. *Prostate cancer and prostatic diseases* 9, 56-61.

Yates, D.R., and Anderson, J.B. (2013). Screening for Prostate Cancer. In *Prostate Cancer: A Comprehensive Perspective*, A. Tewari, ed. (London: Springer-Verlag), pp. 333-346.

Yates, L.R., and Campbell, P.J. (2012). Evolution of the cancer genome. *Nature reviews Genetics* 13, 795-806.

Yoshimoto, M., Joshua, A.M., Chilton-Macneill, S., Bayani, J., Selvarajah, S., Evans, A.J., Zielenska, M., and Squire, J.A. (2006). Three-color FISH analysis of TMPRSS2/ERG fusions in prostate cancer indicates that genomic microdeletion of chromosome 21 is associated with rearrangement. *Neoplasia* 8, 465-469.

Yoshimoto, M., Joshua, A.M., Cunha, I.W., Coudry, R.A., Fonseca, F.P., Ludkovski, O., Zielenska, M., Soares, F.A., and Squire, J.A. (2008). Absence of TMPRSS2:ERG fusions and PTEN losses in prostate cancer is associated with a favorable outcome. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 21, 1451-1460.

Yu, Y.P., Landsittel, D., Jing, L., Nelson, J., Ren, B., Liu, L., McDonald, C., Thomas, R., Dhir, R., Finkelstein, S., *et al.* (2004). Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of

malignancy. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 22, 2790-2799.

Yuan, X., Cai, C., Chen, S., Chen, S., Yu, Z., and Balk, S.P. (2013). Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis. *Oncogene*.