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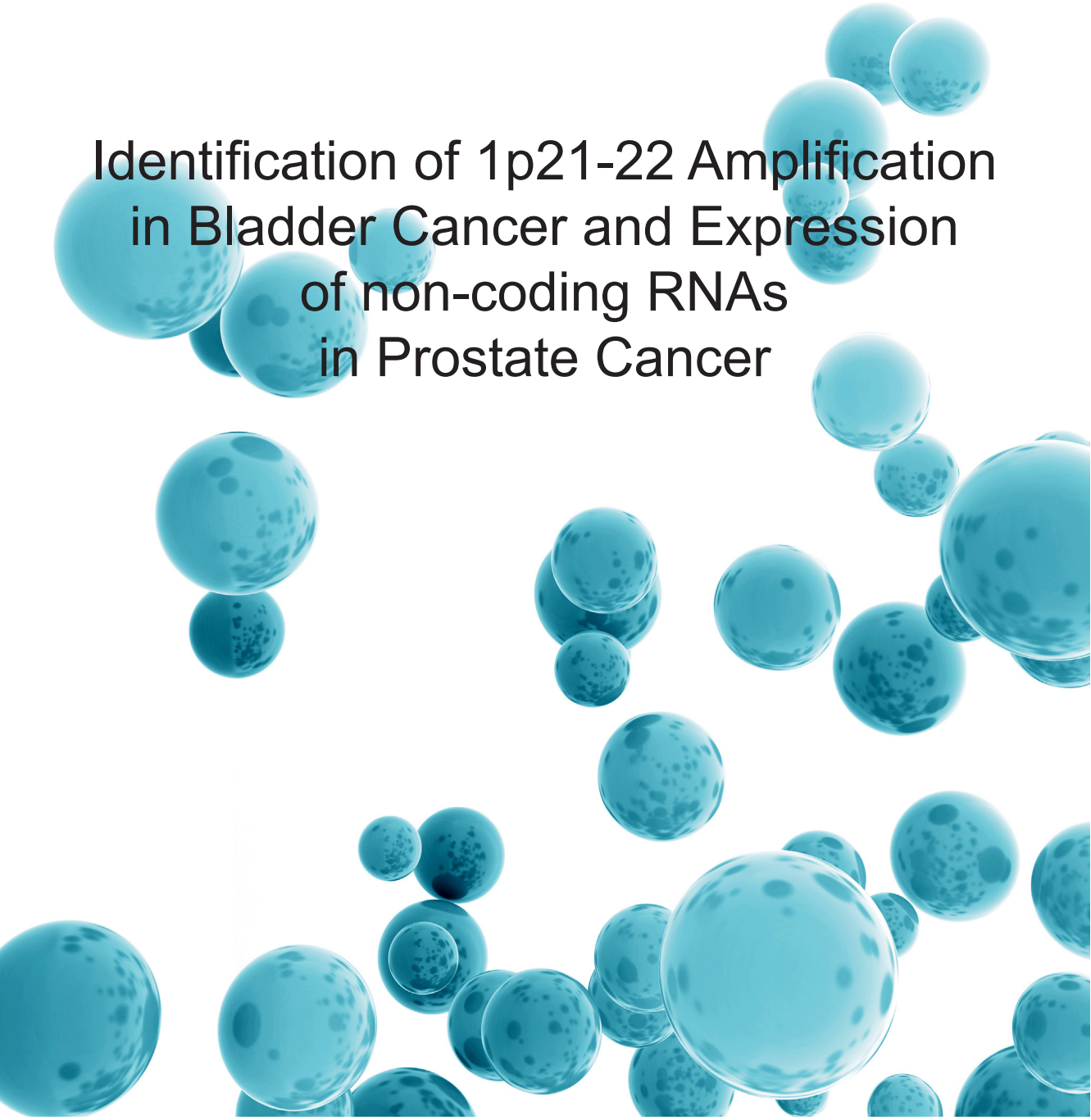
MAURO SCARAVILLI

Identification of 1p21-22 Amplification in Bladder Cancer and Expression of non-coding RNAs in Prostate Cancer



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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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Contents

List of Original Communications

This thesis is based on the following original communications, referred to in the text by Roman numerals:

I. **Mauro Scaravilli**, Paola Asero, Teuvo LJ. Tammela, Tapio Visakorpi, Outi Saramäki. Mapping of the chromosomal amplification 1p21-22 in bladder cancer. BMC Res Notes 2014 7:547.

II. Jayant K. Rane, **Mauro Scaravilli**, Antti Ylipää, Davide Pellacani, Vincent M. Mann, Matthew S. Simms, Matti Nykter, Anne T. Collins, Tapio Visakorpi, Norman J. Maitland. MicroRNA expression profile of primary prostate cancer stem cells as a source of biomarkers and therapeutic targets. Eur Urol 2014 pii:S0302-2838(14)00890-2.

III. **Mauro Scaravilli**, Kati P. Porkka, Anniina Brofeldt, Matti Annala, Teuvo LJ. Tammela, Guido W. Jenster, Matti Nykter, and Tapio Visakorpi. MiR-1247-5p is overexpressed in castration resistant prostate cancer and targets MYCBP2. The Prostate 2015 doi:10.1002/pros.22961.

IV. Michael Olvedy*, **Mauro Scaravilli***, Youri Hoogstrate, Tapio Visakorpi, Guido W. Jenster and Elena S. Martens-Uzunova. A comprehensive repertoire of tRNA-derived fragments in prostate cancer. Submitted for publication.

*These authors contributed equally to the work.

Abbreviations

aCGH	array-comparative genomic hybridization
ACTB	beta-actin
AD	androgen deprivation
ADARs	adenosine deaminases acting on RNA
AML	acute myeloid leukemia
AMV	avian myeloblastosis virus
AR	androgen receptor
ARE	androgen response element
ATCC	American Type Culture Collection
BCG	bacillus Calmette-Guérin
BGI	Beijing Genomic Institute
BMP	bone morphogenetic protein
BPH	benign prostatic hyperplesia
cAMP	3',5'-cyclic adenosine monophosphate
CARN	castration-resistant Nkx3.1-expressing cells
CBC	committed basal cell
CD	cluster of differentiation
CGH	comparative genomic hybridization
ChIPSeq	chromatin immunoprecipitation sequencing
Cis	carcinoma *in situ*
CK	cytokeratin
CLAC	cluster along chromosomes
CLL	cronic lymphocytic leukemia
CpG	cytosine-phosphate-guanine
CRPC	castration-resistant prostate cancer
CSC	cancer stem cell
CTP	cytidine triphosphate
DAPI	4,6-diamidino-2-phenylindole
DHEA	didehydroepiandrosterone
DHT	dihydrotestosterone
DLBCL	diffuse large B-cell lymphoma
DNA	deoxyribonucleic acid
dUTP	deoxyuridine-triphosphate
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition

ERSPC	European Randomized Study of Screening for Prostate Cancer
FISH	fluorescence *in situ* hybridization
GBM	glioblastoma multiforme
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GEO	Gene Expression Omnibus
GWAS	genome-wide association study
GTP	guanosine triphosphate
HCC	hepatocellular carcinoma
hESC	human embryonic stem cell
HGPIN	high-grade prostatic intraepithelial neoplasia
HIV	human immunodeficiency virus
HMG	high-mobility group
Ig	immunoglobulin
LBD	ligand binding domain
LN	lymph node
lncRNA	long non-coding RNA
LOH	loss of heterozygosity
LSC	lukemia stem cell
MET	mesenchymal-epithelial transition
MIBC	muscle invasive bladder cancer
miRNA	microRNA
mRNA	messenger RNA
MRE	miRNA response element
NAP	normal adjacent prostate
ncRNA	non-coding RNA
NEPC	neuroendocrine prostate cancer
NGS	next-generation sequencing
NMIBC	non-muscle invasive bladder cancer
NOD/SCID	non-obese diabetic/SCID
PC	prostate cancer
PCR	polymerase chain-reaction
PIA	proliferative inflammatory atrophy
PIN	prostatic intraepithelial neoplasia
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PSA	prostate-specific antigen
qRT-PCR	quantitative, reverse transcription PCR
RISC	RNA-induced silencing complex
RLC	RISC loading complex
RMA	robust multi-array average
RNA	ribonucleic acid

RNASeq	RNA sequencing
RRBS	reduced representation bisulfite sequencing
rRNA	ribosomal RNA
SC	stem cell
SCI	spinal chord injury
SCID	severe combined immuno-deficient
SD	standard deviation
sncRNA	small non-coding RNA
snoRNA	small nucleolar RNA
SNP	single nucleotide polimorphism
SSC	somatic stem cell
TAC	transit amplifying cell
TIC	tumor-initiating cell
TNM	tumor-node-metastisis
tRNA	transfer RNA
tRF	tRNA-derived RNA fragment
TUR	transurethral resection
TURP	transurethral resection of the prostate
UCSC	University of California Santa Cruz
UGS	urogenital sinus
UTR	untranslated region
WES	whole-exome sequencing
WGS	whole-genome sequencing

Abstract

Cancer is a complex disease, caused by the accumulation of genetic alterations in normal cells. The consequence of these genetic alterations is the disruption of normal cell number homeostasis and uncontrolled cell proliferation. Understanding the molecular mechanisms behind tumorigenesis is essential to identify aggressive and possibly lethal form of the disease as well as to plan effective cancer therapeutic strategies.

Urinary bladder cancer is the most common malignancy of the urinary tract. Most of the tumors arise from the epithelium lining the inside of the urinary bladder (urothelial carcinomas). Squamous cell carcinomas represent fewer than 5% of bladder cancer cases. About 75% of the cases are superficial at diagnosis and the remaining 25% of cases show muscle invasion. Bladder cancer is a heterogeneous disease that is characterized by different genetic alterations, leading to diverse pathways of cancer development and progression. Many of these genetic alterations consist of region-specific gains and losses of DNA copy number. Regions of DNA copy number gain or amplification commonly harbor oncogenes, whereas deleted regions harbor tumor suppressor genes.

In this study, array-comparative genomic hybridization (aCGH) was performed in bladder cancer clinical samples and cell line models, revealing a common amplification at chromosomal region 1p21-22. The minimal region of the amplification was mapped to a region of approximately one Mb in size, containing 11 known genes. The highest amplification was found in the SCaBER squamous cell carcinoma cell line. Four genes, *TMED5*, *DR1*, *RPL5* and *EVI5*, showed significant overexpression in the SCaBER cell line compared to all other samples tested. *DR1* was found to be the most significantly overexpressed in the SCaBER cell line. According to published clinical sample cohorts, *DR1* is also overexpressed in superficial and infiltrating bladder cancers.

Prostate cancer (PC) is the second most commonly diagnosed cancer among males worldwide and the most frequently diagnosed malignancy in developed countries. The heterogeneity of histologic and clinical features of PC is well known,

but the mechanisms underlying the heterogeneity are not understood. Deeper understanding of the molecular mechanisms of PC tumorigenesis is needed to discover more specific biomarkers of aggressive form of the disease.

Recently, there has been increasing attention on the role of microRNAs (miRNAs) in cancer development. Several expression-profiling studies have provided evidence of aberrant expression of miRNAs in prostate cancer and have highlighted the potential use of specific miRNA expression signatures as prognostic or predictive markers.

Similarly to other solid tumors, it is at present unclear whether prostate cancer is organized hierarchically into populations of cells with different proliferative potentials, as cancer stem cell (CSC) model suggests. Several studies have used flow cytometry-based approaches to isolate putative prostate stem cells. Here, genome-wide miRNA expression analysis was performed on patient-derived, stem-like cells (SC), transit-amplifying cells and committed basal (CB) cells. These cell populations were enriched from briefly cultured primary prostate epithelial cells. Each cell subpopulation showed a distinct miRNA expression profile, regardless of its pathologic status. MiR-548c-3p was found to be overexpressed approximately fivefold in SCs, compared with CBs. Functional studies of miR-548c-3p overexpression in CBs showed increased dedifferentiation to a more stem-like phenotype. MiR-548c-3p was also found to be significantly upregulated in CRPC-derived epithelial cells compared with BPH-derived epithelial cells, suggesting that this miRNA is a functional biomarker for PC aggressiveness.

To identify novel, differentially expressed miRNAs, the expression data obtained from recent deep-sequencing experiments on pools of clinical specimens were analyzed. miR-1247-5p, miR-1249, miR-1269a, miR1271-5p, miR-1290, miR-1291 and miR-1299 showed differential expression in malignant samples compared to benign samples and were selected for validation by qRT-PCR.

Significant up-regulation of miR-1247-5p was found in castration-resistant prostate cancer (CRPC) compared to non-malignant prostate. The expression of miR-1247-5p was subsequently studied in PC cell lines where an up-regulation of this miRNA was observed in the androgen-independent PC-3 line. According to on-line target prediction tools *MYCBP2* (myc-binding protein 2) is a high-scoring potential target of miR-1247-5p. The down-regulation of *MYCBP2* at both mRNA and protein levels was demonstrated by the overexpression of miR-1247-5p in PC-

3 and LNCaP models. Next, MYCBP2 was confirmed as a target of miR-1247-5p using luciferase reporter assay.

Several high-throughput sequencing studies in human cancers have recently led to the discovery of additional groups of non-coding RNAs. Next to miRNAs, the most abundant non-coding RNAs in prostate cancer cell lines were found to be fragments derived from tRNAs, termed tRNA-derived RNA fragments (tRFs). The characteristic and abundant expression of the fragments, as well as their precise sequence, indicate that these molecules are not random products of tRNA degradation. However, the precise role of tRFs is unclear. In this study, the expression of tRFs in normal adjacent prostate and different stages of PC was analyzed by RNA-sequencing. A total of 598 unique tRFs were identified, many of which appear to be deregulated in cancer samples compared to controls. Most of the identified tRFs are derived from the 5' and 3' end of mature cytosolic tRNAs, but tRFs produced from pre-tRNA trailers and leaders were also found, as were tRFs from mitochondrial tRNAs. The 5'-derived tRFs comprised the most abundant class of tRFs and represented the major class among upregulated tRFs, whereas 3'-derived tRFs types were dominant among downregulated tRFs in PC. The expression of three tRFs (tRF-544, tRF-315 and tRF-562) was validated in PC using qRT-PCR. Interestingly, the normalized expression ratio of tRF-315 and tRF-544, derived from $tRNA^{Lys}$ and $tRNA^{Phe}$ respectively, emerged as a good indicator of progression-free survival and as a candidate prognostic marker.

In conclusion, a novel amplification, which may harbour important oncogenes, was identified in bladder cancer. In addition, several differentially expressed non-coding RNAs were discovered in prostate cancer. These RNAs may be important drivers of prostate tumorigenesis and putative biomarkers of aggressive form of the disease.

Tiivistelmä

Syöpä on monimutkainen sairaus, jonka aiheuttavat normaaliin soluun kertyvät geneettiset muutokset. Tällaiset geneettiset muutokset häiritsevät normaalien solujen homeostaasia ja johtavat hallitsemattomaan solujen lisääntymiseen. Näiden muutosten tunteminen on tärkeää, jotta voitaisiin kehittää entistä tehokkaampia syövän hoitomuotoja ja diagnostisia menetelmiä tappavan tautimuodon tunnistamiseksi.

Virtsarakon syöpä on yleisin virtsateiden maligniteetti. Useimmat virtsarakon syövät syntyvät välimuotoisesta epiteelistä (uroteliaaliset karsinoomat). Alle 5 % virtsarakon syöpätapauksista on levyepiteeliperäisiä. Virtsarakon syöpä on heterogeeninen sairaus, jolle ovat ominaisia erilaiset geneettiset muutokset, jotka johtavat eri polkuja syövän kehittymiseen ja etenemiseen. Monet näistä geneettisistä muutoksista vaikuttavat geenien kopiolukumäärään johtaen onkogeenien monistumaan ja kasvurajoitegeenien häviämään.

Tässä tutkimuksessa käytettiin sirupohjaista, vertailevaa genomista hybridisaatiomenetelmää (aCGH) geenikopiolukumäärän analysoimiseksi kliinisissä rakkosyöpänäytteissä sekä solulinjoissa. Yhdeksi uudeksi monistuma-alueeksi tunnistettiin kromosomialue 1p21-22. Työssä kartoitettiin ko. aluetta tarkemmin ja osoitettiin, että ns. minimaalinen monistuma-alue käsitti yhden miljoonan emäsparin DNA-jakson sisältäen yhteensä 11 tunnettua geeniä. Korkein monistuma-aste löytyi SCaBER-levyepiteelisyöpäsolulinjasta. Näistä neljä geeniä, TMED5, DR1, RPL5 ja EVI5, yli-ilmeni SCaBER-solulinjassa, DR1 kaikkein eniten. Julkisten tietokantojen perusteella DR1 yli-ilmenee myös kliinisissä näytteissä, jotka edustavat yleistä virtsarakkosyövän histologista tyyppiä.

Eturauhassyöpä on miesten toiseksi yleisin diagnosoitu syöpä maailmassa ja yleisin kehittyneissä maissa. Taudin histologisen ja kliinisen käyttäytymisen monimuotoisuuden taustalla olevia mekanismeja tunnetaan huonosti. Eturauhassyövän molekyylitason mekanismien tunteminen mahdollistaisi uusien taudin aggressiivisuutta kuvastavien biomarkkerien löytämisen.

Viime aikoina on yhä enemmän kiinnitetty huomiota mikroRNA:iden (miRNA) merkitykseen syövän kehittymisessä. Useat tutkimukset ovat osoittaneet, että miRNA-ilmentymistasot ovat muuntuneita eturauhassyövässä. Onkin mahdollista, että niitä voitaisiin käyttää ennusteellisina biomarkkereina.

Kuten muissa syövissä, on epäselvää, koostuuko eturauhassyöpä eri solupopulaatioista, joilla on erilainen jakaantumiskyky, kuten syövän kantasolumallit ennustavat. Useissa tutkimuksissa on käytetty virtaussytometriaa eturauhasen kantasolujen eristämiseen. Tässä tutkimuksessa tehtiin genominlaajuinen miRNA-ilmentymisen analyysi potilaista peräisin olevista kantasolun kaltaisista soluista (SC), välivaiheen soluista ja sitoutuneista (CB) soluista. Jokainen solualapopulaatio poikkesi toisistaan miRNA-ilmentymisprofiililtaan riippumatta siitä, oliko kyseessä syöpänäyte vai ei. MiR-548c-3p:n havaittiin yli-ilmentyvän noin viisinkertaisesti SC-soluissa verrattuna CB-soluihin. Toiminnallisissa tutkimuksissa miR-548c-3p:n yli-ilmentyminen CB-soluissa vähensi erilaistumista kohti kantasoluilmiasua. MiR-548c-3p oli myös merkittävästi yli-ilmentynyt kastraatioresistentistä eturauhassyövästä (CRPC) eristetyissä soluissa verrattuna eturauhasen hyvänlaatuisesta liikakasvusta (BPH) eristettyihin epiteelisoluihin. Tämä viitaa siihen, että miR-548c-3p voisi olla eturauhassyövän aggressiivisuuden biomarkkeri.

Tunnistaaksemme uusia, eri tavoin ilmentyviä miRNA:ita, syväsekvensoimme joukon kliinisiä eturauhassyöpänäytteitä. MiR-1247-5p, miR-1249, miR-1269a, miR1271-5p, miR-1290, miR-1291 ja miR-1299 ilmentyivät eri lailla syövässä ja normaalissa eturauhasessa. Niinpä näitä tutkittiin laajemmassa materiaalissa qRT-PCR-menetelmällä. MiR-1247-5p ilmentyi merkittävästi enemmän CRPC-näytteissä verrattuna ei-maligniin eturauhaseen. Ennusteohjelmien perusteella miR-1247-5p:n yksi kohdegeeni voisi olla MYCBP2 (myc:iä sitova proteiini 2). miR-1247-5p:n yli-ilmentyminen PC-3- ja LNCaP-syöpäsolulinjoissa johti MYCBP2:n ilmentymisen laskuun sekä mRNA- että proteiinitasolla. Lusiferaasireportterikoe vahvisti, että MYCBP2 on miR-1247-5p:n kohde.

Useissa uuden sukupolven sekvensointitutkimuksissa on syövissä löydetty uusia ryhmiä ei-koodaavia RNA:ita. Eturauhassyöpäsolulinjoissa miRNA:iden jälkeen toiseksi yleisin ryhmä ei-koodaavia RNA:ita ovat tRNA:ista peräisin olevat fragmentit (tRFs). Niiden ominaisuudet, runsas ilmentyminen ja tarkka sekvenssi viittaavat siihen, että nämä molekyylit eivät ole satunnaisia tuotteita tRNA:n hajoamisesta. tRF:ien tarkka rooli on kuitenkin edelleen epäselvä. Tässä

tutkimuksessa tRF:ien ilmentymistä analysoitiin potilaan kudosnäytteistä. Tutkimuksessa tunnistettiin yhteensä 598 erilaista tRF:ää, jotka näyttivät ilmentyvän eri lailla syövässä kuin normaalissa kudoksessa. Suurin osa tunnistetuista tRF:ista on peräisin kypsän sytosolisen tRNA:n 5'-ja 3'-päistä, mutta myös muita fragmentteja löytyi. 5'-päästä peräisin olevat tRF:t olivat eniten yli-ilmentyneitä ja 3'-päästä peräsin olevat ali-ilmentyneitä syövässä. Kolmen tRF:n ilmentymiserot varmennettiin qRT-PCR:llä. Normalisoitu $tRNA^{Lys}$:stä ja $tRNA^{Phe}$:stä peräisin olevien tRF-315:n ja tRF-544:n ilmentyvyyssuhde ennusti hyvin taudin etenemistä.

Yhteenvetona tutkimuksessa löydettiin uusi monistuma-alue, jossa saattaa sijaita rakkosyövän kehityksen kannalta tärkeitä onkogeenejä. Lisäksi tunnistettiin useita eturauhassyövässä poikkeavasti ilmentyviä ns. ei-koodaavia RNA:ita. Nämä saattavat olla mekanistisesti tärkeitä eturauhassyövän kehityksen kannalta ja mahdollisia syövän aggressiivisuuden markkereita.

1 Introduction

Prostate cancer and urinary bladder cancer are the most common urological malignancies in developed countries.

Urinary bladder cancer is the fourth most common cancer in men and it is approximately three times more common in males than in females (Jemal *et al.*, 2011). The majority of bladder cancer cases (75%) are non-muscle invasive (NMIBC) at diagnosis. NMIBC is treated by trans-urethral resection of the tumor (TUR-T), followed by chemotherapy or intravesical immunotherapy. The remaining 25% of the cases present at diagnosis with muscle invasive bladder cancer (MIBC). If the tumor is still confined to the bladder, these patients are primarily treated with radical cystectomy. However, 50% of these cases will progress to metastatic disease (Babjuk *et al.*, 2011; Sawhney *et al.*, 2006). Progression from minimally invasive to deeply invasive cancer is concurrent with the acquisition of genomic alterations, which increase the malignant potential of cancer cells. Bladder cancer is a heterogeneous disease with a unique natural history characterized by a highly variable clinical course. The clinical heterogeneity suggests an underlying heterogeneity of genetic alterations leading to different pathways of cancer development and progression (Wolff, 2007). Many of the genetic alterations found in bladder cancer consist of region-specific gain or loss of DNA copy number, which can lead to the identification of key genes involved. Various approaches have been extensively used to study urothelial carcinoma and identify altered genes, including cytogenetics, fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), loss of heterozygosity (LOH) and, more recently, array-CGH (aCGH) (Hoglund, 2012).

Prostate cancer (PC) is the most frequently diagnosed cancer among males in developed countries (Jemal *et al.*, 2011). Although surgery and/or radiation therapy are effective treatments for early-stage disease, 30-40% of cases will progress to advanced disease. For advanced disease, androgen deprivation is initially highly efficient, but patients will eventually develop castration-resistant prostate cancer (CRPC), which remains incurable (Scher and Sawyers, 2005). There has recently

been increasing interest on the role of non-coding RNAs in the molecular mechanisms of cancer development. Non-coding RNAs are a class of small RNA molecules that are not translated into proteins and are involved in the regulation of many cellular processes (Esteller, 2011). MicroRNAs (miRNAs) function in the negative regulation of gene expression. Overexpressed miRNAs may act as oncogenes as they can repress tumor suppressor genes or apoptosis-related genes and in a similar fashion, downregulated miRNAs may function as tumor-suppressors, downregulating the expression of oncogenes or proliferation-related genes (Zhang *et al.*, 2007). However, clinical translation of miRNAs as biomarkers and/or therapeutic targets remains limited, likely due to the heterogeneity and discrepancies in PC miRNA expression profiles (Coppola *et al.*, 2010).

Several high-throughput sequencing studies in human cancers have recently led to the discovery of additional groups of non-coding RNAs. Next to miRNAs, the most abundant non-coding RNAs in prostate cancer cell lines were found to be fragments derived from tRNAs, termed tRNA-derived RNA fragments (tRFs) (Lee *et al.*, 2009). The characteristic and abundant expression of the fragments, as well as their precise sequence, indicate that these molecules are not random products of tRNA degradation. The precise role of tRFs is unclear.

The cancer stem cell (CSC) model is based on the hypothesis that cells within a tumor are organized hierarchically into clonally derived populations with different proliferative potentials. In this model, the cancer stem cell population is characterized by the ability to self-renew and to generate the diverse populations that constitute the tumor (Jordan *et al.*, 2006; Reya *et al.*, 2001).

It has previously been shown that cells with stem-like phenotype can be isolated from prostate cancer tissues, using cell-surface markers. These putative prostate cancer stem cells (SCs) can be distinguished from cells with more limited proliferative capacity, termed transit amplifying cells (TACs), as well as from basal cells committed to differentiation, termed committed basal cells (CBCs) (Collins *et al.*, 2005; Richardson *et al.*, 2004).

2 Review of the Literature

2.1 Molecular mechanisms of cancer development

2.1.1 The biology of cancer

Cancer is a common disease, with 14.1 million new cases and 8.2 million deaths reported worldwide in 2012 (Torre *et al.*, 2015). In recent years, cancer mortality incidence has decreased significantly in more developed countries, but the number of cancer diagnoses is predicted to increase steadily in the future, due to the growth and aging of the population and an increasing prevalence of cancer risk factors (Torre *et al.*, 2015).

In physiological conditions, the cells of the human body divide in a controlled manner, usually in response to specific mitogenic growth signals. Almost every tissue appears to contain a pool of adult stem cells, also referred to as somatic stem cells (SSCs) or tissue-specific stem cells. These cells are undifferentiated and divide at a controlled rate to renew themselves and to further differentiate into tissue-specific cells, guaranteeing tissue homeostasis and regeneration. The somatic stem cells represent only a portion of the total number of cells within a tissue, whereas most of the cells are partly or fully differentiated and characterized by a limited replicative potential (Biteau *et al.*, 2011; Hombach-Klonisch *et al.*, 2008).

In cancer cells, the control of cell division is disrupted, leading to a typical cancer phenotype, characterized by abnormal proliferation, growth signal independence, apoptosis evasion, sustained angiogenesis, invasion and destruction of adjacent tissue and, eventually, the spreading of cancer cells to other parts of the body via blood or lymphatic vessels (metastasis) (Hanahan and Weinberg, 2011).

Cancer is a genetic disease, in which the abnormal control of cell proliferation is caused by the accumulation of mutation events in the genome and consequently changes in gene expression. Mutations can arise spontaneously, due to the intrinsic infidelity of the DNA replication machinery. Mutations can also be caused by

environmental exposure to physical or chemical agents termed mutagens (somatic mutations) and, in some cases, can be directly inherited (germ-line mutations), leading to an increased risk of developing the disease (genetic predisposition or susceptibility) (Stratton *et al.*, 2009). However, the current knowledge of the mechanistic base of somatic mutations in human cancers is limited. In a recent key study, a new algorithm was developed to extract mutational signatures from catalogues of somatic mutations. Almost five million somatic substitutions and small insertions/deletions were compiled from a catalogue of over seven thousand primary cancers, showing a highly variable prevalence of somatic mutations between and within cancer classes. This variability is likely attributable to differences in the duration of the cellular lineage between the zygote and the development of the cancer.

Moreover, most individual cancer genomes were found to exhibit more than one mutational signature and variable combinations of signatures were observed. Some signatures contributed few events to most cancers, whereas others contributed a large number of events to only a few cancer types. Certain signatures were also found to be associated with the age at cancer diagnosis and with known mutagenic exposures, revealing high degree of complexity and diversity of mutational processes underlying the development of cancer (Alexandrov *et al.*, 2013). In another landmark study, the lifetime risk of cancer of a specific tissue was found to be significantly and positively correlated with the average number of divisions taking place in the adult stem cell pool of that specific tissue (Tomasetti and Vogelstein, 2015). The correlation was stronger than any other environmental or inheritable factor, leading to the conclusion that stochastic effects of DNA replication in adult stem cells play a major role in the accumulation of genetic alterations in the genome. Once the cancer has arisen, the acquired mutations are inherited by daughter cells after each replicative event. The genetic changes responsible for increasing the fitness of cancer cells are positively selected in a process defined as somatic evolution (Crespi and Summers, 2005).

2.1.2 Tumor suppressors and oncogenes

Two categories of genes, termed tumor suppressors and oncogenes are targets of many of the mutation events responsible for cancer initiation. These genes can be

further classified based on the specific function of the encoded proteins. *Gatekeeper* genes are directly responsible for maintaining the control of cell cycle and thus the balance of cell number in a renewing cell population, by regulating cell division and apoptosis (Kinzler and Vogelstein, 1997; Pearson and Van der Luijt, 1998). *Caretaker* genes are not responsible for controlling cell growth directly, but have the fundamental function of maintaining genomic integrity and stability through effective repair of DNA damage (Levitt and Hickson, 2002). Mutations in caretaker genes usually lead to an accelerated mutation rate and consequently higher risk of cancer initiation. *Landscaper* genes do not exert their function in the cancer tissue itself, but are active in the surrounding stroma. Mutations in landscapers induce dysregulation of stromal cells, which in turn can promote cancerous growth of the adjacent tissue (Bissell and Radisky, 2001; Michor *et al.*, 2004).

Tumor suppressor genes encode proteins responsible for inhibiting and/or controlling cell proliferation. As the main effect of a tumor suppressor is inhibitory, one copy of the gene is generally sufficient to guarantee the functionality of the protein, therefore, these genes are recessive, and two mutational events that affect both alleles are required to inactivate them (i.e., the two-hit hypothesis) (Knudson, 1971).

An example of a gatekeeper and one of the first tumor suppressor genes to be identified is *RB1* (retinoblastoma), discovered in the malignant tumor of the retina (Cavenee *et al.*, 1983; Friend *et al.*, 1986; Knudson, 1971). *RB1* is dysfunctional in many human tumors (Murphree and Benedict, 1984) and inherited mutations in one of the alleles of the gene confer cancer susceptibility (Kleinerman *et al.*, 2005). The protein encoded by *RB1*, termed pRb, prevents the E2F transcription factors from activating the genes responsible for the initiation of S-phase in the cell cycle, thereby preventing DNA replication and cell division (Dyson, 1998; Leone *et al.*, 1998; Nevins, 1998).

An example of a caretaker gene is the tumor suppressor *ATM*, identified in the autosomal recessive disorder ataxia telangiectasia (Savitsky *et al.*, 1995). The gene encodes a serine/threonine kinase involved in the response to double strand breaks in DNA (Shiloh, 2003). ATM phosphorylates and consequently activates key proteins responsible for DNA repair (Kastan and Lim, 2000). *ATM* mutations are associated with higher risk of several types of cancer (Angele *et al.*, 2003; Gumy-Pause *et al.*, 2004; Thorstenson *et al.*, 2003). The *TP53* tumor suppressor gene can

be considered both a gatekeeper and caretaker, because of the multiple roles of its protein product (Oren and Rotter, 1999). *TP53* encodes a protein named p53, which can be activated by multiple stress factors, including DNA damage-induced cellular stress. p53 subsequently binds specific sequences in the DNA, repressing or activating its target genes (Levine, 1997; Vogelstein *et al.*, 2000; Vousden and Lu, 2002). As a caretaker, p53 triggers the apoptotic response, eliminating cells with potentially harmful genetic alterations (Fritsche *et al.*, 1993). For this essential function, *TP53* has been defined as "the guardian of the genome" (Lane, 1992). As a gatekeeper, p53 induces cell cycle arrest at the G1/S transition phase in response to stress. This arrest can become permanent, resulting in cellular senescence (Hofseth *et al.*, 2004; Levine, 1997). The mutation or inactivation of *TP53* are common features in tumorigenesis and have been described in most human cancers (Hollstein *et al.*, 1991; Levine *et al.*, 1991).

Oncogenes encode proteins that are, in general, responsible for cell growth. In physiological conditions, they function by promoting cell division and replication to guarantee tissue renewal. If mutated, these genes can become abnormally activated and acquire the ability to induce uncontrolled proliferation and cancer (Croce, 2008). Mutations or alterations in oncogenes are dominant. A single event, affecting only one of the alleles, is enough to alter the functionality of the gene. Currently, there is not yet a single accepted standard of oncogene classification, as the products of oncogenes can exert different roles in promoting cell growth. Typical categories of oncogene products are growth factors and their receptors, signal transducers, transcription factors and apoptosis regulators. The Ras proteins, encoded by three ubiquitously expressed oncogenes (*HRAS*, *KRAS* and *NRAS*), represent an example of signal transducers that are very frequently mutated in human cancers (Lowy and Willumsen, 1993). It is estimated that 20% of all human tumors harbor mutations in one of the three *RAS* genes (Bos, 1989). RAS proteins belong to a class of small GTPases that can respond to extra-cellular signals, such as growth factors (Campbell *et al.*, 1998). Growth factors bind growth-factor receptors, usually tyrosine-kinases, which in turn recruit guanine nucleotide exchange factors (GEFs), which are responsible for exchanging the RAS-bound GDP with GTP (Reuther and Der, 2000). GTP-bound RAS becomes activated and binds effector enzymes, which phosphorylate and activate mitogen-activated protein kinases (MAPKs). In turn, MAPKs regulate transcription factors controlling cell proliferation and survival. RAS

mutations constitutively activate the GTPase function, inducing uncontrolled proliferation (Leevers *et al.*, 1994; Marais *et al.*, 1995; Pruitt and Der, 2001).

ERBB2 is one of the most extensively studied oncogenes in human cancer and encodes a member of the family of epidermal growth factor receptors (EGFRs) (Stern, 2000). The binding of the growth factor to the receptor (ERBB2) induces its tyrosine-kinase activity, resulting in the activation of mitogenic signaling pathways, such as the MAP kinase and PI3K/AKT pathways (Rubin and Yarden, 2001). The overexpression of the *ERBB2* gene has been found in several human cancers and occurs in approximately 18 to 20% of breast cancer cases (Owens *et al.*, 2004; Slamon *et al.*, 1987).

MYC (also known as *CMYC*) is a well-studied example of an oncogene and encodes a transcription factor. Interestingly, mutations in the coding sequence of the gene are rarely found in cancer and were discovered only in Burkitt´s lymphoma (Bhatia *et al.*, 1994). Mutations that affect *MYC* are usually associated with chromosomal translocations leading to increased gene expression (Dalla-Favera *et al.*, 1982). *MYC* is overexpressed in approximately 50% of human cancers. The most common alteration responsible for the overexpression of the gene is locus amplification (Vita and Henriksson, 2006). The higher dosage of MYC protein induces cell proliferation by upregulating cyclins and ribosomal RNAs and by down-regulating pro-apoptotic proteins (Schmidt, 1999).

The founding member of the *BCL-2* gene family was the first anti-apoptotic oncogene to be discovered (Tsujimoto *et al.*, 1985). *BCL-2* genes encode both proapoptotic and antiapoptotic protein members. The altered expression of the anti-apoptotic members is observed in many human cancers and leads to effective inhibition of cell death, induced by growth factor deprivation, hypoxia or oxidative stress, increasing the proliferative potential of the cells (Yip and Reed, 2008). Several mechanisms can deregulate the expression of *BCL-2* genes, including gene structure or copy number alteration and the loss of endogenous microRNAs (miRNAs) that downregulate *BCL-2* (Cimmino *et al.*, 2005).

2.1.3 Chromosomal alterations in cancer

Aberrant gene function in tumor suppressors and oncogenes can result from different types of genetic alterations, including point mutations, polymorphisms,

copy number and genome structure alterations, and epigenetic changes. The mechanisms responsible for altered gene function vary between different human tumors and often between tumors originating from the same tissues. Moreover, the prevalence of somatic mutations can differ greatly across tumor types (Albertson *et al.*, 2003). The genetic heterogeneity of cancer represents one of the most challenging aspects in the design of effective therapeutic strategies.

Point mutations are changes in the DNA sequence that affect only one or a few nucleotides and include substitutions of one nucleotide for another and insertions or deletions of small parts of DNA. Genome structure alterations are large-scale mutations in the chromosomal structure and include amplifications, deletions, translocations and inversions. Chromosomal amplifications lead to multiple copies of a certain region of the genome, affecting the dosage of the genes included within the region. As an example, the overexpression of the oncogene *ERBB2* is often caused by the amplification of its coding region (Starczynski *et al.*, 2012).

Chromosomal deletions lead to loss of a certain region of the genome and consequently the genes encoded within it. Typically, the deletion of the functional allele in carriers of a mutated version of tumor suppressor genes is a common event in cancer and is referred to as loss of heterozygosity (LOH). Examples of frequently deleted tumor suppressors in cancer are *PTEN* (Li *et al.*, 1997), *TP53* (Baker *et al.*, 1990), *BRCA1* and *BRCA2* (Nagai *et al.*, 1994).

Chromosomal translocations and inversions change the physical orientation and order of genes harbored in the affected regions, often leading to the juxtaposition of previously separated genetic region and potentially forming new functional entities, termed fusion genes.

The first fusion gene discovered in cancer was described in chronic myelogenous leukemia and involves a reciprocal translocation of chromosome 9 and 22 (Philadelphia chromosome), leading to the generation of the oncogenic fusion gene *BCR-ABL*. *ABL* encodes a mitogen-activated tyrosine-kinase, which becomes constitutively active due to the fusion, driving proliferation (Clarkson *et al.*, 2003).

Advances in DNA sequencing technologies have enabled the study of genome-wide genetic changes in cancer samples. In a recently published study, in which next-generation sequencing (NGS) technologies were used to investigate chromosomal alterations in cancer, a new phenomenon was discovered. Specifically, it was found that tens to hundreds of genomic rearrangements likely occur in a single, catastrophic cellular event (Stephens *et al.*, 2011). The phenomenon is termed chromothripsis

(chromosome shuttering) and gives rise to an alternative view of tumorigenesis, in which several cancer-triggering mutations may be acquired at the same time. This process is in contrast to the above-mentioned paradigm of gradual accumulation of genetic changes, with profound implications in cancer diagnosis and treatment.

Chromothripsis was identified in a chronic lymphocytic leukemia patient, presenting 42 genomic rearrangements, all involving only the long arm of chromosome 4. The subsequent analysis of high-resolution copy number profiles of 746 cancer cell lines revealed complex rearrangements limited to single chromosomes or a few chromosomes in at least 2-3% of all cancers (Stephens *et al.*, 2011). The features of chromothripsis suggest that the chromosome(s) or chromosomal region(s) involved shatter into tens or hundreds of fragments in a single event, likely when they are condensed for mitotic cell division, and are subsequently reassembled incorrectly by the DNA-repair machinery. The observation that the copy number state of the affected chromosome(s) varies only between just one and two copies enforces the hypothesis that these rearrangements are not acquired gradually. Currently, the mechanism(s) responsible for chromothripsis remain unknown, but three possibilities are proposed. Pulses of ionizing radiation could strike chromosomes affecting only specific regions while they are condensed. Alternatively, chromothripsis could be the result of telomere dysfunction, which is already known to promote chromosomal abnormalities, such as end-to-end chromosome fusions and anaphase bridges (Titen and Golic, 2008). Anaphase bridges appear to be involved in the formation of micronuclei containing fragmented DNA (Crasta *et al.*, 2012; Pampalona *et al.*, 2010). Finally, chromosomes might be shattered as a result of aborted programmed cell-death (apoptosis) (Tubio and Estivill, 2011).

2.1.4 Non-coding RNAs

The human genome contains approximately 20-25.000 coding genes, defined as DNA sequences that can be transcribed and subsequently translated into proteins. This number represents only a small fraction (approximately 2%) of the total DNA. The remaining 98% of the genome does not encode proteins but contains a large number of genes that are transcribed into non-coding RNAs (Alexander *et al.*, 2010; Bertone *et al.*, 2004; Eddy, 2001; ENCODE Project Consortium *et al.*,

2007). Non-coding RNAs are RNA molecules that exert their function directly, without translation into proteins. Some non-coding genes are transcribed into RNA molecules with very important and basic biological functions, such as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs). rRNAs are essential structural RNAs that, combined with proteins, form the ribosomes. Ribosomes are large molecular machines that provide the site for protein synthesis. tRNAs are the adaptor molecules that physically link the sequence in the coding messenger RNAs (mRNAs) to the growing polypeptide during protein synthesis, therefore directly translate the language of nucleic acid triplets (codons), into amino acids.

Recently, the rapid evolution of RNA microarrays and RNA deep sequencing technologies have revealed thousands of non-coding RNA molecules (ncRNAs) that can be grouped into two major categories, based on the length of their transcripts. Small ncRNAs are less than 200 bp long, whereas long ncRNAs (lncRNAs) are longer than 200 bp.

These molecules have emerged as a very important part of cell physiology as their function is related to not only housekeeping but also to gene regulation at both pre- and post-transcriptional level. It is currently clear whether ncRNAs are key factors in maintaining proper cellular function, therefore, increasing effort has been invested to investigate their role in human diseases, including cancer (Esteller, 2011).

2.1.4.1 microRNAs

The most frequently studied and better-characterized small ncRNAs are microRNAs (miRNAs). It is currently estimated that the human genome contains approximately 2500 different mature miRNAs, according to the latest version (release 21, June 2014) of miRBase, an electronic miRNA database (Kozomara and Griffiths-Jones, 2014).

The first miRNA was discovered in 1993 in *C. elegans* as a small RNA molecule, named lin-4, capable of regulating the nematode development, by negatively regulating the expression of the coding gene *lin-14* (Lee *et al.*, 1993).

miRNAs are 20-23 nucleotides long, endogenous, single-stranded, small ncRNAs that regulate gene expression at the post-transcriptional level by targeting mRNAs. In most cases, the interaction between the miRNA and the target gene(s) results in

a negative regulation, therefore, the net effect is a reduction in the level of the protein(s) encoded by the target mRNA(s) (Bartel, 2004).

miRNAs are transcribed from their coding genes by either RNA polymerase II or RNA polymerase III (Borchert *et al.*, 2006; Lee *et al.*, 2004). The direct transcript of a miRNA gene is referred to as pri-miRNA. As for protein-coding genes, the expression of a given miRNA can be controlled by transcription factors, introducing several layers of gene expression control (O'Donnell *et al.*, 2005). The pri-miRNA is a double-stranded 60-70 nucleotide precursor with a hairpin structure. The pri-miRNA is post-transcriptionally edited by adenosine deaminases acting on RNA (ADARs). ADARs are responsible for modifying adenosine (A) into inosine (I) and therefore changing the final sequence of the miRNA from the original coding sequence (Blow *et al.*, 2006). pri-miRNAs are processed by the nuclear microprocessor complex, which consists of the RNAase enzyme Drosha and the protein DGCR8, also known as Pasha (partner of Drosha). The processor endonucleolytically cleaves the 5`and 3`terminals of the hairpin to form a pre-miRNA, which is subsequently exported to the cytoplasm by Exportin-5 (Denli *et al.*, 2004; Yi *et al.*, 2003). The pre-miRNA is processed by the RISC (RNA-induced silencing complex) loading complex (RLC), a multiprotein complex composed of the RNAase Dicer, Tar RNA binding protein (TRBP), protein activator of PKR (PACT) and Argonaute-2 (Ago2). The RLC cleaves the pre-miRNA into a single-stranded mature miRNA, which subsequently remains associated with Ago proteins to form the active RISC (Fig. 1). RISC is responsible for the gene silencing effect of miRNAs by directing the binding of the miRNA to a miRNA response element (MRE) in the target gene(s) (Gregory *et al.*, 2005). In most case, the binding relies on the complementarity of the sequence of the miRNA with the 3´-UTR region of the mRNA, although binding to different regions of mRNAs has also been described (Hausser and Zavolan, 2014). The degree of complementarity of the binding has been shown to influence the nature of the inactivation of the target mRNA(s). A perfect complementarity results in cleavage of the target gene(s) by Ago proteins (Yekta *et al.*, 2004), whereas near-perfect complementarity leads to interference with mRNA translation. In animals, miRNAs are in most cases perfectly complementary to their targets only in a small region, termed the seed region (nucleotides 2-7 in the 5´-end of the miRNA). However, miRNAs are not perfectly complementary in the other portions of the sequence, resulting in imperfect binding (Brennecke *et al.*, 2005; Saxena *et al.*, 2003). Recent studies

suggest the presence of several mechanisms of mRNA inactivation by miRNAs in animals, although the precise dynamics of the involved molecular events are not clearly understood. In *C. elegans*, *lin-4* reduces the level of the protein encoded by *lin-1*4 without affecting the expression level of the corresponding mRNA (Seggerson *et al.*, 2002). Experimental evidence shows that in this case, the inactivation is achieved after the initiation of translation, via premature dissociation of the ribosomes from the nascent polypeptide chain (Petersen *et al.*, 2006). Conflicting results in other studies indicate that miRNAs inhibit the translation of mRNAs during the initiation phase, by interfering with the mRNA cap structure and/or with the function of the cap-binding complex eIF4F (Mathonnet *et al.*, 2007). Moreover, miRNAs can direct their targets to the cellular 5-to-3 mRNA decay pathway, where mRNAs are first deadenylated by the CAF1–CCR4–NOT deadenylase complex, and then decapped by the decapping enzyme DCP2. These events eventually lead to mRNA degradation and therefore affect the expression levels of the target mRNAs (Behm-Ansmant *et al.*, 2006; Eulalio *et al.*, 2009) (Fig. 1).

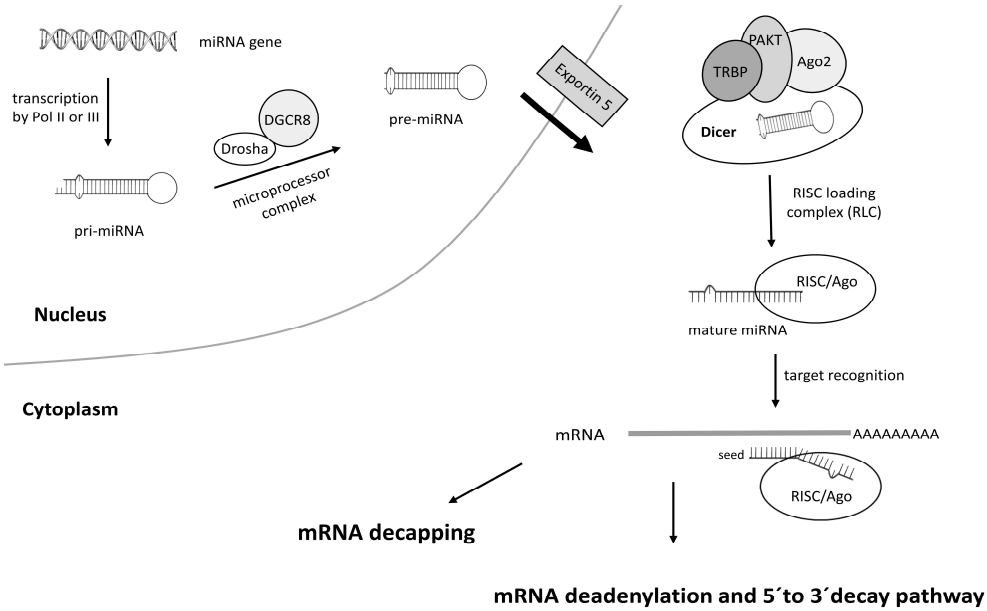

Figure 1. miRNA biogenesis and mechanism(s) of action in animals. miRNAs are in most cases perfectly complementary to their targets only in a small region, termed the seed region. Recent studies suggest the presence of several mechanisms of mRNA inactivation, although the precise dynamics of the involved molecular events are not clearly understood (modified from Lin and Gregory, 2015).

Bioinformatics tools that examine the complementarity of miRNA sequences with the 3´- UTR regions of mRNAs have predicted that each miRNA can recognize hundreds of different mRNA targets (Rajewsky, 2006). Indeed, proteomic studies have confirmed the effect of a single miRNA on hundreds of targets (Baek *et al.*, 2008). In addition, the 3'-UTR of a given mRNA may contain binding sites for various miRNAs. Moreover, miRNAs can function as both gene expression switches, dramatically decreasing the levels of proteins encoded by the target mRNAs, and fine-tuners, inducing moderate gene expression changes (Mukherji *et al.*, 2011). Therefore, gene expression regulation by miRNAs represents a complex network, affecting nearly every function of the cell biology.

Recent advances in microarray technologies provided valuable tools for large profiling studies in cancer biology, revealing that miRNAs are aberrantly expressed in tumor samples compared to controls and initiating a large effort to characterize the function of dysregulated miRNAs in cancer. Moreover, it has been shown that human miRNA genes are frequently located in chromosomal fragile sites, which are associated with cancer (Calin *et al.*, 2004). In principle, a down-regulated miRNA that targets a proto-oncogene can be considered a tumor-suppressor miRNA and an overexpressed miRNA that targets a tumor suppressor gene can effectively function as an oncogene (oncomiR) (Zhang *et al.*, 2007). In addition, miRNA expression signatures have been proven to clearly cluster solid tumors, based on their tissue of origin, highlighting the possible role of miRNAs as cancer biomarkers (Calin and Croce, 2006).

The first evidence of direct miRNA involvement in cancer was discovered in chronic lymphocytic leukemia (CLL). Hemizygous and/or homozygous deletions of the chromosomal region 13q14 occur in more than half of CLL cases and constitute the most frequent chromosomal abnormality in CLL (Mertens *et al.*, 2009). Two tumor suppressor miRNA genes, encoding miR-15a and miR-16-1 were found within the deleted region and allelic loss was clearly correlated with the down-regulation of miR-15a and miR-16-1 expression (Calin *et al.*, 2002). Further studies demonstrated that miR-15a and miR-16-1 expression is inversely correlated with *BCL-2* expression in CLL and that both miRNAs negatively regulate Bcl2 at the post-transcriptional level, reducing its anti-apoptotic activity (Cimmino *et al.*, 2005). Among the most extensively investigated tumor suppressor miRNAs are the members of the *let-7* family. The let-7 miRNA was first identified in *C. elegans*, in which a mutation in *let-7* gene was found to cause a lack of terminal differentiation and over-proliferation (Reinhart *et al.*, 2000). Currently, 10 mature members of the let-7 family have been identified in humans (Roush and Slack, 2008) and are frequently downregulated or deleted in several human malignancies (Wang *et al.*, 2012). Let-7 is known to directly and negatively regulate the expression of the Ras proteins (H-Ras, K-Ras and N-Ras) (Johnson *et al.*, 2005), as well as that of the oncogenic protein high-mobility group A (HMGA2) (Mayr *et al.*, 2007). HMGA2 regulates gene expression by altering the structure of chromatin or by direct protein-protein interactions with transcription factors (Sgarra *et al.*, 2004). Moreover, it has been shown that let-7 directly regulates Myc expression by binding to its 3' UTR. An interesting example of oncomiR is miR-155 which is upregulated in several

hematopoietic malignancies (Eis *et al.*, 2005) as well as in breast (Iorio *et al.*, 2005), lung (Yanaihara *et al.*, 2006) and pancreatic cancer (Greither *et al.*, 2010). The gene encoding miR-155 was first identified as a common proviral DNA insertion site in lymphomas induced by the avian leucosis virus (Tam *et al.*, 1997).

Currently, more than 100 genes, including crucial tumor suppressors, are confirmed to be targeted by miR-155. Among these target genes, two have been identified in breast cancer, including the negative regulator of cytokine signal transduction SOCS1 (suppressor of cytokine signaling 1) (Jiang *et al.*, 2010) and the pro-apoptotic transcription factor FOXO3a (Forkhead box O3) (Kong *et al.*, 2010). In pancreatic cancer, the proapoptotic stress-induced p53 target gene and p53 modulator *TP53INP1* (tumor protein 53-induced nuclear protein 1) has been determined to be a miR-155 target gene (Gironella *et al.*, 2007).

The miR-17-92 cluster, also known as *oncomir-1*, is one of the most potent oncogenic miRNAs in human cancers (He *et al.*, 2005). This cluster was initially identified due to its genomic amplification and elevated expression in multiple hematopoietic malignancies, including diffuse large B-cell lymphomas (DLBCLs), mantle cell lymphomas and Burkitt's lymphomas (Ota *et al.*, 2004). The pri-miR transcript derived from the *miR-17-92* gene contains six tandem stem-loop hairpin structures that can generate six different mature miRNAs, miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1 (Tanzer and Stadler, 2004). Each of these miRNAs can affect the expression of hundreds of coding genes, with the net result of promoting proliferation, inhibiting differentiation, increasing angiogenesis and sustaining cell survival. Recent studies of the miR-17-92 cluster have led to the conclusion that its biological functions are mediated by the downregulation of a large number of mRNAs, the precise set of which varies with cell type and context. Known targets of miR-17-92 include the negative regulator of Akt/PKB signaling and tumor suppressor PTEN (phosphatase and tensin homologue), the tumor suppressive transcription factor E2F1 and the cyclin dependent kinase inhibitor CDKN1A (p21), which is a negative regulator of the G1-S checkpoint cell cycle progression (Olive *et al.*, 2010).

Although, as mentioned above, individual miRNAs can effectively function as tumor suppressors or oncogenes, several studies have revealed that miRNA expression can be globally altered in some cancers. Global miRNA dysregulation has been associated with the aberrant expression of previously mentioned key components of miRNA processing machinery, such as Drosha and Dicer,

suggesting that disrupted miRNA biogenesis might have a causative role in tumorigenesis (Lin and Gregory, 2015).

After the first report of miRNA expression in serum in 2008 (Chim *et al.*, 2008), there has been increasing attention on miRNA detection in the blood circulatory system. Several studies have provided evidence of differential expression of circulating miRNAs in malignancies compared to healthy controls (Ma *et al.*, 2012). The stability of miRNAs, combined with increasing specificity and sensitivity of detection techniques, make them appealing putative cancer biomarkers for diagnosis and prognosis, confirming the importance of miRNA expression and functional studies in cancer research.

2.1.4.2 tRNA fragments

Transfer RNAs (tRNAs) are ubiquitous in all living organisms. Mature cytosolic tRNAs are 75-80-nt non-coding RNAs characterized by a typical secondary structure usually referred to as a cloverleaf. This structure consists of three hairpin loops and a terminal helical stem. The three loops are termed D-, T- and anticodon loops. The anticodon loop contains the triplets that are complementary to the codons in the mRNAs. Codon degeneracy in the genetic code means that up to five different tRNAs, each carrying a different anticodon sequence, can translate all the codons for a single amino acid (i.e., tRNA isoacceptors).

tRNA molecules are first synthesized as precursor pre-tRNAs, which contain a 5`-leader and a 3´-trailer. The leader is removed by endoribonuclease P (RNase P) and the trailer sequence is trimmed by endonuclease Z (RNase Z, encoded by *ELAC2*). The tRNA is subsequently prepared to accept the amino acid by the addition of a 3´terminal trinucleotide (5`-CCA-3`). This step is performed by the CCA-adding tRNA nucleoidyl transferase, TRNT1 (Goodenbour and Pan, 2006; Kirchner and Ignatova, 2015).

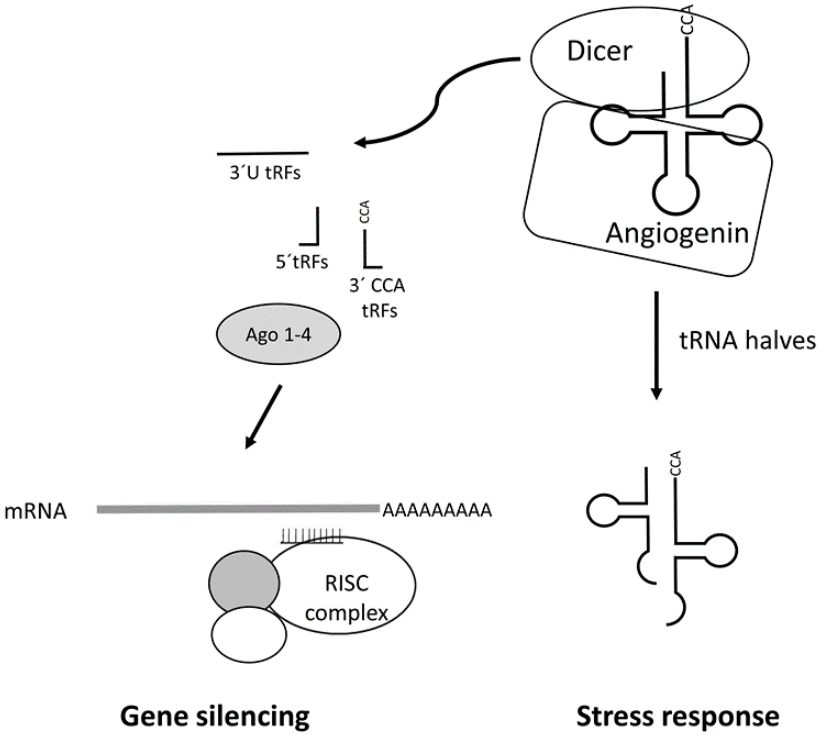

Figure 2. Proposed mechanisms of biogenesis and cellular functions of tRNA halves and tRFs (modified from Martens-Uzunova *et al.*, 2013).

Recently, several studies based on next-generation sequencing technologies (NGS) have investigated the small-RNA fraction in various organisms (Kawaji *et al.*, 2008; Yeung *et al.*, 2009). Interestingly, a significant number of sequences were found to derive from mature or precursor tRNAs, representing a series of tRNA-derived fragments of different sizes. The abundance of these small RNA species and ubiquitous expression gave rise to the question whether the fragments are primarily random tRNA degradation products or true biological entities with specific functions.

Since the discovery of the fragments, several different terminologies have been used to define them, although there is currently no accepted standard nomenclature. tRNA fragments can, however, be distinguished based on their length and are classified in two major groups: tRNA halves and tRNA-derived RNA fragments or tRFs.

tRNA halves are also termed tiRNA (stress-induced small RNAs) and are fragments derived from the cleavage of full length, mature tRNA at the anticodon loop. This cleavage generates 3´ and 5´ fragments, each corresponding to half of the original tRNA. The length of the tRNA halves ranges from 30 to 35 nt (Thompson and Parker, 2009).

tRNA fragments were described already in the 1990s in *E. coli* as being generated in response to T4 bacteriophage infection (Levitz *et al.*, 1990). Several studies have subsequently shown that tRNA halves are produced as the result of a conserved response to stress in eukaryotes. In the ciliate *Tetrahymena thermophila* (Lee and Collins, 2005) and in the filamentous fungus *Aspergillus fumigatus* (Jochl *et al.*, 2008) the anticodon loop cleavage of tRNA molecules has been observed in response to amino acid starvation. In addition, *Saccharomyces cerevisiae* contains a small RNA population consisting primarily of tRNA halves, and their levels are most pronounced during oxidative stress conditions (Thompson *et al.*, 2008). In this yeast, tRNA cleavage was proven to be unrelated to the degradation of unprocessed or mismodified tRNAs, given that the level of fragments was constant in strains that are defective for tRNA processing. A similar increase in tRNA halves was found in *Arabidopsis thaliana* and in *HeLa* cells in response to oxidative stress. Heat-shock, hypoxia and hypothermia were also shown to trigger both the increased cleavage of tRNA and the elevated production of tRNA halves in mammalian cells (Fu *et al.*, 2009). Moreover, the levels of full-length tRNAs do not significantly decline as a result of the generation of tRNA halves (Lee and Collins, 2005; Thompson *et al.*, 2008). The cleavage of tRNA is catalyzed in mammals by the site-specific nuclease angiogenin and recent reports have demonstrated that angiogenin-induced tRNA halves promote stress-granule formation and translational repression. As a result, tRNA halves can directly inhibit protein synthesis (Emara *et al.*, 2010; Ivanov *et al.*, 2011; Sobala and Hutvagner, 2013; Yamasaki *et al.*, 2009) (Fig. 2).

In a recent sequencing study performed on prostate cancer cell lines, the second most abundant class of small RNAs after miRNAs was found to be tRFs, which are derived from precise processing at the 5` or 3` end of mature or precursor tRNAs

(Lee *et al.*, 2009). tFRs are smaller than tRNA halves, ranging in size from 17 to 26 nt. tRFs can be further divided to form three categories based on the region of the tRNA they are derived from. Specifically, tRF-5s are derived from the 5` end of the mature full tRNA and were found to be the most abundant, whereas tRF-3s are derived from the 3´ end of the full mature tRNA and include the 5`-CCA-3` acceptor sequence at their 3´ end. Finally, tRF-1s are derived from the 3´ trailer of the precursor tRNA which extends beyond the 3´ end of the respective mature tRNA form.

The characteristic and abundant expression of specific fragments and their precise sequence indicate that they are not random products of tRNA degradation. Moreover, a specific fragment named tRF-1001, which was found to be one of the most abundantly expressed, was shown to increase proliferation in the human colorectal carcinoma cell line HCT116. This fragment is generated in the cytoplasm from pre-tRNA by the nuclease RNase Z, encoded by *ELAC2*, which was previously shown to be a prostate cancer susceptibility gene.

In a second high-throughput sequencing study performed on HeLa cells, the most abundant RNA reads were found to match sequences from known tRNAs, with a preferred sequence length of 19 nt (Cole *et al.*, 2009). To quantitatively evaluate how many of the matching fragments could be considered putative specific products (i.e., rather than the result of random degradation) a simple processing score (S_p) was defined as the number of reads matching a certain RNA region divided by the length of the matching region. Interestingly, tRNA reads showed the highest mean S_p of all the non-coding RNA. In the same study, the generation of tRFs derived from $tRNA^{Gln}$ was shown to be dependent on the ribonuclease Dicer (Fig. 2).

A Dicer-dependent small tRNA fragment was also described in mouse embryonic stem cells (Babiarz *et al.*, 2008) and tRFs were found to be associated with Ago proteins in several reports. The function of tRFs is currently unknown. However, the evidence of Dicer-dependent processing and their association with Ago proteins suggest that tRFs could target mRNAs in a manner similar to miRNAs and could play a role in the regulation of gene expression.

One of the first reports of tRF involvement in gene silencing was a small ncRNA sequencing study of the HIV-1-infected human monocyte cell line U1 and the human T-cell line MT4 (Yeung *et al.*, 2009). HIV-1 uses the human $tRNA^{Lys}$, $tRNA^{Pro}$ and $tRNA^{Trp}$ as primers for the initiation of reverse transcription and viral

DNA synthesis. In this study, a highly abundant 18-nt tRF, termed PBSncRNA, was found to originate from the double-stranded hybrid formed by the $tRNA^{Lys}$ and the primer binding site (PBS) of HIV. The hybrid is processed *in vitro* by Dicer and can associate with Ago2, triggering gene silencing as a mechanism of viral defense against the host. Moreover, knock-down of PBSncRNA with a synthetic antagomir increased the replication potential of HIV in infected cells.

More recently, a tRF cloned from human mature B-cells and termed CU1276 was found to possess the functional characteristics of a miRNA, including Dicer-dependent generation, association with Ago proteins and the ability to down-regulate mRNA transcripts in a sequence-specific manner (Maute *et al.*, 2013). CU1276 is expressed in normal B-cells, but it appears to be down-regulated in B-cell lymphoma. In this study, CU1276 was shown to target replication protein A1 (RPA1), which plays an essential role in several cellular processes in DNA metabolism, including replication, recombination and DNA repair. Consequently, the expression of this tRNA-derived miRNA in lymphoma suppresses proliferation and modulates the molecular response to DNA damage.

These results suggest an important role of tRFs in cell biology and their potential association with cancer.

2.2 Cancer stem cells

2.2.1 Cancer stem cell model in hematopoietic and solid tumors

The cancer stem cell model is based on the hypothesis that cancer growth is driven by a specific subpopulation of tumor cells, defined as cancer stem cells (CSCs). The CSC model therefore implies that a tumor, like a normal tissue, is composed of a heterogeneous group of cells and organized hierarchically into clonally derived populations with different proliferative potentials. Tumor growth and propagation depends on a pool of stem-like cells at the apex of the hierarchy, with these cells being characterized by an ability to self-renew and to generate the diverse cells that constitute the tumor (Jordan *et al.*, 2006; Reya *et al.*, 2001).

In contrast, the clonal evolution model of cancer is based on the hypothesis that most cancer cells within a tumor are highly tumorigenic, but are characterized by

different genetic and epigenetic features. In this context, clones that possess a growth advantage will be somatically selected and will continue to drive tumor proliferation (Shackleton *et al.*, 2009). CSCs can also be selected based on their growth advantage in a particular environment, thus, the CSC model does not exclude the possibility of clonal evolution of a tumor (Barabe *et al.*, 2007; Marotta and Polyak, 2009).

Inconsistencies in nomenclature in the field have generated confusion over the concept of tumor-initiating cells and CSCs. The two terms have been used interchangeably, although the cell of origin of a cancer is distinct from a cancer stem cell. The cell of origin is defined as the normal cell type from which a tumor arises following oncogenic transformation, whereas CSCs represent the cellular subpopulation that sustains malignant growth within the tumor. The cell of origin, the mutations acquired, and/or the differentiation potential of the cancer cells may determine whether a cancer follows the CSC model (Visvader, 2011) (Fig. 3).

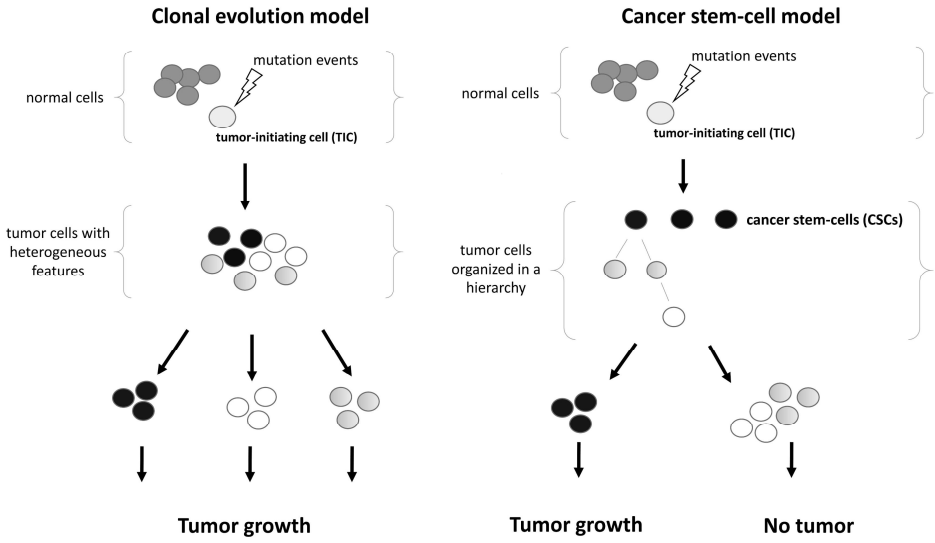

Figure 3. Models of tumor cell proliferation. The clonal evolution model of cancer is based on the hypothesis that most cancer cells within a tumor are highly tumorigenic, but are characterized by different genetic and epigenetic features. The cancer stem cell (CSC) model implies that a tumor is composed of a heterogeneous group of cells and is organized hierarchically into clonally derived populations with different proliferative potentials (modified from Wang and Dick, 2005).

CSCs are practically defined using specific functional assays. The most common method involves the xenotransplantation of populations of primary cancer cells, sorted by flow cytometry, into immunodeficient mice. In this scenario, the CSC model predicts that only a subpopulation of cells within the primary tumor possesses the capacity to initiate new tumor formation *in vivo*. This population is therefore defined as the CSC pool. CSCs can be purified from the bulk tumor and can be enriched by serial xenotransplantation. The clonal model instead predicts that tumor-initiating activity will be found in every cell fraction derived from the primary tumor (Dick, 2003; Wang and Dick, 2005). The implications of the two models for the development of therapeutic approaches in cancer treatment are profound. In the clonal evolution model, it is essential to eliminate the bulk of cancer cell population to achieve therapeutic efficacy, whereas in the CSC model, targeting the stem cells is essential to prevent disease relapse (Al-Hajj *et al.*, 2004).

The existence of cancer stem cells was first proven in acute myeloid leukemia (AML). Transplantation experiments of primary AML cells into severe combined immune-deficient (SCID) and non-obese diabetic/SCID (NOD/SCID) mice showed that only a rare subpopulation of cells, derived from the primary tumor and comprising 0.01 to 1% of the total population, was capable of initiating new growth *in vivo*. These cells were termed SCID leukemia-initiating cells (SL-ICs) and can be effectively separated from the tumor bulk based on their surface markers. SL-ICs are the only $CD34^+$ $CD38^-$ cells in AML and are referred to as leukemia stem cells (LSCs) (Bonnet and Dick, 1997; Lapidot *et al.*, 1994). In this case LSCs show similar phenotype as the normal hematopoietic stem cells (HSCs), with the difference that they are $CD90^-$ and $CD117^-$, whereas HSCs are $CD90^+$ and $CD117^+$ (Blair *et al.*, 1997; Blair and Sutherland, 2000). HSCs and LSCs share certain common molecular features. Recent studies have demonstrated that the *Polycomb group* (*PcG*) gene *Bmi-1* is required for self-renewal of both cell types (Guzman *et al.*, 2001; Lessard and Sauvageau, 2003), but LSCs express the active form of NF-$\varkappa$B, responsible for antiapoptotic activity (Guzman *et al.*, 2001).

Similar approaches were subsequently applied to identify subpopulations with cancer stem-cell properties in solid tumors. In breast cancer, a minor $CD44^+$ $CD24^{-/low}$ $Lineage^-$ cell population was shown to give rise to tumors that could be serially transplanted in NOD/SCID mice, whereas cells with alternative phonotype were not tumorigenic (Al-Hajj *et al.*, 2003). The $CD133^+$ subpopulation in brain tumors (Singh *et al.*, 2004) and colon cancer (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007),

the $CD90^+$ subpopulation in hepatocellular carcinoma (HCC) (Yang *et al.*, 2008) and the $CD44^+$ $CD24^+$ ESA^+ subpopulation in pancreatic cancer (Li *et al.*, 2007) showed similar properties.

2.2.2 Therapeutic implications of cancer stem cells

The CSC model still has many limitations despite the supporting evidence, and many questions remain to be answered to validate the role of CSCs in the molecular mechanisms of cancer initiation and progression.

The cellular origin of CSCs is still undetermined. These cells do not necessarily originate from normal stem cells, but may arise from progenitor cells or even more differentiated cells that have subsequently acquired self-renewal capacity through mutation (Bu and Cao, 2012). The percentage of CSCs contained in tumors appears to be highly variable. Although the relative proportion of CSCs might be determined by the particular characteristics of the individual tumor, CSC fractions from solid malignancies remain highly impure populations, reflecting technical differences in the sample preparation (Kern and Shibata, 2007; Visvader and Lindeman, 2008). Rather than representing exclusive features of actual primary self-renewing cells, the markers used for CSC isolation could reflect the ability of certain cells to survive purification procedures or alternatively to initiate tumor growth in the mouse environment (Marotta and Polyak, 2009). The mouse injection assay used for CSC identification may have serious limitations due to the transplantation of human cancer cells into a specific site in the host (Kelly *et al.*, 2007; Kennedy *et al.*, 2007). Moreover, as transplanted cells originally existed in a complex microenvironment that is not accurately modeled by the assay, a lack of important accessory cells may affect the tumorigenic capacity of transplanted cells from solid tumors (Hill, 2006). Furthermore, the characterization and identification of CSCs in a specific tumor may be limited by the intrinsic genetic instability of most cancers, which is responsible for continuous cell diversification (Nguyen *et al.*, 2012).

Nevertheless, recent studies have provided evidence supporting potential application of therapeutic strategies based on targeted CSCs eradication.

The case of leukemia represents a well-studied example. A fundamental problem in treating leukemia derives from LSC resistance to conventional chemotherapy. Human AML stem cells transplanted in immunodeficient mice have been reported

to engraft within the osteoblast-rich area of the bone marrow and to enter a quiescent state, an event that is responsible for the acquired resistance to chemotherapy (Guan *et al.*, 2003; Ishikawa *et al.*, 2007). The specific targeting of the apoptosis and proliferation regulator promyelocytic leukemia protein (PML) tumor suppressor in CML was shown to disrupt the maintenance of the quiescent LSCs by sensitizing them to pro-apoptotic stimuli (Ito *et al.*, 2008). Moreover, the naturally occurring parthenolide (PTL), a sesquiterpene lactone found as the major active component in Feverfew (*Tanacetum parthenium*), was reported to selectively ablate primitive AML leukemia cells without affecting normal stem and progenitor cells (Guzman *et al.*, 2005).

Strategies directed at eradicating CSCs have been investigated in solid tumors as well. Studies in human glioblastoma showed that $CD133^+$, stem-like, tumor-initiating precursors in glioblastoma multiforme (GBM) are more resistant to irradiation, both *in vivo* and *in vitro*, than $CD133^-$ cells. This effect is mediated through the preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity (Bao *et al.*, 2006). Bone morphogenetic proteins (BMPs), particularly BMP4, were shown to trigger a significant reduction in GBM cells. BMP4 exposure of GBM cells depleted the $CD133^+$ cell fraction and transient *in vitro* exposure of $CD133^+$ cells to BMP4 reduced their capacity to initiate new tumors in transplanted mice (Piccirillo *et al.*, 2006). Therapeutic resistance of CSCs has also been reported in breast cancer, in which chemotherapy was shown to selectively enrich for self-renewing $CD44^+$ $CD24^{-/low}$ breast cancer cells. The results showed that 74% of tumor cells from chemotherapy-treated patients were $CD44^+$ $CD24^{-/low}$ compared to only 9% of cells in untreated patients (Yu *et al.*, 2007). The same study also demonstrated let-7 miRNA downregulation in self-renewing cells and infection with a let-7-lentivirus led to reductions in (i) proliferation, (ii) mammosphere formation, (iii) the proportion of undifferentiated cells *in vitro*, and (iv) tumor formation and metastasis in transplanted NOD/SCID mice.

2.3 Bladder cancer

2.3.1 Bladder cancer pathology and risk factors

Urinary bladder cancer is the most common malignancy of the urinary tract, with an estimated 386.000 new cases and 150.000 deaths worldwide (Jemal *et al.*, 2011). Bladder cancer is the fourth most common cancer in men in Western countries and is approximately 3 times more common in males than in females. An average of 90 to 95% of malignant bladder tumors arise from the urothelium (i.e., the epithelium lining the inside of the urinary bladder) and are thus termed urothelial carcinomas. The remaining 5-10% of cases include adenocarcinoma, squamous cell carcinoma and other rare histological types (Reuter, 2006). Squamous cell carcinoma (SCC) of the bladder represents less than 5% of bladder cancer cases and is usually diagnosed in patients who suffered spinal cord injury (SCI) and who have a history of a prolonged use of indwelling catheters (Navon *et al.*, 1997). Inflammation caused by chronic urinary tract irritation, either from bacterial infections or bladder calculi is reported as the main cause of SCC (Shokeir, 2004). SCC represents 59% of bladder cancer cases in countries with endemic bilharziasis, an infectious disease affecting agricultural communities and caused by the parasite *Schistosoma haematobium*, with the highest number of cases reported in Egypt (Mostafa *et al.*, 1999).

Tobacco smoke is the most important environmental risk factor for bladder cancer (Stewart *et al.*, 2008; van der Meijden, 1998). Occupational exposure to aromatic amines, typically found in industrial aniline dyes, has also been correlated with an increased risk of developing bladder cancer (Golka *et al.*, 2004), as have arsenic and chloride contamination in drinking water (Chiou *et al.*, 2001; Villanueva *et al.*, 2003). However, only a fraction of cases exposed to a known carcinogen will eventually develop bladder cancer, suggesting a role for genetic variation as a contributing factor in determining risk. Recent genome-wide association studies have been performed and considered at least one thousand cases and one thousand controls, with hundreds of thousands of single-nucleotide polymorphisms (SNPs) being examined for association with the disease (Chung and Chanock, 2011). Polymorphisms in two carcinogen-detoxifying genes, *N*-acetyltransferase 2 (*NAT2*) and glutathione *S*-transferase-μ1 (*GSTM1*) were found to confer

increased bladder cancer risk. SNPs associated with moderate risk of bladder cancer were discovered for several candidate genes, including *MYC*, *TP63*, prostate stem cell antigen (*PSCA*), telomerase reverse transcriptase (*TERT*)–CLPTM1-like (*CLPTM1L*), fibroblast growth factor receptor 3 (*FGFR3*), transforming, acidic coiled-coil containing protein 3 (*TACC3*), *NAT2*, chromobox homologue 6 (*CBX6*), apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3A (*APOBEC3A*), cyclin E1 (*CCNE1*) and UDP glucuronosyltransferase 1 family, polypeptide A complex locus(*UGT1A*) (Dudek *et al.*, 2013).

The majority of bladder cancer cases (75%) are non-muscle invasive (NMIBC) at diagnosis. NMIBC is treated by trans-urethral resection of the tumor (TUR-T), followed by perioperative intravescical chemotherapy instillation. In patients with high risk of progression or recurrence, intravescical immunotherapy is used, which consists of the serial administration of Bacillus Calmette-Guérin (BCG). BCG is an attenuated mycobacterium that triggers an intense local immune activation in the bladder. It has been shown that BCG provides significantly better prophylaxis of tumor recurrence over TUR alone in high-risk superficial urothelial carcinoma (Cookson and Sarosdy, 1992; Shelley *et al.*, 2001). However, some patients are still at risk of recurrence and progression to muscle-invasive bladder cancer (MIBC) (van den Bosch and Alfred Witjes, 2011).

The remaining 25% of cases present at diagnosis with MIBC (TNM stage T2 or higher), and are primarily treated with radical cystectomy if the tumor is still confined to the bladder. However, 50% of these cases will progress to metastatic disease (Sawhney *et al.*, 2006).

2.3.2 Chromosomal alterations in bladder cancer

Bladder cancer is a heterogeneous disease with a unique natural history characterized by a highly variable clinical course. Patients with superficial and invasive tumors can have remarkably different 5-year survival rates (Lee and Droller, 2000). Therefore, clinical parameters of the disease, including tumor grade and shape, location, and presence of carcinoma in situ (Cis) are of limited value as prognostic markers (Holmang *et al.*, 1995). The clinical heterogeneity suggests an underlying heterogeneity of genetic alterations leading to different pathways of cancer development and progression. Although there is no established specific

chromosomal alteration for bladder cancer, several studies have revealed considerable variability in the degree of alteration at the chromosomal level (Wolff, 2007). The spectrum of alterations can also vary depending on the grade of differentiation and the tumor stage, with low-stage, low-grade tumors generally showing fewer aberrations than more aggressive tumors (Fadl-Elmula, 2005; Sandberg, 2002). This result suggests that bladder cancer progression may in fact be driven by the accumulation of chromosomal changes and a better understanding of the molecular mechanisms that lead to tumor formation and progression is therefore needed to identify more aggressive tumors and improve survival rates.

Many of the genetic alterations found in bladder cancer consist of region-specific gains or losses of DNA copy number, which can lead to the identification of the key genes involved. Regions of DNA copy number gain or amplification commonly harbor oncogenes, whereas deleted regions harbor tumor suppressor genes. Various approaches have been used extensively to study urothelial carcinoma and identify altered genes, including cytogenetics, fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), loss of heterozygosity (LOH) and, more recently, array-comparative genomic hybridization (aCGH). CGH produces a map of DNA sequence copy number as a function of chromosomal location throughout the entire genome. Differentially labeled sample DNA and reference DNA sequences are co-hybridized to normal chromosome spreads and regions of gain or loss of DNA sequences (e.g., deletions, duplications, or amplifications), considered to be changes in the ratio of the intensities of the two fluorochromes (Weiss *et al.*, 1999). The resolution of conventional CGH was limited to regions on the order of 3 Mb (Lichter *et al.*, 2000), and it was not possible to resolve regional single-copy DNA gains or losses. The further development of aCGH allowed high-resolution copy number analyses. In aCGH, the metaphase chromosomes are replaced by cloned DNA fragments of approximately 100–200 kb of which the exact chromosomal location is known (Oostlander *et al.*, 2004). The resolution becomes limited only by the physical size of the clones used in the array (Pinkel *et al.*, 1998; Veltman *et al.*, 2002), facilitating the identification of single-copy changes.

As has been shown by conventional cytogenetic studies, one of the most common features of early bladder cancer is the loss of the entire chromosome 9 or portions of it (Sandberg, 1992). Deletions at chromosome 9 are found in more

than half of all bladder tumors (Fadl-Elmula, 2005). Monosomy and deletion of 10q have also been described using cytogenetics (Smeets *et al.*, 1987) and FISH (Wang *et al.*, 1994). LOH and mapping studies on both arms of the chromosome 9 have provided a list of candidate tumor suppressor genes, that are likely involved in the initiation of tumorigenesis. These genes include *CDKN2B* and *CDKN2A* at 9p21, which encode the negative regulators of cell cycle p15 and p16 (Packenham *et al.*, 1995; Williamson *et al.*, 1995); *PTCH* and *TSC1* at 9q22 and 9q34, respectively (Aboulkassim *et al.*, 2003; Habuchi *et al.*, 1995); and *DBC1* at 9q32 (Habuchi *et al.*, 1998). LOH studies have also lead to the identification of other known and candidate tumor suppressors at 17p (*TP53*) (Sidransky *et al.*, 1991; Williamson *et al.*, 1994), 10q (*PTEN*) (Aveyard *et al.*, 1999; Cairns *et al.*, 1998) and 13q (*RB1*) (Cairns *et al.*, 1991). Aberrations of chromosome 9 are observed in superficial papillary non-invasive tumors (Ta), but in only a small subset of invasive bladder neoplasms. In contrast, carcinoma in situ (Tis) and invasive tumors are characterized by loss-of-function mutations of *TP53*, *RB1* and *PTEN* (Cordon-Cardo, 2008).

Many CGH studies of bladder cancer have been published (Kallioniemi *et al.*, 1995; Richter *et al.*, 1997; Richter *et al.*, 1998; Simon *et al.*, 1998; Voorter *et al.*, 1995; Zhao *et al.*, 1999), leading to the identification of non-random genomic regions of DNA amplification and loss. The identified regions include the overrepresentation of 1q, 3p, 3q, 5p, 6p, 8q and 10p, as well as common amplifications at 1q22-24, 3p24-25, 6p22, 8p12, 8q21-22, 10p12-14, 12q15-21, 13q31-33, Xp11-12 and Xq21-22. Moreover, a high-level of amplification was identified at 17q21 and was associated with the oncogene *ERBB2* (Hovey *et al.*, 1998). The amplification at 11q13 was associated with *CCND1* (Bringuier *et al.*, 1996). The frequent losses of chromosome 9 and 10q regions were also validated in these studies and frequent loss of 8p, 5q and 4q were described. One of the major general findings is that T1 tumors show a larger number of genomic alterations than Ta tumors. Several other genomic changes have been identified by more recent aCGH studies. For example, recurrent homozygous deletions were detected at 8q23.1, 9p21.3, 9q33, 10q23 and11p13. The previously identified gene *CDKN2* was confirmed as an affected gene in 9p21, *DBC1* was confirmed as an affected gene in 9q33 and *PTEN* in 10q23. *TRAF6* and *RAG1* were associated with 11p13. In addition, several high level amplifications were identified and associated with target genes at regions 6p22 (*E2F3*), 8p12 (*FGFR1*), 8q22.2 (*CMYC*) 11q13 (*CCND1, EMS1, INT2*) and 19q13 (*CCNE1*) (Blaveri *et al.*, 2005; Hurst *et al.*, 2004; Veltman *et al.*, 2003).

Taken together, chromosomal alteration studies in bladder cancer show an increased number of aberrations in higher-grade tumors and more aggressive tumors. The association between the genomic profile and the behavior of the tumors has recently been investigated, with the purpose of identifying possible prognostic markers. The loss of 9p21 region has been shown to correlate with the response to BCG treatment (Cai *et al.*, 2010) and an association with disease prognosis was found for loss of the chromosome region 8p23 and concomitant LOH at 9p and 14q (Eguchi *et al.*, 2010; Tzai *et al.*, 2003). In the search for prognostic markers, other approaches have shown the importance of the whole genomic profile and the total number of changes, as opposed to single chromosomal alterations (Blaveri *et al.*, 2005). Moreover, genomic instability in itself, measured by number of metaphase bridges and centrosomes, has been proven an informative marker (Jin *et al.*, 2007; Yamamoto *et al.*, 2004).

Although significant associations have been revealed, large cohort validation studies are lacking and more research is needed to investigate the association between genetic changes and the development and progression of the disease.

2.4 Prostate cancer

2.4.1 Prostate cancer pathology and clinical characteristics

Prostate cancer (PC) is the second most commonly diagnosed cancer worldwide and the most frequently diagnosed malignancy in men in developed countries (Torre *et al.*, 2015). The incidence of PC varies widely internationally, primarily because of substantial differences in diagnosis practices and the adoption of prostate-specific antigen (PSA) testing. PC rates have been continuously increasing in Europe since the adoption of PSA screening (Center *et al.*, 2012).

Histologically, the prostate epithelium can be divided into two distinct cellular layers, termed the basal layer and luminal layer. The luminal layer consists of differentiated, secretory cells, whereas the basal layer consists of low cuboidal epithelium and columnar mucus-secreting cells, separating the luminal cells from the basal membrane. Rare neuroendocrine cells are also found, primarily within the basal layer (van Leenders and Schalken, 2003). PC presents a strikingly luminal

phenotype and is therefore referred to as an adenocarcinoma (Okada *et al.*, 1992). Pathologists often base the diagnosis of cancer on the absence of basal cells markers (Parsons *et al.*, 2001), although recent studies in a murine model have suggested the possible involvement of both luminal and basal cells in tumor initiation (Goldstein *et al.*, 2010; Lawson *et al.*, 2010; Wang *et al.*, 2009). This result suggests either that prostate cancer arises from a luminal cell or that the oncogenic transformation of a basal progenitor results in rapid differentiation of luminal progeny.

Prostatic intraepithelial neoplasia (PIN) is a neoplastic lesion of the epithelium lining the prostatic ducts and acini (Kastendieck, 1980; McNeal and Bostwick, 1986). PIN is classified as low-grade and high-grade based on the cytological characteristics of the secretory cells (Montironi *et al.*, 2007). Based on co-localization and phenotypic and molecular genetic similarities, high-grade PIN (HGPIN) is considered the likely precursor of prostatic carcinoma (Bhatia-Gaur *et al.*, 1999; Bostwick *et al.*, 2004; Montironi *et al.*, 2000; Qian *et al.*, 1995); however, HGPIN is not exclusively associated with cancer and several benign disorders present with HGPIN lesions (Bostwick and Qian, 2004). Recent studies have highlighted the possible role of regenerative lesions, acquired as a consequence of chronic prostatic inflammation, as another possible early stage of prostate carcinogenesis (De Marzo *et al.*, 2007). Histologically, these lesions usually contain either acute or chronic inflammatory infiltrates and are associated with atrophic epithelium or focal epithelial atrophy, showing increased epithelial cell proliferation. The lesions are referred to as proliferative inflammatory atrophy (PIA) (De Marzo *et al.*, 1999). The hypothesis of chronic inflammation caused by inflammatory oxidants as a risk factor for prostate cancer development is supported by epidemiological data, showing significantly lower incidence and mortality rates in Southeast and East Asia than in Europe and the United States (Center *et al.*, 2012), suggesting environmental effects. Although the cause of prostatic inflammation remains unclear, a link between incidence and the consumption of red meat and animal fats has recently been shown (Michaud *et al.*, 2001; Sinha *et al.*, 2009).

PC is a heterogeneous disease, which presents with histologic and anatomic variability (Arora *et al.*, 2004), as well as remarkably different clinical evolution, ranging from indolent disease to rapidly invasive and metastatic carcinoma (De Marzo *et al.*, 2004). The presence of multiple foci of PIN and/or adenocarcinoma is a common finding in a single prostate gland (Djavan *et al.*, 1999; Miller and Cygan, 1994; Villers *et al.*, 1992). Therefore, the Gleason grading system is the most

commonly used classification for histologic grading of prostate malignancies. The system is based on the microscopic evaluation of the glandular architecture. The tissue specimens are given an overall Gleason score, which is based on the sum of primary and secondary grades, representing the two most commonly observed histologic patterns. Each pattern is defined numerically from 1 to 5, indicating the most to least differentiated cellular features, respectively (Epstein, 2010; Mellinger *et al.*, 1967). A highly available and minimally invasive blood test for prostate-specific antigen (PSA) is commonly used for the early detection and treatment response monitoring. PSA is a kallikrein-related serine protease that is physiologically involved in the liquefaction of seminal fluid (Lilja, 1985). Prostate cancer can induce up to a 10^5-fold increase in PSA levels in the circulatory system compared to basal levels. This increase is believed to result from the disruption of prostate architecture in prostate tumors, such as disruption of the basement membrane and a loss of the basal cell layer (Lilja *et al.*, 2008). However, increased levels of PSA can also result from benign conditions, such as benign prostatic hyperplasia (Nadler *et al.*, 1995) and clinically localized, low Gleason grade cancers. This type of prostate cancer may not require intervention, due to its indolent nature (Wolf *et al.*, 2010). Moreover, recently published data from the European Randomized Study of Screening for Prostate Cancer (ERSPC) indicate that the increased rate of diagnosis of indolent tumors is potentially responsible for overtreatment and adverse effects associated with treatment options (Schroder *et al.*, 2009).

Men that present with elevated blood PSA levels or abnormal prostate after clinical evaluation typically undergo prostate biopsy screening and histological evaluation. Treatment options are normally based on the clinical stage and histological grade of prostate cancer. Active surveillance is a viable option for low-risk, localized disease. Though radical prostatectomy and radiation therapy represent effective treatments for localized, early stage disease, 30-40% of cases will progress to advanced, metastatic disease (Shore, 2014).

PC is highly dependent on androgen hormones (Huggins and Hodges, 2002). The most abundant androgen hormone in males is testosterone, which is primarily synthesized by the testes. Once testosterone enters prostate cells, it is converted into the more potent 5α-dihydrotestosterone (DHT), which binds with high affinity to the androgen receptor (AR) in the cytoplasm. AR is consequently translocated in the nucleus, where it binds androgen response elements (AREs) at

the promoter region of target genes, inducing their transcription and resulting in an overall response of cell growth and survival (Heinlein and Chang, 2004). The role of androgens and AR is essential in both normal prostate development (Imperato-McGinley *et al.*, 1985; Yeh *et al.*, 2002) and prostate cancer development and progression (Heinlein and Chang, 2004). For these reasons, androgen deprivation therapy (AD) is initially highly effective in advanced cases (Ryan and Tindall, 2011; Scher *et al.*, 2004). During the early phase of AD, rapid apoptosis and regression are observed in the tumor; however, most patients will ultimately develop castration-resistant prostate cancer (CRPC). CRPC remains incurable and represents one of the major clinical challenges for disease treatment (Agus *et al.*, 1999; Hotte and Saad, 2010; Scher and Sawyers, 2005). Another major challenge is the absence of reliable prognostic information, necessary to readily distinguish indolent from aggressive cases in patients with low Gleason grade biopsies.

Therefore, a deeper understanding of the molecular mechanisms of prostate cancer tumorigenesis is required for the discovery more specific biomarkers and the development of improved therapeutic strategies.

2.4.2 Molecular biology of prostate cancer

The heterogeneity of the histologic and anatomic features of PC, as well as its variable clinical evolution, reflect the fact that there is no clear model of common, defined genetic events that lead to PC development (Tomlins *et al.*, 2006b). However, susceptibility studies suggest a strong genetic component and extensive effort has led to the identification of several genetic and genomic alterations (both inherited and somatic), involved in PC tumorigenesis (Table 1).

2.4.2.1 Prostate cancer susceptibility

Family history is among the strongest epidemiological risk factors for PC, with relative risk increasing markedly when the number of affected individuals in a family cluster increases and when the age of the affected individuals decreases (Carter *et al.*, 1992; Steinberg *et al.*, 1990). Moreover, monozygotic twins have a fourfold increased concordance rate of prostate cancer compared with dizygotic twins (Carter *et al.*, 1992). A landmark cohort study using the combined data from 44,788 pairs of twins

listed in Swedish, Danish and Finnish twin registries, estimated that 42% of all prostate cancer risk might be explained by inheritable factors (Lichtenstein *et al.*, 2000). A more recent cohort study, performed using novel statistical models and data from the largest twin study of cancer in the world (Nordic Twin Study of Cancer, NorTwinCan), and including 143,467 pairs of twins, revealed an even higher estimate of 57% of prostate cancer heritability (Hjelmborg *et al.*, 2014).

Strong candidate familial PC susceptibility genes, identified by linkage and mutation screenings, include *ELAC2* (elaC ribonuclease Z 2) (Tavtigian *et al.*, 2001), *RNASEL* (2'-5'-oligoadenylate-dependent ribonuclease L) (Carpten *et al.*, 2002), *MSR1* (macrophage scavenger receptor 1) (Xu *et al.*, 2002), *HOXB13* (homeobox protein Hox-B13) (Xu *et al.*, 2013), and the breast cancer susceptibility gene *BRCA2* in early-onset cases (Edwards *et al.*, 2003). However, mutations in these candidate genes are rare and no single susceptibility locus is alone responsible for a large portion of familial prostate cancers.

With the advent of modern genomic tools, the field of cancer susceptibility has greatly expanded and genome-wide association studies (GWAS) have recently been performed to assay hundreds of thousands of common single nucleotide polymorphisms (SNPs). The 1000 Genomes Project, which aimed at building a resource to help explain the genetic contribution to human disease, has sequenced the genomes of 1,092 individuals from 14 populations and revealed an estimated 4 million SNPs with a minor allele frequency of ≥5% per-individual (1000 Genomes Project Consortium *et al.*, 2012). To date, published prostate cancer GWAS have identified 100 prostate cancer risk SNPs, accounting for ~33% of the familial risk of prostate cancer in populations of European ancestry (Al Olama *et al.*, 2014). The SNPs are primarily located in regions not previously associated with prostate cancer risk. Despite some evidence of disease-associated SNPs in coding regions of known genes, the majority of these SNPs are in intronic or intergenic non-coding regions and the molecular mechanism for their function remains unknown (Eeles *et al.*, 2014; Freedman *et al.*, 2011).

2.4.2.2 Candidate genes involved in prostate cancer tumorigenesis and progression.

One of the most commonly reported events in PC is the down-regulation of the π-class glutathione S-transferase gene (*GSTP1*), which is an important multifunctional detoxifying enzyme, that belongs to the glutathione S-transferase family (Lee *et al.*, 1994). GSTP1 inactivates electrophilic carcinogens by conjugation with glutathione (Toffoli *et al.*, 1992). The regulatory sequence near the *GSTP1* gene is commonly inactivated by hypermethylation during the early stages of prostatic carcinogenesis (Brooks *et al.*, 1998; Jeronimo *et al.*, 2004). The hypermethylation is not detected in benign prostatic epithelium (Jeronimo *et al.*, 2001). Reduced expression of *GSTP1* is also described in PIN (Nakayama *et al.*, 2004), whereas PIA lesions generally show high *GSTP1* expression. *GSTP1* promoter methylation has been shown in some cells within PIA regions, suggesting a possible increased chance of progression to high-grade PIN and/or adenocarcinoma (Nakayama *et al.*, 2003), and supporting the hypothesis that inflammation might play an important role in PC initiation. Although the mechanism responsible for the observed hypermethylation is not well understood, the loss of *GSTP1* expression likely increases vulnerability to oxidant carcinogens (Nelson *et al.*, 2001).

The phosphatase and tensin homolog (*PTEN*) gene is a tumor suppressor identified by mapping of the chromosomal region 10q23, which is frequently deleted in PC (Li *et al.*, 1997). In early studies, LOH analysis performed on tumor samples revealed 35-49% of PC cases harboring *PTEN* hemizygous deletions (Feilotter *et al.*, 1998; Muller *et al.*, 2000). Later analysis of *PTEN* status by FISH, confirmed the high frequency of deletions (Yoshimoto *et al.*, 2007). HGPIN samples were found to exhibit lower frequency of PTEN deletion (20%) than primary tumors (Bismar *et al.*, 2011; Yoshimoto *et al.*, 2006a), whereas advanced prostate cancer samples showed higher frequency of both hemizygous and homozygous deletions (Yoshimoto *et al.*, 2007). Moreover, point mutations and promoter methylation have also been reported to affect *PTEN* gene expression and function (Taylor *et al.*, 2010; Whang *et al.*, 1998). The deletion or functional inactivation of PTEN is correlated with poor clinical prognosis, with hemizygous deletions being associated with an increased risk of PC and earlier biochemical relapse after radical prostatectomy; moreover, homozygous deletions of *PTEN* are strongly linked to metastasis and androgen-independent progression (Bismar *et al.*, 2011; Sircar *et al.*, 2009; Squire, 2009). The protein encoded by *PTEN* is a phosphatase that targets phosphatidylinositol-3,4,5-

trisphosphate (PIP3), thereby antagonizing the PI3K/AKT signaling pathway. PI3Ks are a family of intracellular lipid kinases responsible for the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to generate the lipid second messenger PIP3. PIP3 transduces the signal by activating the serine-threonine kinase AKT, resulting in increased cell survival and proliferation (Chalhoub and Baker, 2009).

Another common event observed in early prostate carcinogenesis is the loss of specific regions of chromosome 8p, an event that is observed in 80% of prostate tumors (Chang *et al.*, 1994). LOH at chromosome region 8p21 has been reported in 90% of PC cases and in 60% of PIN (Emmert-Buck *et al.*, 1995; Vocke *et al.*, 1996).

The NK3 transcription factor related locus 1 (*NKX3.1*) homeobox gene maps within the critical region of 8p21 loss (He *et al.*, 1997) and is an important regulator of normal prostate development and prostate tumorigenesis (Bhatia-Gaur *et al.*, 1999). Although LOH at 8p21 progressively increases in frequency with cancer grade, as the remaining *NKX3.1* allele is not mutated, it does not act as a classic tumor suppressor gene (Ornstein *et al.*, 2001). In addition, independently of 8p21 LOH, it has been shown that *NKX3.1* can be regulated epigenetically, through promoter methylation (Asatiani *et al.*, 2005).

Analyses of *NKX3.1* function in engineered mice have shown that its inactivation results in a defective response to oxidative damage (Ouyang *et al.*, 2005). *NKX3.1* expression in human prostate cancer cell lines affects DNA damage response and cell survival after DNA damage, enhancing colony formation and having a minimal effect on apoptosis (Bowen and Gelmann, 2010). These results suggest that *NKX3.1* represents a haploinsufficient tumor suppressor gene that acts as a gatekeeper for prostate cancer initiation (Gelmann, 2003).

Gene	Function	Alteration
GSTP1	Detoxification	Hyper methylation
PTEN	Tumor suppressor, PI3K/AKT pathway antagonist	Deletion, mutation, promoter methylation
NKX3.1	Oxidative damage response	Deletion, promoter methylation
AR	Cell growth and survival	Mutation, amplification, alternative splicing
ERG, ETV1, ETV4, ETV5	Transcription factors, proliferation, differentiation, apoptosis and transformation	Gene fusion, primarily with TMPRSS2
MYC	Transcription factor, proliferation	Overexpression
EZH2	AR coactivator	Overexpression
SKIL	TGF-beta pathway inhibitor	Gene fusion with TMPRSS2
HES6	Transcription cofactor	Gene fusion with DOT1L
FOXA1	Transcription factor, cell cycle progression	Mutation
SPOP	Transcriptional repression	Mutation
HOXB13	Transcription factor	Mutation
MLL2, UTX, ASXL1	Histone and chromatin modification	Mutation
AURKA	Serine/threonine kinase	Overexpression, amplification
MYCN	Transcription factor	Overexpression, amplification
MED12	Transcription regulation	Mutation

Table 1. Candidate genes involved in prostate tumorigenesis and progression, their functions and genetic alterations.

The androgen receptor (*AR*) gene is one of the most frequently altered genes in CRPC. Several *AR* somatic mutations have been reported. Although differing results have been published (Marcelli *et al.*, 2000; Tilley *et al.*, 1996), it is generally accepted that somatic *AR* mutations are very rare in untreated, localized PC, but are detected at a high frequency in castration-resistant and metastatic tumors (Gottlieb *et al.*, 2004; Linja and Visakorpi, 2004). The majority of *AR* mutations are point mutations, which result in single amino acid substitution. Most of the mutations are localized in the ligand-binding domain (LBD) of AR. Frequently, these mutations enable anti-

androgens to function as AR agonists (Gaddipati *et al.*, 1994; Taplin *et al.*, 1999) and/or allow AR activation by the adrenal androgens dehydroepiandrosterone (DHEA) and androstenediol (Suzuki *et al.*, 1993; Taplin *et al.*, 1995).

Several mechanisms have also been shown to cause increased activity of *AR* in CRPC, resulting in signaling activity retention even upon androgen deprivation. The amplification of *AR* was found in approximately one-third of castration-resistant carcinomas, leading to increased expression of the protein (Koivisto *et al.*, 1997; Linja *et al.*, 2001; Visakorpi *et al.*, 1995a). Recent evidence also suggests that androgen levels in androgen-deprived patients are sufficiently high to activate overexpressed AR. Sufficient levels are achieved by intratumoral *in-situ* synthesis (Montgomery *et al.*, 2008), residual androgen production from the adrenal gland (Zhu and Garcia, 2013) and decreased expression levels of the androgen inactivating enzymes CYP3A4, CYP3A5 and CYP3A7 (Mitsiades *et al.*, 2012). Moreover, alternative splicing of the *AR* gene was shown to generate ligand-independent, constitutively active AR variants (Hu *et al.*, 2009; Hu *et al.*, 2012). However, these *AR* alterations are almost exclusively found in metastatic, CRPC, indicating that they likely do not play a role in prostate progression, but rather arise as a mechanism of resistance during treatment (Barbieri *et al.*, 2013).

The most common genetic alteration in prostate tumors is the *TMPRSS2:ERG* gene fusion, present in approximately 20% of HGPIN lesions and 50% of localized prostate cancer (Clark *et al.*, 2008; Mehra *et al.*, 2007b; Mosquera *et al.*, 2008). Earlier studies reported *ERG* as the most commonly overexpressed oncogene in prostate cancer, present in approximately 72% of cases (Petrovics *et al.*, 2005). It was subsequently discovered that the mechanism responsible for this overexpression is the recurrent genomic rearrangement between the first exon(s) of *TMPRSS2* and the *ERG* oncogene (Tomlins *et al.*, 2005). ERG is a member of the ETS family of DNA-binding transcription factors that can function as both transcriptional activators and repressors and play a crucial role in many cellular processes, including proliferation, differentiation, apoptosis, metastasis and transformation (Hollenhorst *et al.*, 2011). *ERG* overexpression is found in both early and late-stage prostate cancer (Soller *et al.*, 2006). More recent analyses have reported *ERG* overexpression, detected by immunohistochemistry, in a much higher percentage of PIN cases, suggesting that the gene fusion is an early event in prostate tumorigenesis (Park *et al.*, 2010). *TMPRSS2* (androgen-regulated trans-membrane protease, serine 2), encodes a serine protease that is secreted by prostate

epithelial cells in response to androgens (Afar *et al.*, 2001). Both *TMPRSS2* and *ERG* are located at the chromosomal region 21q22 and are approximately 3 Mb apart. In most cases, the fusion is the result of a deletion of the DNA sequence separating the genes, but more complex rearrangements, such as translocations, have also been described (Mehra *et al.*, 2007a; Tu *et al.*, 2007; Yoshimoto *et al.*, 2006b). Different DNA breakage points are possible, generating over 20 different variants of *TMPRSS2:ERG* fusions. The most common variant is the products of recombination between exon 1 or 2 of *TMPRSS2* and exon 4 of *ERG* (Clark *et al.*, 2007). The fusion attaches the prostate-specific *TMPRSS2* promoter to the *ERG* open reading frame and is responsible for the androgen-induced up-regulation of *ERG* expression (Tomlins *et al.*, 2005).

Other members of the ETS family of transcription factors were also found to be involved in fusions with *TMPRSS2* (*TMPRSS2:ETS*), although less commonly. These other members include *ETV1* (Hermans *et al.*, 2008b; Tomlins *et al.*, 2005), *ETV4* (Tomlins *et al.*, 2006a) and *ETV5* (Helgeson *et al.*, 2008). The different ETS gene fusions seem mutually exclusive in prostate cancer, but different fusion events can be found within the same tumor in multifocal disease (Mehra *et al.*, 2007a; Rubin *et al.*, 2011). *ERG* is predominantly fused to *TMPRSS2*, but *ETV1*, *ETV4*, and *ETV5* can have several fusion partners that are located on different chromosomes (Hermans *et al.*, 2008a; Hermans *et al.*, 2008b).

The role of *ERG* dysregulation in prostate cancer is not well understood. *ERG* overexpression in vivo in a mouse model results in the development of PIN lesions but not invasive cancer (Klezovitch *et al.*, 2008). Recent ChIP-seq studies have shown an overlap between AR-binding and ERG-binding genomic regions, revealing that ERG can disrupts AR signaling by inhibiting *AR* expression and activity at gene-specific loci. This result suggests that ERG activity could inhibit AR-induced prostatic differentiation and induction of cellular de-differentiation via the activation of the H3K27 methyltransferase polycomb gene *EZH2* (Boyer *et al.*, 2006; Yu *et al.*, 2010). However, *EZH2* was also found to be significantly upregulated in metastatic prostate cancer (Varambally *et al.*, 2002) and recent studies suggest that the oncogenic function of *EZH2* in CRPC is independent of its role as a transcriptional repressor, involving instead its ability to act as a co-activator for critical transcription factors, including the AR itself (Xu *et al.*, 2012). Moreover, the prognostic significance of the *TMPRSS2:ERG* fusion remains controversial and discrepant results have been published. It was previously shown that patients with

higher expression levels of *ERG* presented slower progression rates than patients with tumors without *ERG* overexpression (Petrovics *et al.*, 2005). In contrast, later investigations reported a significant correlation between the *TMPRSS2:ERG* fusion and poor clinical outcome (Attard *et al.*, 2008; Demichelis *et al.*, 2007; Nam *et al.*, 2007), although this finding was not confirmed in subsequent studies (FitzGerald *et al.*, 2008; Gopalan *et al.*, 2009; Saramaki *et al.*, 2008).

Another common alteration in prostate cancer is the somatic amplification of the chromosomal region 8q24. This event has been reported most commonly in advanced tumors and the genomic region is known to harbor the oncogene *MYC*. Gains of the whole chromosome 8 seem more common in early-stage disease, whereas the specific amplification of 8q24 is found more frequently in advanced cases (Jenkins *et al.*, 1997; Nupponen *et al.*, 1998; Sun *et al.*, 2007; Van Den Berg *et al.*, 1995; Visakorpi *et al.*, 1995b). However, in more recent studies, *MYC* mRNA was found to be significantly overexpressed in primary prostate tumors compared to normal prostate or prostatic benign hyperplasia (BPH) samples (Lapointe *et al.*, 2004; Tomlins *et al.*, 2007; Yu *et al.*, 2004). In the absence of gene amplification, immunohistochemically detected MYC protein expression was confirmed to be elevated in cases of both primary adenocarcinoma and HGPIN compared to normal prostate epithelium (Gurel *et al.*, 2008). At a functional level, *MYC* overexpression was reported to induce rapid formation of PIN, followed by invasive adenocarcinoma in a mouse model (Ellwood-Yen *et al.*, 2003), and to immortalize normal human prostate epithelial cells (Gil *et al.*, 2005). Taken together, these findings suggest that *MYC* overexpression plays an important role in prostate tumorigenesis.

The gain at 8q24 may not be directly responsible for *MYC* upregulation and other genes in the same region may explain the significance of the chromosomal alteration (Koh *et al.*, 2010). The *EIF3S3* (eIF3-p40) gene encodes p40, a subunit of the translation initiation factor eIF3 and located at 8q23 and was found to be highly expressed in both breast and prostate cancer cell lines harboring the amplification at 8q23–q24 (Nupponen *et al.*, 2000; Savinainen *et al.*, 2006). Moreover, *RAD21* (double-strand-break repair protein rad21) and *KIAA0196* were shown to be overexpressed in clinical prostate carcinomas and to be amplified in 30-40% of xenografts and hormone-refractory tumors, suggesting that these genes may contribute to the effects of the common 8q23-24 amplification (Porkka *et al.*, 2004). In addition, several genome-wide association studies have shown that

the 8q24 region contains several loci linked to increased prostate cancer risk (Al Olama *et al.*, 2009; Amundadottir *et al.*, 2006; Yeager *et al.*, 2007). Although the risk alleles all cluster within a region which does not seem to contain known annotated genes or miRNAs (Pomerantz *et al.*, 2009), it was recently shown that multiple enhancer elements are present within this region (Jia *et al.*, 2009; Sotelo *et al.*, 2010); these enhancers may in turn alter *MYC* expression. Despite the current level of understanding, the exact mechanisms responsible for *MYC* overexpression in prostate cancer remain unclear.

Recent advances in next-generation sequencing (NGS) technologies have significantly increased the sensitivity and scalability of DNA sequencing, allowing the complete sequencing of entire genomes (whole-genome sequencing, WGS), the sequencing of the coding regions of the genome (whole-exome sequencing, WES) and/or the sequencing of the total RNA content/transcriptome (RNA-sequencing, RNAseq) in a reasonably cost-effective and reliable manner. Moreover, other variations of the technique, such as reduced representation bisulfite sequencing (RRBS) and chromatin immunoprecipitation sequencing (ChIPSeq), are used to study epigenetic features of a genome (methylation and DNA-associated protein binding sites, respectively). These recently developed technologies have generated an unprecedented amount of data, allowing improved understanding of the genetic basis of the clinical variability of prostate cancer, through the discovery of rarer genetic alterations. These discoveries provide a major step towards the establishment of patient-specific, personalized treatment paradigm (Yadav *et al.*, 2015).

Recently performed NGS studies have validated the previously described ETS transcription factor fusion rearrangements, which occur in approximately 50% of PC cases (Berger *et al.*, 2011). These experiments have also led to the discovery of rare SLC45A3-BRAF (solute carrier family 45, member 3-v-raf murine sarcoma viral oncogene homolog B1) and ESRP1-RAF1 (epithelial splicing regulatory protein-1-v-raf-1 murine leukemia viral oncogene homolog-1) gene fusions. The expression of SLC45A3-BRAF or ESRP1-RAF1 in prostate cells was found to induce a tumor phenotype that was sensitive to RAF and mitogen-activated protein kinase kinase (MAP2K1) inhibitors. These data highlight the importance of RAF family gene rearrangements in prostate cancer and suggesting that RAF and MEK inhibitors may be useful treatment options for a subset of tumors (Palanisamy *et al.*, 2010).

Moreover, using transcriptome sequencing, a novel TMPRSS2-SKIL fusion gene was recently identified in 1.1% of prostate cancer samples and 3.7% of cell lines and

xenografts, (Annala *et al.*, 2015). The fusion was shown to cause the overexpression of *SKIL*, which encodes a Ski protein responsible for TGF-beta pathway inhibition via interaction with SMAD proteins (Stroschein *et al.*, 1999). Another rare fusion event in prostate cancer has recently been reported using deep sequencing and involves the histone H3K79 methyltransferase *DOT1L* and *HES6*. The *DOT1L-HES6* fusion gene was found to induce *HES6* overexpression, which was shown to promote androgen-independent growth *in vitro* (Annala *et al.*, 2014). These results are consistent with another study reporting *HES6* up-regulation in aggressive human prostate cancer and increased castration-resistant tumor growth in the absence of AR ligand binding, with HES6 enhancing the transcriptional activity of AR (Ramos-Montoya *et al.*, 2014).

Novel recurrent mutations were recently identified in multiple genes using WES, including the Forkhead transcription factor gene *FOXA1,* which is known to promote cell cycle progression in castration-resistant prostate cancer. Also identified was *MED12*, which encodes a subunit of the mediator complex and the Cyclin-dependent kinase 8 (CDK8) sub-complex. This latter complex regulates basal and stimulus-specific transcriptional programs. In addition, recurrent mutations were found in *SPOP* (speckle-type POZ protein), and these alterations were mutually exclusive with *ERG* rearrangements, suggesting that *SPOP* mutation and ETS fusions may represent early and divergent driver events in prostate carcinogenesis (Barbieri *et al.*, 2012).

A rare but recurrent mutation (G84E) was discovered by targeted sequencing in the homeobox transcription factor gene *HOXB13* and was found to be associated with a significantly increased risk of hereditary prostate cancer (Ewing *et al.*, 2012).

Recurrent mutations in multiple chromatin- and histone-modifying genes were discovered by WES in CRPC. These genes include the H3K4-specific histone methyltransferase *MLL2*, the above-mentioned *FOXA1*, *UTX* (also known as *KDM6A*) and *ASXL1*, both known to interact with AR (Grasso *et al.*, 2012).

Significant overexpression and gene amplification of the serine/threonine protein kinase *AURKA* (aurora kinase A) and the transcription factor N-myc (*MYCN)* were also discovered by RNASeq in the rarer and more aggressive neuroendocrine prostate cancer (NEPC), which more commonly arises after hormonal therapy for prostate adenocarcinoma (Beltran *et al.*, 2011).

2.4.3 miRNAs in prostate cancer

Several studies have recently reported aberrant expression of miRNAs in clinical specimens of prostate cancer, using microarray technology and bead-based flow cytometry. However, the results of these analyses are often controversial and conflicting for several possible reasons, including sample quality, integrity and number; the RNA collection methods; the choice of the healthy reference tissue; contaminating cells; and the specificity of the expression platform used (Coppola *et al.*, 2010). One of the first large-scale, bead-based, flow cytometric miRNA expression profiles of cancer tissue reported a general downregulation of miRNAs in tumors compared to normal tissues (Lu *et al.*, 2005). However, more recent microarray studies showed an overall up-regulation of miRNAs in cancer, including prostate cancer (Ambs *et al.*, 2008; Volinia *et al.*, 2006). Different results were also reported by other groups, who observed a widespread tendency of miRNA down-regulation in prostate cancer (Ozen *et al.*, 2008; Porkka *et al.*, 2007). Taken together, these results indicate that insufficient evidence has been collected for a conclusive miRNA expression profile of prostate cancer.

Nonetheless, several specific miRNAs have been individually studied and confirmed to be functionally involved in prostate cancer tumorigenesis (Table 2).

The *let-7* miRNA family, encoded by the *let-7* gene, was reported to be significantly downregulated in human PC (Nadiminty *et al.*, 2012) and includes ten highly conserved mature miRNAs (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i and miR-98) (Roush and Slack, 2008). Although the role of *let-7* is not fully understood, several studies have shown that *let-7* function as tumor suppressor miRNAs by targeting oncogenes involved in inflammation, cell proliferation, the epithelial to mesenchymal transition (EMT) and cell cycle regulation. In particular, *let-7* can downregulate high mobility group (HMG) HMGA1 and HMGA2 (Rahman *et al.*, 2009), which are chromatin-associated non-histone proteins and have been implicated in differentiation, neoplastic transformation and EMT (Hillion *et al.*, 2009; Reeves *et al.*, 2001; Zhu *et al.*, 2013). Other targets of *let-7* are the cell cycle regulating factor cyclin D2 (*CCND2*) (Dong *et al.*, 2010), the oncogenic transcription factor c-Myc (Sampson *et al.*, 2007), the major regulator of inflammation and prostate cancer progression interleukin-6 (IL6) (Nguyen *et al.*, 2014; Sung *et al.*, 2013) and the oncogenes *NRAS*, *KRAS* and *HRAS* (Johnson *et al.*, 2005). *Let-7* can also indirectly

regulate *AR*, by suppressing its transcriptional activator c-Myc (Nadiminty *et al.*, 2012).

Other well studied miRNAs with tumor suppressive functions in prostate cancer include miR-145, miR-203, miR-205, miR34a, miR-15a/miR-16-1, and miR-101 and miR-193b.

miR-145 has been reported to be downregulated in PC (Suh *et al.*, 2011) and to target the actin bundling protein Fascin Homolog 1 (*FSCN1*), which is involved in cell motility and adhesion during tumorigenesis and metastasis (Fuse *et al.*, 2011). It has also been shown that miR-145 targets *OCT4*, *SOX2* and *KLF4*, which are involved in cellular dedifferentiation and pluripotency (Huang *et al.*, 2012).

miR-34a was shown to be downregulated in $CD44^+$ prostate cancer cells, which are putative cancer stem cells (CSCs) (prostate cancer stem cells are reviewed below). MiR-34a overexpression was found to significantly inhibit prostate cancer metastasis and extend survival *in vivo* in mouse model (Liu *et al.*, 2011). miR-34a was also shown to target *AR* (Kong *et al.*, 2012), *c-Myc* (Yamamura *et al.*, 2012), the cell-cycle regulatory gene *CDK6* (Lodygin *et al.*, 2008) and the anti-apoptotic gene *SIRT1* (Yamakuchi *et al.*, 2008).

miR-15a and miR-16-1 belong to the same cluster at 13q14 and their expression is often downregulated in PC, due to 13q14 deletion (Hyytinen *et al.*, 1999; Porkka *et al.*, 2011). A recent study demonstrated that the level of miR-15a/16-1 is inversely correlated with the expression of the anti-apoptotic gene *BCL-2*, wingless-type 3A (*WNT3A*) and cyclin-D1 (*CCND1*), the latter two of which are involved in proliferation. The restoration of miR-15a and 16-1 was reported to arrest cell growth and induce apoptosis *in vivo* (Bonci *et al.*, 2008). Moreover, miR-15a and miR-16-1 were observed to be downregulated in fibroblasts surrounding the prostate tumors, resulting in increased tumor growth and progression. The mechanism underlying this effect was determined to be reduced post-transcriptional repression of FGF-2 and its receptor FGFR1, which function in both stromal and tumor cells to enhance cancer cell survival, proliferation and migration (Musumeci *et al.*, 2011).

miR-101 was recently described to be inversely correlated with the expression of polycomb gene *EZH2,* which is involved in prostate cancer progression, as described above (Varambally *et al.*, 2002). The expression of *EZH2* in prostate cancer cell lines was shown to be inhibited by miR-101. Conversely, miR-101 expression was shown to decrease in human prostate tumors during cancer

progression, a result that was consistent with the observed increase in *EZH2* expression. In the same study, the loss of one or both miR-101 encoding loci was observed in 38% of clinically localized prostate cancer cells and 67% of metastatic disease cells (Varambally *et al.*, 2008).

miR-203 expression has been reported to be specifically downregulated in bone metastatic PC. In addition, the re-expression of miR-203 in bone metastatic PC cell lines was reported to suppress metastasis by reducing migration and invasion and inducing a reverse mesenchymal-to-epithelial transition (MET). In the same study, miR-203 was shown to target the cell cycle regulator survivin/BIRC5, the bone-specific transcriptional regulators *RUNX2* and *DLX5*, the transcriptional repressors of E-cadherin *ZEB2*, involved in EMT, and the central mediator of TGF-β signaling *SMAD4* (Saini *et al.*, 2011).

miR-205, similarly to miR-203, is involved in PC progression via the targeting of EMT genes. Significantly lower miR-205 expression levels were found in cancer than in normal prostate cell lines and in tumors than in matched normal prostate tissues. miR-205 re-expression in prostate cancer cells was shown to induce cell rearrangements consistent with a mesenchymal-to-epithelial transition (Gandellini *et al.*, 2009). Several putative targets have been described for miR-205, including the epidermal growth factor receptor family member *ERBB3,* the transcription factors *E2F5* and *E2F1*, the serine/threonine kinase *PRKCE* (PKCε) (Gandellini *et al.*, 2009), *BCL2* (Verdoodt *et al.*, 2013) and *ZEB1* (Tucci *et al.*, 2012).

miRNA	Alteration	Targets
let-7	Downregulated	HMGA1, HMGA2, MYC, NRAS, KRAS, HRAS, AR
miR-145	Downregulated	FSCN1, OCT4, SOX2, KLF4
miR-34a	Downregulated	AR, MYC, CDK6, SIRT1
miR-15a/16-1	Deleted	BCL-2, WNT3A, CCND1
miR-101	Deleted	EZH2
miR-203	Downregulated	BIRC5, RUNX2, DLX5, ZEB2, SMAD4
miR-205	Downregulated	ERBB3, E2F5, E2F1, PRKCE, BCL2, ZEB1
miR-193b	Hyper-methylated/Downregulated	CCND1
miR-21	Upregulated	PDCD4, BCL-2, MARCKS
miR-125b	Upregulated	TP53, BBC3, BAK1
miR-221/miR-222	Upregulated	$p27^{Kip1}$, ARHI
miR-148a	Upregulated	CAND1, MSK1
miR-32	Upregulated	BTG2

Table 2. miRNAs functionally involved in prostate cancer and their target genes.

In a screening aimed at identifying epigenetically regulated miRNAs in prostate cancer, miR-193b was described to be significantly downregulated in cancer specimens compared to BPH controls. Moreover, a CpG island located approximately 1 kb upstream of the mature miR-193b locus was found to be densely methylated in the 22Rv1 prostate cancer cell line. Forced miR-193b re-expression was shown to significantly reduce both proliferation rate and the ability of 22Rv1 cells to grow anchorage-independently in a soft agar assay. The same pattern of methylation was found in tumor samples, although not as dense as in 22Rv1 cells, and this pattern was absent in BPH controls. This result suggests that miR-193b function as a tumor suppressive miRNA and is targeted by epigenetic silencing in prostate cancer (Rauhala *et al.*, 2010). In a recently published study,

miR-193b was shown to target cyclin D1 (*CCND1*) in prostate cancer cell lines (Kaukoniemi *et al.*, 2015).

Well studied oncogenic miRNAs (oncomiRs) in prostate cancer include miR-21, miR-125b, miR-221/miR-222, miR148a and miR-32.

Recent studies suggest that miR-21 is regulated by AR and its expression level was found to be elevated in PC. Functional studies showed that miR-21 overexpression was sufficient to rescue androgen-dependent LNCaP cells from androgen-ablated growth arrest and to drive androgen-independent growth. Moreover, elevated miR-21 expression was found to be sufficient to drive androgen-dependent tumor growth in a castrate environment and to induce androgen-independence *in vivo* (Ribas *et al.*, 2009; Ribas and Lupold, 2010). Serum levels of miR-21 were reported to be elevated in CRPC patients and to correlate with serum PSA levels, indicating the possible application of miR-21 as a marker for advanced disease (Zhang *et al.*, 2011). miR-21 has also been reported to be activated by adaptor-related protein complex 1 (AP-1) (Fujita *et al.*, 2008) and STAT-3 (Iliopoulos *et al.*, 2010), both of which are associated with advanced prostate cancer and metastasis (Abdulghani *et al.*, 2008; Kajanne *et al.*, 2009). miR-21 has been shown to target several tumor suppressor genes, including (i) programmed cell death 4 (PDCD4) and maspin, both of which have been implicated in invasion and metastasis (Zhu *et al.*, 2008); (ii) PTEN (Meng *et al.*, 2007); (iii) BCL-2 (Shi *et al.*, 2010); (iv) and myristoylated alanine-rich protein kinase c substrate (MARCKS), which is involved in increased cell mobility and invasiveness (Li *et al.*, 2009).

miR-125b is also an AR-induced miRNA and has been described to be overexpressed in prostate cancer cell lines and in prostate tumors compared with benign prostate epithelial lines and benign prostatic tissues, respectively. Moreover, miR-125b was shown to promote xenograft cell proliferation *in vivo* and to target the pro-apoptotic mediators *TP53*, *BBC3* (Puma) and *BAK1* (Nadiminty *et al.*, 2012; Shi *et al.*, 2007; Shi *et al.*, 2011).

miR-221 and miR-222 belong to the same cluster and have been shown to be upregulated in androgen-independent PC-3 cells. These miRNAs target $p27^{Kip1}$, a cell cycle inhibitor and tumor suppressor (Galardi *et al.*, 2007; le Sage *et al.*, 2007), and *ARHI*, a tumor suppressor that negatively regulates proliferation (Chen *et al.*, 2011).

miR-148a was found to be overexpressed in advanced PC compared to primary tumors (Walter *et al.*, 2013) and to increase proliferation in an *in vitro* model by

targeting the ubiquitin ligase cullin-associated and neddylation-dissociated 1 (CAND1), which is involved in cell cycle regulation and proliferation (Murata *et al.*, 2010). However, another study reported lower miR-148a expression levels in PC3 and DU145 hormone-refractory prostate cancer cells in comparison to normal prostate epithelial cells and LNCaP cells. Transfection with miR-148a was also found to inhibit cell growth, migration and invasion. miR-148a was also found to increase PC-3 sensitivity to the anti-cancer drug paclitaxel by targeting of mitogen- and stress-activated kinase 1 (MSK1)(Fujita *et al.*, 2010). These disparate results indicate the debated role of this miRNA in prostate cancer.

In a recent study, miR-32 was shown to be androgen-regulated and to be highly expressed in CRPC compared to BPH controls. The B-cell translocation gene 2 (*BTG2*), which is involved in several cellular processes, including cell cycle control and apoptosis, was identified as a miR-32 target (Jalava *et al.*, 2012).

Increasing evidence has confirmed the aberrant expression of miRNAs in prostate cancer and has highlighted their functionality in prostate tumorigenesis. However, inconsistencies in miRNA expression in clinical specimens and the complex regulatory mechanisms between miRNAs and their multiple target genes necessitate further clarification of their role and potential use as biomarkers and therapeutic targets.

2.4.4 Prostate cancer stem cells

Similarly to other solid tumors, it is unclear whether prostate cancer is organized hierarchically into clonally derived populations of cells with different proliferative potentials, as posited by the CSC model.

As CSCs may originate from oncogenic transformation of normal prostate epithelial stem cells, several studies have focused on the identification of normal epithelial stem cells within prostate tissue. As mentioned above, the prostatic epithelium consists of multiple cell types, including basal, luminal and more rare neuroendocrine cells. These distinct populations can be classified based on their morphological appearance, location and expression of specific markers. Basal cells are characterized by low/negative AR expression and exclusively express p63, a homologue of the p53 tumor suppressor. Basal cells also express cytokeratins (CKs) CK5 and CK14, but not CK8 or CK18. Luminal cells, in contrast, express

high level of AR, CK8 and CK18, but do not express CK5 or CK14. Moreover, an intermediate cell type characterized by properties of both luminal and basal cells has been described and reported to express CKs of both basal and luminal cells (CK5, 14, 8, and 18). These intermediate cells have been proposed to represent the transient amplifying cell population (Signoretti *et al.*, 2000; Signoretti and Loda, 2007; Uzgare *et al.*, 2004; Wang *et al.*, 2001).

The first line of evidence suggesting the presence of a stem population in the prostate was obtained by castration and testosterone replacement experiments. Following androgen ablation, 90% of the luminal cells, but only a small percentage of basal cells, undergo apoptosis. The prostate epithelium can be regenerated upon androgen restoration and the process can be repeated for many cycles, suggesting that stem cells are able to survive castration and are likely within the basal compartment (English *et al.*, 1987; Evans and Chandler, 1987). Consequently, a hierarchical model was proposed for the prostatic epithelium; specifically, a stem cell in the basal layer generates another stem cell and a multipotent progenitor cell by asymmetric division. The progenitor cell is often referred to as a transient amplifying cell and will differentiate into either an exocrine or neuroendocrine cell (Bonkhoff *et al.*, 1994; Bonkhoff and Remberger, 1996; Isaacs and Coffey, 1989).

The primary approach for the prospective identification of prostate stem cells has been the use of cell-surface markers for the isolation of cell population via flow cytometry and the subsequent analysis of their prostate tissue reconstitution ability in mouse renal grafts (Cunha and Lung, 1978; Xin *et al.*, 2003). Using this assay, several laboratories have enriched populations with stemness properties from the mouse prostate, including Sca-1$^+$ (Burger *et al.*, 2005), Lin$^-$ Sca-1$^+$ CD49f$^+$ (Lawson *et al.*, 2007) and Lin$^-$ Sca-1$^+$ CD133$^+$ CD44$^+$ CD117$^+$ cells (Leong *et al.*, 2008). However, it is not clear whether these populations are exclusively basal and translation from the mouse model to human tissue is often inadequate. The existence of luminal stem cells has also recently been reported. A *p63*$^{-/-}$ prostate containing luminal and neuroendocrine, but not basal cells, was successfully generated from *p63*$^{-/-}$ urogenital sinus (UGS), when grafted into adult male nude mice (Kurita *et al.*, 2004). p63 had been previously described as a marker of basal cells and p63 null mice do not form a functional prostate (Signoretti *et al.*, 2000). The results of the grafting study indicate that basal cells are not essential for prostatic regeneration and suggest the presence of stem cells in the luminal compartment. Moreover, in another study, genetic lineage marking of progenitor cells, followed by analysis of progeny

differentiation *in vivo*, led to the identification of a rare luminal population, termed CARNs (Castration-resistant Nkx3.1-expressing cells), that exhibited stem cell properties (Wang et al., 2009),. In this study, the cells were marked using a genetically engineered mouse line, in which the activity of an inducible Cre recombinase was controlled by the endogenous Nkx3.1 promoter. CARNs were the only cells that expressed Nkx3.1 upon castration and their progeny were found in both luminal and basal compartments, following androgen-mediated prostate regeneration. Single-cell transplantation of CARNs was also shown to induce prostatic duct formation in renal grafts, which generated all three prostate epithelial cell types. These experiments indicate that CARNs are multipotent (Wang *et al.*, 2009).

Several studies have also used flow cytometry-based approaches to isolate putative prostate stem cells from human tissues. In particular, a subpopulation of basal cells, representing approximately 1% of the total basal cell population and expressing high levels of $\alpha_2\beta_1$-integrin was shown to possess a fourfold greater ability to form colonies in vitro than the total basal population and a greater potential to regenerate a fully differentiated prostate epithelium *in vivo* (Collins *et al.*, 2001). It was subsequently reported that further enrichment of prostate stem cells could be achieved by isolating $\alpha_2\beta_1^{hi}$/CD133$^+$ cells, which possess a high *in vitro* proliferative potential and can reconstitute prostatic-like acini in immunocompromised male nude mice (Richardson *et al.*, 2004). In another report, the surface markers Trop2 and CD49f were used to enrich for basal cells that were able to efficiently form spheres *in vitro*, as well as regenerate prostatic tubules *in vivo* (Goldstein *et al.*, 2008).

In a key study aimed at identifying prostate cancer stem cells, $\alpha_2\beta_1^{hi}$/CD133$^+$ cells were assayed to establish whether they are also present in prostate tumors and distinct from the bulk population of cancer cells. CD44$^+$/$\alpha_2\beta_1^{hi}$/CD133$^+$ cells, comprising less than 0.1% of the tumor mass, were found to possess self-renewal and high proliferative potential in colony-forming assay and to differentiate into luminal cells in culture (Collins *et al.*, 2005). Subsequently, other studies investigated prostate cancer stem cells in prostate cancer cell lines and identified CD44$^+$/$\alpha_2\beta_1^{hi}$/CD133$^+$ cells in the DU145 line (Wei *et al.*, 2007), as well as CD44$^+$/CD24$^-$ cells in the LNCaP line (Hurt *et al.*, 2008). CD44$^+$/$\alpha_2\beta_1^{hi}$ cells from human LAPC9 xenografts have also been identified as candidate prostate CSCs (Patrawala *et al.*, 2007).

Despite our level of understanding, controversy remains with respect to both identification and definition of prostate cancer stem cells. Most importantly, tumor initiation has not been demonstrated in xenotransplantation experiments of cell populations derived from primary prostate tumors and the specificity of the cell-surface markers remains unclear. As previously mentioned, CSC nomenclature in the literature is often non-specific, with the concepts of CSC and tumor-initiating cells (TICs) often used interchangeably (Wang and Shen, 2011). More studies are necessary to clearly identify prostate CSC population(s) and to clarify the cellular origin of the disease.

3 Aims of the study

The aims of this study were to characterize a newly discovered chromosomal alteration in bladder cancer and to identify and investigate the role of novel non-coding RNAs in the molecular mechanisms of prostate cancer tumorigenesis. More specifically, the aims of each original communication are as follows:

I. To characterize the recurrent amplification at chromosomal region 1p21-22 in bladder cancer.
II. To obtain a miRNA expression profile in prostate epithelial subpopulations enriched from patient-derived clinical specimens.
III. To validate the expression and study the function of newly identified differentially expressed miRNAs in prostate cancer.
IV. To identify differentially expressed tRNA-derived fragments (tRFs) in clinical samples of prostate cancer.

4 Materials and methods

4.1 Cell lines and clinical samples (I, II, III, IV)

In study I, the bladder cancer cell lines UM-UC-3, TCCSUP, RT4, T24, HT-1376, J82, SCaBER, 5637, HT-1197 and SW780 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured according to the recommended conditions.

Freshly frozen samples from seven bladder cancer tissues were used for this study. The samples were obtained from Tampere University Hospital and include five urothelial carcinomas, one lymphoepithelial carcinoma and one undifferentiated carcinoma. DNA was extracted using DNAzol reagent (Molecular Research Center, Inc. Cincinnati, OH), according to the manufacturer's protocol. The use of the clinical samples was approved by the ethical committee of the Tampere University Hospital.

In study II, prostate biopsy cores were obtained immediately following radical prostatectomy. The site selected for biopsy was based on MRI and ultrasound reports, and any palpable lesions. BPH tissue was obtained from transurethral resection of prostate (TURP) chips. After confirming the pathological status, cultures were established and stem cells (SCs), transient amplifying cells (TAs), and committed basal cells (CBs) were selected as described previously (Collins *et al.*, 2005). Patient prostate tissue samples were obtained after written consent and full ethical approval. All the experiments were performed on cultures at passage 2. Flow cytometry was performed as previously described (Rane *et al.*, 2014). The details of antibodies used for FACS are as follows: CD49f (eBioscience: 11–0495–80, 1:200 for 20 min RT) and CD49b (Serotec: MCA743F, 1:200 for 20 min RT).

In study III, the prostate cancer cell lines PC3, DU145, LNCaP, 22Rv1, and VCaP were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured according to the recommended conditions. HT-1080 cells were a kind gift from Olli Lohi, Tampere Center for Child Health Research. Two sets of clinical samples were used for miRNA expression analysis and were both obtained from

Tampere University Hospital (TAUH). The first set included 54 freshly frozen samples of 5 benign prostate hyperplasia (BPH) samples and 28 untreated primary prostate tumors obtained via radical prostatectomy specimens. In addition, 7 BPH and 14 CRPC tumors were obtained from transurethral resection of the prostate (TURP). The second set included 81 hormonally untreated, freshly frozen PC prostatectomy samples. The samples were confirmed to contain a minimum of 70% cancerous or hyperplastic cells by hematoxylin-eosin staining. The mean age at diagnosis for the second set of samples was 62.1 years (range: 47.4–71.8) and the mean PSA at diagnosis was 11.8 (range: 3.15–51.5). The use of clinical material was approved by the ethical committee of the Tampere University Hospital. Written informed consent was obtained from the subjects donating the samples.

In study IV, the sequencing was performed on 10 libraries that were generated as previously described (Martens-Uzunova *et al.*, 2015). Briefly, each library was constructed from an RNA pool prepared from four individual patient samples with similar pathological or genetic characteristics (Hendriksen *et al.*, 2006). RNA was isolated from several tissue types, including normal adjacent prostate tissue (NAP), prostate tumors with different Gleason score (6, 7, 8) (PCa6, PCa7, PCa8) and metastatic lymph nodes (LN_PCa), all obtained by radical prostatectomy. RNA was also isolated from benign prostate hyperplasia tissue (BPH) obtained by cystoprostatectomy and castration resistant prostate tumors obtained by trans-urethral resection of the prostate (TURP_PCa) (Martens-Uzunova *et al.*, 2015). NAP and BPH samples were used as controls. The clinical parameters of each group are summarized in Table 1 in IV. PC groups with Gleason score 6 were divided into cured and recurrent disease groups or into groups with or without the TMPRSS2-ERG fusion or ETV abnormalities. Sample materials were obtained from the tissue banks of the Erasmus University Medical Center, Rotterdam, The Netherlands (Erasmus MC, Rotterdam, The Netherlands) and Tampere University Hospital (TAUH, Tampere, Finland). The collection and use of patient material was performed according to the national laws concerning ethical requirements and approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act (MEC-2004- 261), and the Ethical Committee of the Tampere University Hospital. The samples were snap-frozen and stored in liquid nitrogen. The Gleason score and percentage of cancer cells were evaluated independently by two pathologists. Only samples with more than 70% tumor cells were used for sequencing library preparation. All the samples that

were used for the control prostate pool contained 0% of tumor cells. Total RNA was extracted using RNABee reagent (Campro Scientific, GmbH, Berlin, Germany) according to the manufacturer´s protocol.

qRT-PCR validation was performed in two separate cohorts. The first cohort (cohort 1) consisted of 65 samples obtained from Erasmus MC, including 48 PC samples and 17 NAP controls. The samples were collected, handled and evaluated as mentioned in the previous paragraph. The second cohort (cohort 2) consisted of 104 hormonally untreated primary prostate tumors from radical prostatectomy specimens obtained from Tampere University Hospital. The samples were confirmed to contain a minimum of 70% cancerous or hyperplastic cells by hematoxylin/eosin staining. A pathologist performed the histological evaluation and Gleason grading for the second set based on hematoxylin/eosin stained slides. Follow-up data were available for 74 of these samples. The use of clinical material was approved by the ethical committee of the Tampere University Hospital. Written informed consent was obtained from the subjects donating the samples. TRI-reagent (Molecular Research Center Inc., Cincinnati, OH, USA) was used to collect total RNA from the freshly frozen clinical samples, according to the manufacturer's instructions. To account for technical variations, the NAP samples from cohort 1 were used as a control in qRT-PCR validation for cohort 2 and were analyzed and normalized independently and separately for each cohort.

4.2 Array comparative genomic hybridization (I)

16K cDNA microarrays were obtained from the Finnish Microarray DNA Centre (http://www.btk.fi/microarray-and-sequencing/) (Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland). The poly-L-lysine coated slides contain approximately 16000 annotated clones, in duplicate, from sequence verified I.M.A.G.E. Consortium cDNA library. Comparative genomic hybridization to microarray (aCGH) was performed as described previously (Saramaki *et al.*, 2006). Briefly, using a BioPrime Labeling Kit (Invitrogen), 2 to 10 µg RsaI-digested (Fermentas UAB, Vilnius, Lithuania) DNA was labeled with Cy5-dCTP, and normal male reference DNA was labeled with Cy3-dCTP (Amersham Biosciences UK Ltd., LittleChalfont, United Kingdom). The sample and reference DNAs were cohybridized overnight at +65°C, under cover slips, to microarray

slides, in a final volume of 38.5 µl of hybridization mix (3.4×SSC, 0.3% SDS, 1.3×Denhardt's (Sigma-Aldrich, St. Louis, MO), and 0.5×DIG Blocking Buffer (Roche Diagnostics, Mannheim, Germany)). After stringent washes, the slides were scanned with a ScanArray4000 confocal laser scanner (Perkin Elmer, Boston, MA). The signal volumes were quantified using the QuantArray software program (Packard Bioscience, Bio- Chip Technology LCC, Billerica, MA). The data were analyzed using the cluster along chromosomes (CLAC) algorithm, as previously described and visualized using CGH-Miner software (Wang *et al.*, 2005).

4.3 Fluorescence in situ hybridization (I)

Human genome PAC/BAC clones were purchased from Invitrogen™ Corporation. The list of clones is shown in Table 1 in I and the indicated chromosome positions are were determined according to the UCSC (University of California Santa Cruz) Genome Browser, February 2009 assembly (GRCh37/h19). The clones were labeled with digoxigenin-dUTP (Roche Diagnostics) or Alexa Fluor®-dUTP (Invitrogen™) by nick-translation. A pericentromeric probe for chromosome 1 labeled with FITC-dUTP was obtained from Roche. The metaphase slides from the bladder cancer cell lines were prepared using standard techniques. The slides were denatured in 70% formamide/2xSSC at 70°C for 2 min and dehydrated in an ascending ethanol series. Hybridization was performed over night at 37°C. After stringent washes, the slides were stained with antidigoxigenin-rhodamine (Roche Diagnostics) for the digoxigenin-labeled probes and embedded in an antifade solution (Vectashied, Vector Laboratories, Burlingame, CA, USA) containing 4,6-diamidino-2-phenylindole (DAPI) as a counter stain. The stained slides were analyzed on an epifluorescence microscope (Olympus) and the acquired images were processed using Image-Pro® image-processing software (Media Cybernetics). A total of 50 nuclei were considered for statistical analysis of the FISH signals in each experiment. An amplification was defined as a locus-specific probe/centromere ratio >2. In each experiment the hybridization efficiency of the locus-specific and centromeric probes was evaluated using the 5637 bladder cancer cell line as a triploid control.

4.4 Microarrays (I, II)

4.4.1 Gene expression microarray (I)

Total RNA from bladder cancer cell lines was collected and extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The samples were subsequently amplified and hybridized using the Agilent whole genome oligo microarray platform (Agilent Technologies, Palo Alto, CA, USA) and Xpress Ref ™ Human Universal Reference Total RNA (SuperArray Bioscience Corporation) was used as a reference. The resulting data files from Agilent Feature Extraction Software (version 9.5.1.1) were imported into the Agilent GeneSpring GX software (version 11.0) for further analysis. A fold-change cutoff of 2 was used to determine differential gene expression.

4.4.2 miRNA microarray (II)

Total RNA was extracted using miRVana kit (Life Technology, Paisley, UK). The microarray was performed using an Agilent V3 miRNA microarray (Agilent Technologies Inc., Santa Clara, USA). The array data were processed using Agilent Feature extraction software. The raw data were mapped to the latest human genome release and zero or negative intensities were replaced with the lowest positive intensity values. The data were quantile normalized and RMA summarized. For each comparison, p values were determined using Student's two-tailed *t*-test and the Wilcoxon rank sum test. The microarray data are deposited at Gene Expression Omnibus (GEO) (accession number: GSE59156).

4.5 Cell transfections (II, III)

In study II, the cells were transfected with a 50 nM miScript miRNA mimic for miR-548c-3p (Qiagen GmbH, Hilden, Germany) and appropriate controls (Qiagen GmbH, Hilden, Germany) using Oligofectamine (Life Technology, Paisley, UK). The transfected cells were washed with phosphate-buffered saline (PBS) twice after

8 hours to minimize the cellular toxicity of transfection reagents. All the analyses were performed 72 hours after transfection.

In study III, the cells were transfected with 20 nM or 100 nM of human miRVana TM microRNA mimic for miR-1247–5p or a negative control (Thermo Fisher Scientific/Ambion, Waltham, MA). The INTERFERin™ transfection reagent (Polyplus-transfection, Illkirch, France) was used according to the manufacturer's instructions.

4.6 Real time, quantitative PCR (I, II, III, IV)

In study I, total RNA from bladder cancer cell lines, extracted as described above, was reverse transcribed using random hexamer primers and AMV reverse transcriptase (Thermo Scientific). Quantitative Real Time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) and a BioRad CFX96 ™ Real-Time PCR Detection System. Each sample was run in duplicate and the expression values were normalized against those of TATA-binding protein (TBP). The primer sequences are shown in Table 2 in I.

In study II, total RNA was extracted using miRVana kit (Life Technology, Paisley, UK). miRNA expression was assessed either using miRNA TaqMan probes (Life Technology, Paisley, UK) or miScript primer assays (Qiagen GmbH, Hilden, Germany).

qRT-PCR for mRNA analysis was performed using TaqMan probes (Life Technology, Paisley, UK): LCN2: Hs01008571_m1, CEACAM6: Hs03645554_m1, NF-kß1: Hs00765730_m1, WNT5A: Hs00998537_m1, RPLP0: Hs99999902_m1, ID2: Hs04187239_m1, PROM1: Hs01009250_m1, and SOX2: Hs01053049_s1.

In study III, TaqMan® microRNA assays (Thermo Fisher Scientific, Waltham, MA) were used to study the expression of selected miRNAs, according to the manufacturer's protocol. The analysis was performed on CFX96 qPCR equipment (Bio-Rad Laboratories, Hercules, CA), and the raw expression data were normalized against RNU6B. The expression analysis of MYCBP2 and SOX9 was performed using Maxima TM SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA) on the same equipment. Specific primers for MYCBP2 and SOX9 were designed based on the Internet database Primer Bank:

MYCBP2_for 5´-GGGGACGGATTCTACCCAG-3´ and MYCBP2_rev 5´-ATTGAGCGCAGCGGTATAAAT-3´; SOX9_for 5´-AGCGAACGCACATCAAGAC-3´ and SOX9_rev 5´-CTGTAGGCGATCTGTTGGGG-3´. The raw expression data were normalized against TBP (TBP_for 5´-GAATATAATCCCAAGCGGTTTG-3´ and TBP_rev 5´-ACTTCACATCACAGCTCCCC-3´).

In study IV, total RNA extracted from clinical samples was reverse transcribed using an Exiqon miRCURY Universal cDNA Synthesis kit for first strand cDNA synthesis. This step adds a poly-A tail to RNA templates and the cDNA strand is subsequently synthesized using a poly-T primer. The provided UniSp6 spike-in RNA was added to the reverse transcription reaction to control for the quality of RNA isolation and efficiency of the reaction. A calibrator sample was also included for the analysis of the results. The amplification was performed using Exiqon miRCURY LNA™ SYBR® Green Master Mix and specific LNA™ primers were designed by Exiqon for each of the tRNA fragments. The names of the fragments with the target sequences are shown in Table 2 in IV. The plates were run on an Applied Biosystems ABI 7900 thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) and on Bio-Rad CFX96 Real Time System (Bio-Rad Laboratories, Hercules, CA, USA). The data were analyzed using the ΔΔCT method and the expression of each tRF was normalized against the small nucleolar RNA SNORD38B (Reference gene primer set 2039, Exiqon, Vedbaek, Denmark). The statistical significance of qPCR expression data was assessed using the Mann-Whitney U test. The log-rank test was used to compare progression-free survival distributions of the tumor samples. P-values lower than 0.05 were considered statistically significant. The statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla CA, USA, www.graphpad.com).

4.7 Western blot (III)

Total proteins were extracted from cell lines using RIPA lysis buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 4% polyacrylamide gel. The proteins were subsequently wet-transferred to Whatman™ nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). The membranes were incubated for 2 hr with a rabbit polyclonal antibody against MYCBP2 (ab86078,

Abcam, Cambridge, UK), a rabbit polyclonal against SOX9 (ab26414, Abcam, Cambridge, UK), a mouse monoclonal against vinculin as a loading control for MYCBP2 (ab18058, Abcam, Cambridge, UK) and a mouse monoclonal against pan actin as a loading control for SOX9 (NeoMarkers, Freont, CA). After washing, the membranes were incubated with secondary antibodies (anti-rabbit IgG-horseradish peroxidase-conjugated and anti-mouse IgG-horseradish peroxidase-conjugated (Dako, Glostrup, Denmark)) and the protein bands were visualized using Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA). The optical density of the protein bands was quantified using ImageJ, image processing and analysis software (http://imagej.nih.gov/ij/). The values for the MYCBP2 bands were normalized against vinculin, and the values for the SOX9 bands were normalized against pan-actin. Each experiment was performed in duplicate.

4.8 Luciferase assay (III)

The luciferase assay was performed using SwitchGear Genomics GoClone reporter constructs that were co-transfected with a LightSwitch miRNA mimic and non-targeting control (SwitchGear Genomics, Menlo Park, CA), according to the manufacturer's instructions. Briefly, HT-1080 human fibrosarcoma cells were seeded overnight to yield 90% confluence in a 96-well plate. The cells were subsequently co-transfected using the DharmaFECT Duo transfection reagent (Thermo Fisher Scientific, Waltham, MA) with 30 ng/ml of individual GoClone reporter vectors (3´-UTR sequence for MYCBP2; 3´-UTR for ACTB (beta-actin); random 3 0 -UTR; empty vector control) and 100 nM of the miR-1247–5p mimic or non-targeting control,. Each transfection was repeated for a total of eight replicates per sample. The next day, 100ml of the LightSwitch Assay Solution was added to each well of co-transfected cells and the luciferase signal was measured on a Wallac EnVision TM 2104 multilabel plate reader luminometer (Perkin Elmer, Waltham, MA), using the settings described in the protocol. The difference in luciferase signal intensity for miR-1247–5p transfected cells was calculated for each construct versus the non-targeting control. Data from housekeeping, random and empty constructs were used to control for non-UTR-specific treatment effects.

4.9 Cell irradiation and clonogenic recovery assay (II)

The cells were irradiated using a RS2000 X-Ray Biological Irradiator, which contains a Comet MXR-165 X-Ray Source (Rad-Source Technologies Inc., Suwanee, GA, USA). A dose of 2, 5 or 10 Gy was administered. The cells were stained with Trypan Blue stain (Sigma-Aldrich, Dorset, England). The live cells were counted using a Neubauer's hemocytometer. Clonogenic recovery assays were performed as previously described (Rane *et al.*, 2014).

4.10 Sequencing of tRNA fragments (IV)

RNA pools were outsourced for library construction and sequencing to BGI (Beijing Genomics Institute, Beijing, China). Briefly, total RNA samples were size-separated on denaturing polyacrylamide gel. RNA fragments in the size range of 15-35 nt were recovered from the gel and used for the preparation of sequencing libraries. The libraries were sequenced by Illumina deep sequencing. The tRNA database used to map the reads was constructed from the Genomic tRNA Database (http://gtrnadb.ucsc.edu/) as previously described (Hoogstrate *et al.*, 2015; Martens-Uzunova *et al.*, 2015). Shortly, tRNA genes with identical sequences were merged into single entries. Intronic sequences in tRNAs were removed, to allow mapping of tRFs derived from mature, spliced tRNAs. Genomic tRNAs in the database were modified by extending the 3'-ends with a single "CCA" sequence. Sequencing reads were mapped to tRNA database using CLC-Bio Genomics Workbench (Aarhus, Denmark). Subsequently, tRFs were predicted using the FlaiMapper program and a tRF database was constructed (Hoogstrate *et al.*, 2015).

The clinical parameters of the samples are listed in Table 1 in IV. The final read counts used for the expression analysis were generated by mapping the sequencing reads to the tRF database. tRFs derived from 5'-pre-tRNA leaders (5'U-tRFs) and 3'-pre-tRNA trailers (3'U-tRFs) were identified by mapping the sequencing reads to a tRNA reference database in which the genomic sequence of each tRNA gene was extended by 50 bp on both sides. The length, position and type of tRF were calculated from the sum of the read counts of the following groups: NAP, PCa6_cur, PCa6_nofusion, PCa6_TERG, PCa6_recur, PCa7_recur, PCa8_recur, TURP_PCa, and LN_PCa. To identify differentially expressed tRFs, read counts were normalized

as “parts per million” and Kal’s Z-test on the observed proportions, followed by Bonferroni correction was subsequently performed. The determined adjusted p-values that were lower than 0.05 were considered significant.

4.11 Statistical analysis (I, II, III, IV)

The following tests were used to determine statistical significance of the results. In study II, all the data are representative of three or more experiments. The errors are the standard deviation (SD) of the mean. The significance was determined using Student’s two-tailed t-test. In study III, significant differences of the qRT-PCR results were evaluated using the Mann–Whitney U-test using GraphPad Prism statistics software (GraphPad Software Inc., La Jolla, CA). Student’s t-test was used to evaluate the statistical significance of the luciferase assay. Spearman’s rank correlation was used to compare reference genes for qRT-PCR normalization. The statistical tests for studies I and IV are described above in the respective methods section.

5 Results

5.1 Mapping of the chromosomal amplification at 1p21-22 in bladder cancer (I)

In this study, aCGH was performed on seven bladder cancer clinical samples, obtained from Tampere University Hospital and on six bladder cancer cell lines (HT-1197, 5637, RT4, T24, SW780 and SCaBER). The clinical specimens included five urothelial carcinomas of the bladder, one lymphoepithelial carcinoma and one undifferentiated carcinoma. The CLAC-analysis of the aCGH data (Wang *et al.*, 2005) showed a region of increased copy number at chromosome region 1p21-22, comprising a total of 2 Mb. The chromosomal alteration was present in five out of the seven clinical samples and in all the listed cell lines, except for HT-1197. An example of CLAC consensus plot for one of the samples (cell line 5637) is shown in Fig. 4.

As described above in the review of the literature, many of the genetic alterations found in bladder cancer consist of region-specific gains or losses of DNA copy number, which allows the identification of the position of key genes (Hoglund, 2012). Regions of DNA copy number gain or amplification commonly harbor oncogenes, whereas deleted regions harbor tumor suppressor genes. Therefore, we performed a series of fluorescence *in situ* hybridization (FISH) experiments to identify the minimal region of amplification at chromosome 1p21-22 and the genes harbored within the region. The bladder cancer cell lines SCaBER, HT-1376, UM-UC-3, TCCSUP, RT4, J82, T24 and 5637 were assayed with human PAC/BAC clones, spanning a region of approximately 6 Mb at 1p21-22.

Most of the cell lines did not show significant chromosomal changes in the region, except HT-1376 and SCaBER (Table 1 in I). The urothelial carcinoma cell line HT-1376 showed a copy number gain in a region of approximately 5 Mb, whereas the squamous cell carcinoma cell line SCaBER showed a chromosomal amplification between chromosome positions 92,940,318 and 93,828,148, which represents the minimal amplified region (Table 1 and Fig. 2 in I). The UCSC (University of

California Santa Cruz) Genome Browser database, Feb. 2009 assembly (GRCh37/hg19), was queried for known human coding genes harbored between 92,940,318 and 93,828,148. A total of 11 genes are located within the amplicon and nine are known protein-coding genes (Table 3 in I).

To verify whether the gene amplification is associated with overexpression of the genes located within the amplicon, gene expression microarray was performed using the same bladder cancer cell lines. Interestingly, SCaBER squamous cell carcinoma cells, which harbor the high-level amplification, were also the only cells to show significant overexpression of four of the nine coding genes, namely, *EVI5*, *RPL5*, *TMED5* and *DR1* (Fig. 2 in I).

The expression of these genes was validated using qRT-PCR and the results of the gene expression microarray were confirmed, with the SCaBER cells showing the highest expression levels (Fig. 3 in I). Next, the Oncomine™ online database (http://www.oncomine.org) was queried to assess the expression of the four selected genes in published clinical sample datasets of bladder cancer vs. normal controls. Statistically significant ($P<0.0001$) upregulation of *DR1* was found in clinical samples of both superficial and infiltrating bladder cancer, compared to normal bladder tissue, and *TMED5* showed significant upregulation in superficial bladder cancer compared to normal. Neither *RPL5* nor *EVI5* showed changes in the same dataset (Sanchez-Carbayo *et al.*, 2006) (Fig. 4 in I).

5.2 Expression profile of miRNA in primary prostate cancer stem cells (II)

In this study, miRNA expression profiles were generated from prostate epithelial subpopulations, enriched from patient-derived specimens. As mentioned above in the review of the literature, considerable discrepancy and heterogeneity is present in previous reports investigating differential expression of miRNAs in prostate cancer (Coppola *et al.*, 2010). One of the major characteristics of miRNAs is their marked tissue specificity and involvement in organ development (Lee *et al.*, 2005; Lu *et al.*, 2005; Sempere *et al.*, 2004). One of the reasons for these inconsistencies may be that each cell-type has specific miRNA expression patterns, which are subject to changes in relation to the cellular differentiation status. Therefore, cell-

type specific and differentiation-specific signatures might contribute to the significant variations in the published data on miRNA expression.

As previously described, it has been shown that cells with stem-like phenotypes can be isolated from prostate cancer tissues based on cell-surface markers. In particular, it has been reported that $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$ cells, comprising less than 0.1% of the tumor mass, possess self-renewal and high proliferative potential in colony-forming assay and can differentiate to luminal cells in culture. These putative prostate cancer stem cells (SCs) can be distinguished from cells with more limited proliferative capacity, termed transit amplifying cells (TACs), and are characterized by a $CD44^+/\alpha_2\beta_1^{hi}/CD133^-$ phenotype. A third cell population includes basal cells committed to differentiation, and are therefore termed committed basal cells (CBCs), characterized by the phenotype $CD44^+/\alpha_2\beta_1^{low}$ (Collins *et al.*, 2001; Collins *et al.*, 2005; Richardson *et al.*, 2004).

To isolate the different subpopulations from clinical specimens, prostate epithelial cells derived from 5 BPH samples, 5 treatment-naïve primary tumors (PCa), 3 CRPC cases and 1 primary prostate epithelial cell (PrEC) sample, were briefly cultured and separated based on the above-mentioned surface markers. A miRNA microarray was performed on each cell subtype from each sample. The microarray data were validated by examining the expression of 11 randomly selected miRNAs, using qRT-PCR. Principal component analysis (PCA) performed on the microarray data, revealed a distinct miRNA expression profile in each subpopulation, regardless of the pathological status (Fig. 1a in II). Notably, the magnitude and extent of differential miRNA expression in the samples was found to be significantly higher in SCs compared to CBCs, than in PCa compared to BPH, or CRPC compared to BPH. These results suggest that the differentiation status of prostate epithelial cells is the primary determinant of miRNA expression profile (Fig.1b in II). PCA is a mathematical algorithm that simplifies complex data sets by reducing the dimensionality of the data while retaining most of the original variation. This process is accomplished by identifying new variables, termed principal components, that account for as much of the variance in the original variables as possible, while remaining mutually uncorrelated and orthogonal. In this way, each sample can be represented by relatively few metrics, making it possible to visualize similarities and differences in a plot (Ringner, 2008).

In addition to subpopulation-specific miRNA expression, a prostate epithelial SC signature was found to be conserved in BPH, PCa and CRPC samples, suggesting

that miRNAs may regulate SC properties, regardless of pathological status. A significant 60% overlap of miRNA expression was also found between SCs and previously published expression data of unfractionated CRPC samples. Interestingly, PCa-cancer stemlike cell (CSC) and CRPC-CSC signatures were identified and effectively distinguished from normal SC signatures (Table 1 in II). Moreover, conserved prostate SC miRNA signatures were found to share an miRNA expression pattern with human embryonic SCs (hESCs) (Leonardo *et al.*, 2012).

To validate the relevance of the obtained expression profiles, the role of one selected differentially expressed miRNA (miR-548-3p) was further investigated. miR-548-3p was found to be significantly overexpressed in SCs compared with CBCs and its overexpression has been previously associated with poor survival of PC patients (Taylor *et al.*, 2010) (Fig. 1c in II). The functional effects of miR-548-3p overexpression were evaluated in CBCs, transfected with miR-548-3p precursor. Dedifferentiation to a stem-like phenotype was observed, with a significant increase in colony-formation (Fig. 1d in II) and increased expression of the prostate epithelial stem cell proteins CD49b (integrin β_2) and CD49f (integrin β_6) (Fig.1 e in II). Moreover, significantly increased expression of the SC-specific genes *NFKB* (Birnie *et al.*, 2008), *ID2* (Lasorella *et al.*, 2014), *PROM1* (CD133) (Richardson *et al.*, 2004) and *SOX2* (Rybak and Tang, 2013) was observed, with a concomitant reduction of the CBC-specific genes *CEACAM6*, *WNT5A* and *LCN2* (Rane *et al.*, 2014). In addition, higher live cell count was obtained in miR-548-3p overexpressing CBCs after exposure to 5-Gy radiation (Fig. 1f in II).

Gene ontology analysis (GO) of potential miR-548-3p targets, predicted using the miRWalk algorithm (available at http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), was performed, and the results suggested that miR-548c-3p might be involved in SC maintenance and cell cycle regulation.

Lastly, miR-548-3p was found to be significantly upregulated in uncultured CRPC-derived epithelial cells compared to BPH-derived epithelial cells (Fig. 1g in II).

In a recent independent study, the over-expression of miR-548c-3p was shown to decrease doxorubicin-induced DNA damage in a cervical cancer cell line (Srikantan *et al.*, 2011) and miR-548-3p serum levels were also described to be significantly higher in CRPC patients compared to low-risk PC patients (Nguyen *et al.*, 2013).

Taken together, these results indicate that miR-548c-3p is a putative diagnostic and prognostic candidate marker for improving CRPC patient management. Clinical validation in a larger patient cohort is now necessary to establish the prognostic and/or therapeutic relevance of this miRNA. Moreover, this study provides a foundation towards understanding of key miRNA expression changes during prostate epithelial differentiation. The overlap between the miRNA expression patterns of hESCs, prostate epithelial SCs, and unfractionated CRPCs indicates that embryonic signaling machinery is activated in the terminal stages of PC. More studies are needed to clarify the role of prostate cancer stem cells in the development and progression of the disease.

5.3 Overexpression of miR-1247 in prostate cancer (III)

In recent studies, the Agilent microarray platform was used to profile the expression of miRNAs in clinical samples of primary PC and CRPC (Jalava *et al.*, 2012; Martens-Uzunova *et al.*, 2012). However, the arrays contained probes for only 723 miRNAs. To identify recently discovered, differentially expressed miRNAs, that are not included in the mentioned arrays, the expression data obtained from recent deep-sequencing experiments were analyzed. RNA-sequencing was performed on a total of eleven pools of clinical specimens, each pool containing four samples from control or malignant prostate tissue (Martens-Uzunova *et al.*, 2015). miR-1247-5p, miR-1249, miR-1269a, miR1271-5p, miR-1290, miR-1291 and miR-1299 showed differential expression in malignant samples compared to controls and were selected for validation by qRT-PCR in two sets of clinical specimens, described above in the Materials and Methods.

When performing qRT-PCR experiments, variation in the amount of starting material, sample preparation and RNA extraction, as well as in reverse transcription efficiency, can introduce errors. Therefore, technical variations between the reactions must be corrected. This is usually achieved by normalizing the raw expression values to an endogenous control gene. The endogenous control needs to be accurately validated and its expression is required to be relatively constant and abundant in the particular sample set used in the experiment (Carlsson *et al.*, 2010; Peltier and Latham, 2008). However, uncertainty remains over the selection of

appropriate reference genes for miRNA expression studies. The current convention is to normalize to small-nucleolar RNAs (commonly RNU6B, RNU44, RNU24 or RNU48), which are ≈70-nt non-coding RNAs involved in site-specific nucleotide modification of ribosomal RNAs as well as pre-mRNA splicing (Kiss, 2002). Recent evidence suggests that snoRNAs are dysregulated in cancer and therefore might not be suitable as references for the normalization of expression analyses that compare cancer to normal tissue (Mourtada-Maarabouni *et al.*, 2009). Moreover, the expression of some of these snoRNAs has also recently been correlated with tumor pathology and prognosis, suggesting that their use as reference genes can introduce a bias (Gee *et al.*, 2011). In this study, the four mentioned, commonly used reference genes were assessed in the specific sample sets used for miRNA expression analysis. The qRT-PCR expression data for five control miRNAs (miR-17-5p, 32-5p, 96-5p, 141-5p and 182-5p) normalized with the four RNUs, were compared with previously generated, published microarray data (Jalava *et al.*, 2012; Martens-Uzunova *et al.*, 2012) and RNA deep-sequencing of individual samples (Ylipaa *et al.*, 2015), for the same miRNAs. The normalization of qRT-PCR using RNU6B generated consistent results between all three compared platforms (Fig. S1 and S2 in III). Moreover, the expression of RNU44, RNU24 and RNU48 was subsequently studied in the same sample set, using RNU6B as a reference gene. All three snoRNAs showed significant differential expression in cancer samples compared to controls. In particular, in our sample sets, all these snoRNAs were upregulated in cancer cases, confirming RNU6B as the most stably expressed reference gene in our sample cohort (Fig.S3 in III). In the above-mentioned miRNA expression microarray studies, the clinical samples obtained by radical prostatectomy and those obtained by trans-urethral resection of the prostate (TURP) showed different overall miRNA expression patterns (Jalava *et al.*, 2012; Martens-Uzunova *et al.*, 2012). Therefore, the two types of samples were also analyzed separately in the present study and miRNA expression was compared only between cancer and control samples within the same type.

The expression of the selected miRNAs (miR-1247–5p, miR-1249, miR-1269a, miR-1271–5p, miR-1290, miR-1291, and miR-1299) was first studied using qRT-PCR in the first sample set (Fig. 1A and Fig. S4 in III). miR-1247-5p showed the highest differential expression and was significantly upregulated in CRPC samples compared to benign prostatic hyperplasia (BPH) (Fig. 1A and in III and Fig. 4). In

the second sample set, consisting of 81 primary PCs obtained by radical prostatectomy, miR-1247-5p did not show an association with the Gleason score, the pathological stage of the disease (Fig. 1B in III), or the prognosis (data not shown). To further assess the significance of the differential expression of miR-1247, qRT-PCR was performed on the prostate cancer cell lines PC-3, DU145, LNCaP, 22Rv1, and VCaP. Interestingly, miR-1247-5p showed highest expression in the androgen-independent PC-3 cells (Fig. 2A in III). This finding, together with the significant overexpression in CRPC samples, suggest that miR-1247-5p alteration might have a role during the advanced stages of the disease. Of the other studied miRNA candidates, miR-1290 showed a slight reduction in expression in PC samples, compared to BPH, although no significant difference was found in CRPC samples compared to BPH (Fig.S4D in III).

To identify putative target genes for miR-1247-5p in prostate cancer, online tools for target prediction (TargetScan 6.2 (http://www.targetscan.org) and miRanda (http://microrna.org)) were queried. In both queries, *MYCBP2* was identified as highest-scoring putative target. *MYCBP2* encodes a very large 510 kDa E3-ubiquitin ligase, also known as protein associated with myc (PAM). MYCBP2 was originally identified as a protein that interacts directly with the transcriptional activating domain of the transcription factor Myc (Guo *et al.*, 1998). However, there is no strong evidence that MYCBP2 protein is functionally associated with MYC. The expression of *MYCBP2* was studied in the first panel of clinical samples and was found to be significantly down-regulated in CRPC compared to BPH controls, showing an inverse correlation with miR-1247-5p expression in the same samples (Fig. 2B in III and Fig. 4). An inverse correlation of *MYCBP2* and miR-1247-5p expression was also found in the prostate cancer cell lines PC3, DU145, LNCaP, 22Rv1, and VCaP (Fig. 2A in III). To further investigate the effect of miR-1247-5p on *MYCBP2* expression, cell line models of miR-1247-5p overexpression were generated by transiently transfecting PC-3 and LNCaP cells with miR-1247 precursor and control. LNCaP and PC-3 cells were selected due to their intrinsically high and low levels of miR-1247-5p, respectively, as shown in Fig. 2A in III. The overexpression of miR-1257-5p upon transient transfection of precursor was confirmed by qRT-PCR with a scrambled RNA sequence used as a control. In both models, a significant reduction in *MYCBP2* mRNA levels was observed in miR-1247-5p-overexpressing cells (Fig. 3A and B in III). Moreover, PC-3 cells were

assayed for MYCBP2 protein expression levels by western blotting and the down-regulation of MYCBP2 was confirmed (Fig. 3C in III and Fig. 4).

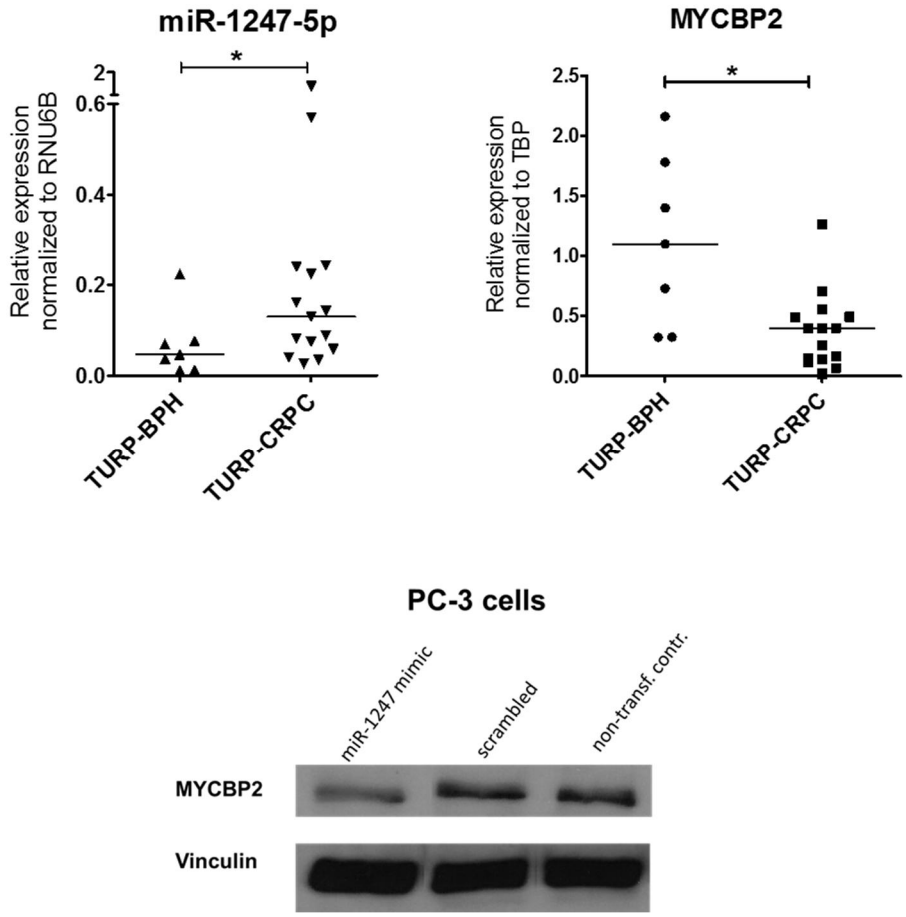

Figure 4. qRT-PCR analysis miR-1247-5p and *MYCBP2* expression in clinical samples of castration-resistant prostate cancer (CRPC) and benign prostatic hyperplasia (BPH) controls, both obtained by trans-urethral resection of the prostate (TURP). Western blotting of protein from the androgen-independent prostate cancer cell line PC-3 showing MYCBP2 protein downregulation upon miR-1247-5p transient overexpression.

Lastly, to investigate the interaction between miR-1247–5p and the putative target *MYCBP2*, a luciferase reporter assay was performed in HT-1080 human fibrosarcoma cells co-transfected with (i) a construct vector expressing the 3´-UTR of the *MYCBP2* transcript downstream of the luciferase gene and (ii) a miR-1247–5p mimic or negative control, as described in the Materials and Methods. Vectors expressing the 3´-UTR of the housekeeping gene *ACTB* (beta-actin) or random sequences were used to control for non-specific interactions of the miR-1247–5p mimic. A significant reduction in the luciferase signal was observed in cells co-transfected with the *MYCBP2* 3´-UTR vector and the miR-1247–5p mimic compared to controls (Fig. 3D in III), suggesting that *MYCBP2* is indeed targeted by miR-1247-5p in prostate cancer.

Interestingly, miR-1247-5p was recently reported to downregulate the expression of the transcription factor *SOX9* in isolated human chondrocytes, via non-canonical binding at the coding region of the *SOX9* gene, instead of the 3´-UTR. Moreover, miR-1247-5p itself was also shown to be down-regulated by SOX9 in a negative feedback loop (Martinez-Sanchez and Murphy, 2013).

However, *SOX9* elevation in the prostate has been shown to promote proliferation and to cooperate with PTEN loss to induce tumor formation (Thomsen *et al.*, 2010) and prostate cancer invasion (Wang *et al.*, 2008). In addition, dysregulation of a *SOX9*-dependent pathway, due to *SOX9* hyperactivation, has recently been shown to induce senescence bypass and tumor invasion in prostate cancer (Wang *et al.*, 2013). Therefore, to test whether miR-1247-5p overexpression affects *SOX9* levels in prostate cancer, *SOX9* expression was studied in PC-3 and LNCaP cells that transiently overexpress miR-1247-5p. Although a slight, non-significant reduction was found in *SOX9* mRNA levels, western blotting performed in PC-3 cells showed no changes at the protein level upon miR-1247-5p overexpression, suggesting that *SOX9* is not the primary target of miR-1247-5p in prostate cancer (Fig.S5 in III). More studies will be needed to investigate possible interaction between *SOX9* and miR-1247-5p in prostate cancer.

5.4 Expression of tRNA-derived fragments (tRFs) in prostate cancer (IV)

In this study, the composition and expression of tRFs was analyzed in an extended cohort of clinical prostate cancer samples, including normal adjacent prostate (NAP) and benign prostatic hyperplasia (BPH) controls, by deep sequencing. The cohort was previously described in study III (Martens-Uzunova *et al.*, 2015) and the clinical parameters of the samples are listed in Table 1 in IV.

The deep sequencing revealed tRFs derived from all 21 cytosolic tRNA isotypes, including selenocystein. A heatmap representing the variable relative abundances of the detected tRFs per isotype is shown in Fig. 1A in IV. The most abundant fragments were found to derive from $tRNA^{Ala}$ and $tRNA^{Lys}$. Moreover, variable amounts of tRFs were found to be generated from 15 out of 20 mitochondrial tRNAs (Fig.1A in IV). To reliably quantify the expression of tRFs, the RNA-sequencing reads require precise assignment of the exact position on the precursor transcript, a process that is hampered by the lack of proper annotation. To correctly determine the boundaries of tRFs in this dataset, the computational algorithm Fragment Location Annotation and Identification Mapper (FlaiMapper) was applied. The algorithm allows the extraction and annotation of the locations of tRFs by peak detection using the start and end position densities, followed by filtering and a reconstruction process (Hoogstrate *et al.*, 2015). The results of the FlaiMapper detection were subsequently filtered to merge identical sequences deriving from multiple precursor tRNAs into single entries (Supplementaty Fig. 2 in IV). A total of 598 unique cytosolic tRFs were identified with this method, whereas mitochondrial tRFs were excluded from further analysis due to unreliability in the automated prediction (Supplementary Fig.3). Most of the 598 tRFs were found to be between 15 and 23 nucleotides in length, with 40% being 19 nucleotides long (Fig. 1B in IV).

To further study their characteristics, the start and end position frequencies on the precursor tRNAs were analyzed (Fig. 1D and E in IV). In concordance with a recent report on tRF expression in prostate cancer cell lines (Lee *et al.*, 2009), most of the tRFs were found to originate from the 5´- and 3´- ends of the precursor tRNAs (Fig. 1C), when defined as follows:

1. 5´-derived tRFs, when the 3'-end nucleotide is at position ≤40 on the precursor tRNA sequence
2. 3´-derived tRFs, when the first 5'-nucleotide is at position ≥30 on the precursor tRNA sequence

However, based on the start and end position peaks, five different types of tRFs could be classified (Fig. 1F in IV):

1. 5e-tRFs, with start position in the first nucleotide of the 5'-end of the tRNA
2. D-tRFs, with a start position between nucleotides 12-23 and overlapping the D-loop
3. A-tRFs, with a start position between nucleotides 31-39 and overlapping the anticodon loop
4. V-tRFs, with a start position between nucleotides 45-49 and overlapping the variable loop
5. 3e-tRFs, with a start position between nucleotides 50-60 and overlapping the T loop

The number of unique fragments for each of the five types, generated from all the precursor tRNAs, appeared to be comparable (fragment uniqueness), whereas the relative abundance in the sample set showed a notable 75% of the total being of 5e-tRF type (Fig. 1G in IV).

Moreover, tRFs deriving from 5´-pre-tRNA leaders (5´U-tRFs) and 3´-pre-tRNA trailer (3´U-tRFs) were also detected (Supplementary Fig.4 in IV), although at lower expression levels than other types (data not shown).

As described in the review of the literature, several studies have recently provided evidence of aberrant expression of tRFs in cancer (Martens-Uzunova *et al.*, 2012; Maute *et al.*, 2013). To investigate whether tRFs are dysregulated in the analyzed samples, the expression levels of the detected fragments were compared between cancer samples and non-malignant controls. The overall expression levels of small non-coding RNAs (including tRFs, miRNAs and snoRNAs) was compared between NAP and BPH libraries (Supplementary Fig. 5 in IV). Interestingly, tRFs showed low correlation between the two sample groups, with increased expression in the BPH library, whereas the other sncRNAs showed similar expression levels. The result suggests that tRFs might be specifically overexpressed in BPH; these samples were therefore excluded from further analysis, and NAP samples were used as control.

A total of 110 tRFs were found to be differentially expressed in cancer compared to controls (Fig. 2 in IV). The number of differentially expressed tRFs was variable in samples with different stages of PC and 12 fragments were commonly differentially expressed in recurrent PC samples with Gleason scores 6, 7 and 8. Moreover, most of the up-regulated tRFs were found to belong to the 5e-tRF type, whereas most of the down-regulated tRFs were found to belong to the 3e-tRF type, suggesting they might exert different functions (Fig. 3 A-D in IV).

Six tRFs were selected for further validation. All were found to be commonly differentially expressed in recurrent PC with Gleason scores of 6, 7 and 8. According to the deep-sequencing data, four fragments were up-regulated and two were down-regulated in cancer compared to NAP, (Fig. 3 H-J, Table 2 and Supplementary Table 5 in IV).

The validation was performed by qRT-PCR in two distinct clinical sample cohorts obtained from the Erasmus Medical Center, Rotterdam, The Netherlands (cohort 1) and from Tampere University Hospital (cohort 2). A detailed overview of the cohorts is described above in the Materials and Methods.

tRF-544 (derived from $tRNA^{PheGAA}$) was confirmed to be significantly down-regulated in recurrent PC samples compared to both NAP controls and cured PC samples in cohort 1. In cohort 2, down-regulation was observed in PC samples with Gleason scores higher than 7 and in PC samples with more advanced pathological stage, suggesting a possible association between the aberrant expression and more aggressive or late-stage disease (Fig 4A in IV). The differential expression of tRF-544 was also confirmed in a recently published independent deep-sequencing study (Fig. 5) performed on a sub-set of the PC samples from Tampere University Hospital (Ylipaa *et al.*, 2015). tRF-315 (derived from $tRNA^{LysCTT}$) was significantly upregulated in PC in cohort 2 compared to NAP controls, and the difference in expression remained significant when the NAP controls were compared to any subgroup of PC samples (Fig. 4A in IV).

tRF-562 (derived from $tRNA^{GlyTCC}$) was significantly down-regulated in recurrent PC sample compared to NAP in cohort 1 and in advanced stage PC samples compared to NAP in cohort 2 (Fig. 4A in IV).

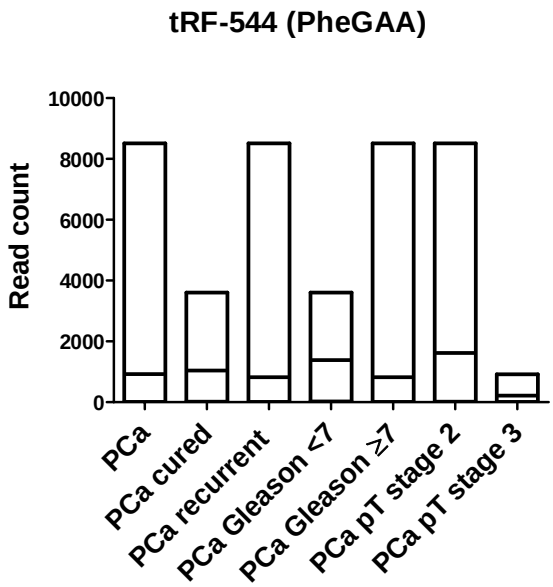

Figure 5. Expression of tRF-544 in a second independent deep-sequencing study performed on a subset of PC samples from Tampere University Hospital (Ylipaa *et al.*, 2015).

As tRF-544 and tRF-315 showed opposing expression patterns in more advanced tumor samples, the ratio tRF-315/tRF-544 was calculated for both cohorts. The results showed statistically significant differences, clearly distinguishing high- from low-grade PC samples, cured from recurrent cases and higher from lower pathological stage. Moreover, a higher expression ratio was significantly associated with poorer progression-free survival and a shorter time to disease relapse (Fig. 4B and C in IV), suggesting that the tRF-315/tRF-544 ratio might represent a candidate biomarker for disease progression.

6 Discussion

6.1 1p21-22 amplification in bladder cancer

In study I, a recurrent amplification at chromosome region 1p21-22 was characterized, using bladder cancer cell line models. The amplification was first identified in clinical samples of urothelial carcinoma and fine-mapped in a total of 8 bladder cancer cell lines. No high level amplification was identified in any of the cell line models of urothelial carcinoma, whereas it was present in the SCaBER squamous cell carcinoma cell line, spanning a minimum amplified region of approximately 1 Mb. This evidence suggests that the locus 1p21-22 may be altered in a subset of cancer cases. The amplified region is known to harbor several protein coding genes, four of which (*DR1*, *EVI5*, *TMED5* and *RPL5*) showed high expression levels in the SCaBER line compared to all the other models tested, indicating that the chromosomal alteration might be responsible for the aberrant expression.

Interestingly, Oncomine™ database interrogation also showed *DR1*, *EVI5*, *TMED5* and *RPL5* to be significantly coamplified in brain (George *et al.*, 2007; Kotliarov *et al.*, 2006; Northcott *et al.*, 2009), colon (Kurashina *et al.*, 2008) and lung cancer (Ramos *et al.*, 2009) as well as melanoma (Maser *et al.*, 2007). These results indicate that the amplification of 1p21-22 could be an alteration involved in the tumorigenesis of several cancers. Moreover, a recent genome-wide association study conducted by the International Multiple Sclerosis Genetic Consortium (IMSGC) identified a number of putative multiple sclerosis (MS) susceptibility variants at position 1p22. Twenty-one SNPs positively associated with MS were located at the *GFI-EVI5-RPL5-FAM69A* locus (Alcina *et al.*, 2010).

In addition, based on the highest expression level obtained from the gene expression microarray and qRT-PCR validation, *DR1* was the most relevant amplification target in bladder cancer among the examined genes.

DR1 is also known as *NC2beta* and has been shown to bind *DRAP1* to repress RNA polymerase II gene transcription (Mermelstein *et al.*, 1996). Despite targeting

the general transcription machinery, only a subset of mRNAs have been shown to respond to *DR1/DRAP1* inhibition (Geisberg *et al.*, 2001) and an opposite, transcription inducing effect, of DR1/DRAP1 has also been shown for some mRNAs, suggesting a specific regulatory effect (Cang and Prelich, 2002).

Further studies are necessary to assess the functional significance of *DR1* amplification and overexpression in bladder cancer.

6.2 non-coding RNAs in prostate cancer

In studies II, III and IV, expression analysis of non-coding RNAs was performed in both cell line models and clinical samples of prostate cancer. As described above, non-coding RNAs have recently emerged as a very important component of the cell physiology, given that their function is related to not only housekeeping but also gene expression regulation.

Study II represents the first comprehensive overview of miRNA expression changes during prostate epithelial differentiation obtained by miRNA microarray analysis in sorted epithelial subpopulations from patient-derived material. The main finding of the study is the identification of distinct expression profiles in each of the subpopulations studied, regardless of the pathologic status. Specifically, a greater extent and higher magnitude of differential expression was observed in stem cells (SC) versus committed basal cells (CB) than in cancer versus benign controls. This result suggests that the differentiation state of prostate epithelial cells plays a relevant role in the modulation of miRNA expression and provides a possible explanation for the heterogeneous and often contradicting data on miRNA expression profiles of unfractionated prostate tumors. Moreover, the analysis of prostate epithelial subpopulations has provided several novel prostate cancer stem cell-specific (PC-CSC) and CRPC stem-cell specific (CRPC-CSC) miRNA candidates. In addition, an overlap between the miRNA expression patterns of human embryonic stem cells (hESCs), prostate epithelial SCs and unfractionated CRPCs was revealed, indicating that embryonic signaling machinery is activated in the terminal stages of PC.

MiR-548c-3p emerged as a relevant PC-CSC miRNA that is significantly overexpressed in CRPC samples. In a recent independent study, miR-548c-3p overexpression was shown to decrease doxorubicin-induced DNA damage in a cervical cancer cell line (Srikantan *et al.*, 2011) and miR-548-3p serum levels were

also described to be significantly higher in CRPC patients than in low-risk PC patients (Nguyen *et al.*, 2013).

The results of the functional studies performed on miR-548c-3p indicate this miRNA is a putative diagnostic and prognostic candidate for improving CRPC patient management. Clinical validation in a larger patient cohort is now necessary to establish the prognostic and/or therapeutic relevance of this miRNA and more studies are necessary in the future to clarify the role of prostate cancer stem cells in the development and progression of the disease.

A recent RNA-sequencing study performed on clinical samples of malignant prostate cancers and non-malignant controls (Martens-Uzunova *et al.*, 2015) revealed the aberrant expression of several previously unstudied miRNAs in prostate cancer as well as non-coding RNAs derived from tRNAs, termed tRNA-derived RNA fragments (i.e., tRFs).

The expression of selected miRNA candidates was validated in study III using qRT-PCR in cell line models and prostate cancer clinical samples. Overall, the newly identified miRNAs showed a relatively low expression level in the samples tested and most of them did not present significantly aberrant expression in cancer samples compared to controls. miR-1247-5p was selected for further analysis as the only miRNA with significantly higher expression levels in advanced stages of the disease. The qRT-PCR validation confirmed significant overexpression of miR-1247-5p in CRPC clinical samples and in the androgen-independent cell line PC-3.

In contrast with our findings in prostate cancer, a recent *in situ* hybridization expression profile of miR-1247 using a pancreatic cancer tissue microarray, showed miR-1247 down-regulation in cancer tissues compared to matched benign controls. High levels of miR-1247 expression were also correlated with higher overall and recurrence free survival in pancreatic cancer patients. Neuropilin1 (NRP1) and Neuropilin2 (NRP2) were identified as direct targets of miR-1247 in pancreatic cancer (Shi *et al.*, 2014).

MiR-1247 has also been earlier described to be aberrantly hypermethylated and down-regulated in colorectal cancer and to suppress cell growth and migration (Yan *et al.*, 2011). Epigenetic silencing of miR-1247 has also been found in hepatocellular carcinoma (Anwar *et al.*, 2013). These results indicate the uncertain role for miR-1247 in different cancer types and the need for further investigations.

MYCBP2 was identified in study II as a target of miR-1247-5p in prostate cancer. This protein has been previously described to be highly expressed in peripheral and

central neurons (Yang *et al.*, 2002), where it has been shown to regulate neuronal outgrowth and synaptogenesis by regulating the cAMP (Scholich *et al.*, 2001), Smad4 (McCabe *et al.*, 2004), mTOR (Han *et al.*, 2008) and p38 MAPK-signaling pathways (Nakata *et al.*, 2005). Although the role of *MYCBP2* in cancer is currently unknown, recent data revealed the existence of a novel biological phenomenon in tumors termed cancer-related axonogenesis and neurogenesis (Ayala *et al.*, 2008). In experiments performed in prostate cancer, nerve density was shown to be increased in cancer and in preneoplastic areas of the prostate compared to non-malignant areas, confirming that cancer cells can induce neurite outgrowth. Further studies are necessary to investigate the potential role of both miR-1247-5p and *MYCBP2* in prostate cancer.

As described above, recent advancements in NGS technologies have led to the discovery of nearly ubiquitous RNA fragments of different sizes that are derived from mature or precursors tRNAs (tRFs). The abundance of these small RNA species and their specific expression profiles gave rise to the question of whether the fragments are primarily random tRNA degradation products or true biological entities with specific functions.

Study IV provides a comprehensive analysis of tRF composition and expression in PC clinical samples with different clinic-pathological characteristics. The novelty of the study is represented by the generation of a PC tRF database. The proper annotation of the tRF sequences was achieved using the fragment detection algorithm FlaiMapper, and the data were subsequently filtered to merge identical sequences deriving from multiple precursor tRNAs into single entries. After this correction, a total of 598 unique fragments were identified, 110 of which showed aberrant expression in cancer versus control. Moreover, the majority of identified tRF fragments originated from the 5´ or 3´ends of the mature tRNAs, in concordance with a previous report on tRF expression in prostate cancer cell lines (Lee *et al.*, 2009).

The results generated in the study IV dataset demonstrated that 32 of 36 5'-tRFs that were previously described by Lee *et al.* were detectable in the clinical samples used in study IV. This confirmatory result further supports the hypothesis that tRFs in prostate cancer are discrete biological entities produced by defined molecular mechanisms.

Moreover, a prevalence of 5`-tRFs was observed within tRFs that are up-regulated in prostate cancer samples, whereas 3´-tRFs are more prevalent within tRFs that are

down-regulated in prostate cancer, suggesting different molecular mechanisms for different tRF types and different roles in tumorigenesis.

Recently, 5'-tRFs were found to inhibit the translation of reporter genes *in vitro* and *in vivo*. The effect does not require complementary target sites in the reporter sequence but does require a universally conserved "GG" dinucleotide in the tRF (Sobala and Hutvagner, 2013), which is a common feature of ~75% of the upregulated 5´-tRFs described in study IV.

Selected differentially expressed tRFs were also validated by qRT-PCR in two independent cohorts of clinical samples of prostate cancer, using custom designed primers. Three fragments, namely tRF-544 (derived from tRNAPheGAA), tRF-315 (derived from tRNALysCTT) and tRF-562 (derived from tRNAGlyCTT) were confirmed to be significantly deregulated in cancer samples versus NAP controls. Interstingly, tRF-544 was consistently downregulated in samples obtained from patients with recurrent disease, whereas tRF-315 was consistently upregulated in the same cases. The normalized expression ratio of these two fragments significantly and consistently distinguished cancer samples, based on Gleason score, pathological stage, recurrence and progression-free survival.

In conclusion, these results highlight the potential role of tRFs as biomarkers for prostate cancer diagnosis and prognosis. However, the specific role of tRFs remains unclear and more studies will be needed in the future to clarify their function in both physiologic and pathologic conditions.

7 Conclusions

The primary findings and conclusions of the study are as following:

I. The minimal region of the 1p21-22 amplification was fine-mapped to approximately 1 Mb and was found to harbor 11 known human genes. The highest level of amplification was observed in the SCaBER cell line model of squamous cell carcinoma of the bladder. Four genes, *TMED5*, *DR1*, *RPL5* and *EVI5*, showed significant overexpression in the SCaBER cell line compared to all the other samples tested. Moreover, the Oncomine database analysis confirmed the statistically significant upregulation of *DR1* in a set of superficial and infiltrating bladder cancer samples compared to normal bladder, suggesting *DR1* as a putative selected gene in the amplification.

II. The analysis of the genome-wide miRNA microarray of prostate cancer epithelial subpopulations demonstrated that each subpopulation shows a distinct miRNA expression profile, regardless of its pathologic status. The miRNA miR-548c-3p was found to be overexpressed approximately fivefold in prostate epithelial stem cells compared with CBs and its overexpression has been associated with poor survival of PC patients. Functional studies of miR-5498c-3p overexpression in CB cells resulted in dedifferentiation to a more stem-like phenotype. Moreover, miR-548c-3p was found to be significantly upregulated in CRPC-derived epithelial cells compared with BPH-derived epithelial cells. Together, the results demonstrate the importance of miR-548c-3p as a diagnostic and prognostic candidate biomarker to improve CRPC management.

III. Recently published deep-sequencing projects identified several putatively differentially expressed miRNAs in prostate cancer. Using prostate cancer clinical samples obtained from Tampere University Hospital, a significant upregulation of the miRNA miR-1247-5p was validated in CRPC samples compared to benign controls. The expression of miR-1247-5p was subsequently studied in prostate cancer cell line models showing significant upregulation in the androgen-insensitive, bone metastasis-derived PC-3 cells

compared to all other cell lines. Online analysis of target prediction programs for miR-1247 revealed the *MYCBP2* (myc-binding protein 2) transcript to be a high-score potential target. Functional *in vitro* studies were performed on prostate cancer cell lines, confirming the down-regulation of MYCBP2 at the mRNA and protein level. In addition, an interaction between miR-1247-5p and the 3´-UTR of *MYCBP2* was shown using a luciferase assay. Moreover, *MYCBP2* down-regulation was found in clinical samples of CRPC. *MYCBP2* was confirmed as target gene for miR-1247-5p in prostate cancer.

IV. A total of 598 unique tRFs were identified in clinical samples of prostate cancer, 110 of which were found to be deregulated in PC when compared to NAP controls. Most of the detected tRFs were found to derive from the 5' and 3' ends of the precursor tRNAs. The 5`- tRFs were found to be the most abundant type of tRFs and represented the majority of the upregulated tRFs; the 3'-tRFs were dominant among the downregulated tRFs. The aberrant expression of three tRFs in PC was further validated using qRT-PCR. The ratio of the two fragments derived from $tRNA^{LysCTT}$ and $tRNA^{PheGAA}$ emerged as a good indicator of progression-free survival and as a candidate prognostic marker.

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References

1000 Genomes Project Consortium Abecasis, G.R. Auton, A. Brooks, L.D. DePristo, M.A. Durbin, R.M. Handsaker, R.E. Kang, H.M. Marth, G.T. and McVean, G.A. 2012. An integrated map of genetic variation from 1,092 human genomes. Nature 491:7422:56-65.

Abdulghani, J. Gu, L. Dagvadorj, A. Lutz, J. Leiby, B. Bonuccelli, G. Lisanti, M.P. Zellweger, T. Alanen, K. Mirtti, T. Visakorpi, T. Bubendorf, L. and Nevalainen, M.T. 2008. Stat3 promotes metastatic progression of prostate cancer. Am.J.Pathol. 172:6:1717-1728.

Aboulkassim, T.O. LaRue, H. Lemieux, P. Rousseau, F. and Fradet, Y. 2003. Alteration of the PATCHED locus in superficial bladder cancer. Oncogene 22:19:2967-2971.

Afar, D.E. Vivanco, I. Hubert, R.S. Kuo, J. Chen, E. Saffran, D.C. Raitano, A.B. and Jakobovits, A. 2001. Catalytic cleavage of the androgen-regulated TMPRSS2 protease results in its secretion by prostate and prostate cancer epithelia. Cancer Res. 61:4:1686-1692.

Agus, D.B. Cordon-Cardo, C. Fox, W. Drobnjak, M. Koff, A. Golde, D.W. and Scher, H.I. 1999. Prostate cancer cell cycle regulators: response to androgen withdrawal and development of androgen independence. J.Natl.Cancer Inst. 91:21:1869-1876.

Al Olama, A.A. Kote-Jarai, Z. Berndt, S.I. Conti, D.V. Schumacher, F. Han, Y. Benlloch, S. Hazelett, D.J. Wang, Z. Saunders, E. Leongamornlert, D. Lindstrom, S. Jugurnauth-Little, S. Dadaev, T. Tymrakiewicz, M. Stram, D.O. Rand, K. Wan, P. Stram, A. Sheng, X. Pooler, L.C. Park, K. Xia, L. Tyrer, J. Kolonel, L.N. Le Marchand, L. Hoover, R.N. Machiela, M.J. Yeager, M. Burdette, L. Chung, C.C. Hutchinson, A. Yu, K. Goh, C. Ahmed, M. Govindasami, K. Guy, M. Tammela, T.L. Auvinen, A. Wahlfors, T. Schleutker, J. Visakorpi, T. Leinonen, K.A. Xu, J. Aly, M. Donovan, J. Travis, R.C. Key, T.J. Siddiq, A. Canzian, F. Khaw, K.T. Takahashi, A. Kubo, M. Pharoah, P. Pashayan, N. Weischer, M. Nordestgaard, B.G. Nielsen, S.F. Klarskov, P. Roder, M.A. Iversen, P. Thibodeau, S.N. McDonnell, S.K. Schaid, D.J. Stanford, J.L. Kolb, S. Holt, S. Knudsen, B. Coll, A.H. Gapstur, S.M. Diver, W.R. Stevens, V.L. Maier, C. Luedeke, M. Herkommer, K. Rinckleb, A.E. Strom, S.S. Pettaway, C. Yeboah, E.D. Tettey, Y. Biritwum, R.B. Adjei, A.A. Tay, E. Truelove, A. Niwa, S. Chokkalingam, A.P. Cannon-Albright, L. Cybulski, C. Wokolorczyk, D. Kluzniak, W. Park, J. Sellers, T. Lin, H.Y. Isaacs, W.B. Partin, A.W. Brenner, H. Dieffenbach, A.K. Stegmaier, C. Chen, C. Giovannucci, E.L. Ma, J. Stampfer, M. Penney, K.L. Mucci, L. John, E.M. Ingles, S.A. Kittles, R.A. Murphy, A.B. Pandha, H. Michael, A. Kierzek, A.M. Blot, W. Signorello, L.B. Zheng, W. Albanes, D. Virtamo, J. Weinstein, S. Nemesure, B. Carpten, J. Leske,

C. Wu, S.Y. Hennis, A. Kibel, A.S. Rybicki, B.A. Neslund-Dudas, C. Hsing, A.W. Chu, L. Goodman, P.J. Klein, E.A. Zheng, S.L. Batra, J. Clements, J. Spurdle, A. Teixeira, M.R. Paulo, P. Maia, S. Slavov, C. Kaneva, R. Mitev, V. Witte, J.S. Casey, G. Gillanders, E.M. Seminara, D. Riboli, E. Hamdy, F.C. Coetzee, G.A. Li, Q. Freedman, M.L. Hunter, D.J. Muir, K. Gronberg, H. Neal, D.E. Southey, M. Giles, G.G. Severi, G. Breast and Prostate Cancer Cohort Consortium (BPC3) PRACTICAL (Prostate Cancer Association Group to Investigate Cancer-Associated Alterations in the Genome) Consortium COGS (Collaborative Oncological Gene-environment Study) Consortium GAME-ON/ELLIPSE Consortium Cook, M.B. Nakagawa, H. Wiklund, F. Kraft, P. Chanock, S.J. Henderson, B.E. Easton, D.F. Eeles, R.A. and Haiman, C.A. 2014. A meta-analysis of 87,040 individuals identifies 23 new susceptibility loci for prostate cancer. Nat.Genet. 46:10:1103-1109.

Al Olama, A.A. Kote-Jarai, Z. Giles, G.G. Guy, M. Morrison, J. Severi, G. Leongamornlert, D.A. Tymrakiewicz, M. Jhavar, S. Saunders, E. Hopper, J.L. Southey, M.C. Muir, K.R. English, D.R. Dearnaley, D.P. Ardern-Jones, A.T. Hall, A.L. O'Brien, L.T. Wilkinson, R.A. Sawyer, E. Lophatananon, A. UK Genetic Prostate Cancer Study Collaborators/British Association of Urological Surgeons' Section of Oncology UK Prostate testing for cancer and Treatment study (ProtecT Study) Collaborators Horwich, A. Huddart, R.A. Khoo, V.S. Parker, C.C. Woodhouse, C.J. Thompson, A. Christmas, T. Ogden, C. Cooper, C. Donovan, J.L. Hamdy, F.C. Neal, D.E. Eeles, R.A. and Easton, D.F. 2009. Multiple loci on 8q24 associated with prostate cancer susceptibility. Nat.Genet. 41:10:1058-1060.

Albertson, D.G. Collins, C. McCormick, F. and Gray, J.W. 2003. Chromosome aberrations in solid tumors. Nat.Genet. 34:4:369-376.

Alcina, A. Fernandez, O. Gonzalez, J.R. Catala-Rabasa, A. Fedetz, M. Ndagire, D. Leyva, L. Guerrero, M. Arnal, C. Delgado, C. Lucas, M. Izquierdo, G. and Matesanz, F. 2010. Tag-SNP analysis of the GFI1-EVI5-RPL5-FAM69 risk locus for multiple sclerosis. Eur.J.Hum.Genet. 18:7:827-831.

Alexander, R.P. Fang, G. Rozowsky, J. Snyder, M. and Gerstein, M.B. 2010. Annotating non-coding regions of the genome. Nat.Rev.Genet. 11:8:559-571.

Alexandrov, L.B. Nik-Zainal, S. Wedge, D.C. Aparicio, S.A. Behjati, S. Biankin, A.V. Bignell, G.R. Bolli, N. Borg, A. Borresen-Dale, A.L. Boyault, S. Burkhardt, B. Butler, A.P. Caldas, C. Davies, H.R. Desmedt, C. Eils, R. Eyfjord, J.E. Foekens, J.A. Greaves, M. Hosoda, F. Hutter, B. Ilicic, T. Imbeaud, S. Imielinski, M. Jager, N. Jones, D.T. Jones, D. Knappskog, S. Kool, M. Lakhani, S.R. Lopez-Otin, C. Martin, S. Munshi, N.C. Nakamura, H. Northcott, P.A. Pajic, M. Papaemmanuil, E. Paradiso, A. Pearson, J.V. Puente, X.S. Raine, K. Ramakrishna, M. Richardson, A.L. Richter, J. Rosenstiel, P. Schlesner, M. Schumacher, T.N. Span, P.N. Teague, J.W. Totoki, Y. Tutt, A.N. Valdes-Mas, R. van Buuren, M.M. van 't Veer, L. Vincent-Salomon, A. Waddell, N. Yates, L.R. Australian Pancreatic Cancer Genome Initiative ICGC Breast Cancer Consortium ICGC MMML-Seq Consortium ICGC PedBrain Zucman-Rossi, J. Futreal, P.A. McDermott, U. Lichter, P. Meyerson, M. Grimmond, S.M. Siebert, R. Campo, E.

Shibata, T. Pfister, S.M. Campbell, P.J. and Stratton, M.R. 2013. Signatures of mutational processes in human cancer. Nature 500:7463:415-421.

Al-Hajj, M. Becker, M.W. Wicha, M. Weissman, I. and Clarke, M.F. 2004. Therapeutic implications of cancer stem cells. Curr.Opin.Genet.Dev. 14:1:43-47.

Al-Hajj, M. Wicha, M.S. Benito-Hernandez, A. Morrison, S.J. and Clarke, M.F. 2003. Prospective identification of tumorigenic breast cancer cells. Proc.Natl.Acad.Sci.U.S.A. 100:7:3983-3988.

Ambs, S. Prueitt, R.L. Yi, M. Hudson, R.S. Howe, T.M. Petrocca, F. Wallace, T.A. Liu, C.G. Volinia, S. Calin, G.A. Yfantis, H.G. Stephens, R.M. and Croce, C.M. 2008. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. Cancer Res. 68:15:6162-6170.

Amundadottir, L.T. Sulem, P. Gudmundsson, J. Helgason, A. Baker, A. Agnarsson, B.A. Sigurdsson, A. Benediktsdottir, K.R. Cazier, J.B. Sainz, J. Jakobsdottir, M. Kostic, J. Magnusdottir, D.N. Ghosh, S. Agnarsson, K. Birgisdottir, B. Le Roux, L. Olafsdottir, A. Blondal, T. Andresdottir, M. Gretarsdottir, O.S. Bergthorsson, J.T. Gudbjartsson, D. Gylfason, A. Thorleifsson, G. Manolescu, A. Kristjansson, K. Geirsson, G. Isaksson, H. Douglas, J. Johansson, J.E. Balter, K. Wiklund, F. Montie, J.E. Yu, X. Suarez, B.K. Ober, C. Cooney, K.A. Gronberg, H. Catalona, W.J. Einarsson, G.V. Barkardottir, R.B. Gulcher, J.R. Kong, A. Thorsteinsdottir, U. and Stefansson, K. 2006. A common variant associated with prostate cancer in European and African populations. Nat.Genet. 38:6:652-658.

Angele, S. Romestaing, P. Moullan, N. Vuillaume, M. Chapot, B. Friesen, M. Jongmans, W. Cox, D.G. Pisani, P. Gerard, J.P. and Hall, J. 2003. ATM haplotypes and cellular response to DNA damage: association with breast cancer risk and clinical radiosensitivity. Cancer Res. 63:24:8717-8725.

Annala, M. Kivinummi, K. Leinonen, K. Tuominen, J. Zhang, W. Visakorpi, T. and Nykter, M. 2014. DOT1L-HES6 fusion drives androgen independent growth in prostate cancer. EMBO Mol.Med. 6:9:1121-1123.

Annala, M. Kivinummi, K. Tuominen, J. Karakurt, S. Granberg, K. Latonen, L. Ylipaa, A. Sjoblom, L. Ruusuvuori, P. Saramaki, O. Kaukoniemi, K.M. Yli-Harja, O. Vessella, R.L. Tammela, T.L. Zhang, W. Visakorpi, T. and Nykter, M. 2015. Recurrent SKIL-activating rearrangements in ETS-negative prostate cancer. Oncotarget 6:8:6235-6250.

Anwar, S.L. Albat, C. Krech, T. Hasemeier, B. Schipper, E. Schweitzer, N. Vogel, A. Kreipe, H. and Lehmann, U. 2013. Concordant hypermethylation of intergenic microRNA genes in human hepatocellular carcinoma as new diagnostic and prognostic marker. Int.J.Cancer 133:3:660-670.

Arora, R. Koch, M.O. Eble, J.N. Ulbright, T.M. Li, L. and Cheng, L. 2004. Heterogeneity of Gleason grade in multifocal adenocarcinoma of the prostate. Cancer 100:11:2362-2366.

Asatiani, E. Huang, W.X. Wang, A. Rodriguez Ortner, E. Cavalli, L.R. Haddad, B.R. and Gelmann, E.P. 2005. Deletion, methylation, and expression of the NKX3.1 suppressor gene in primary human prostate cancer. Cancer Res. 65:4:1164-1173.

Attard, G. Clark, J. Ambroisine, L. Mills, I.G. Fisher, G. Flohr, P. Reid, A. Edwards, S. Kovacs, G. Berney, D. Foster, C. Massie, C.E. Fletcher, A. De Bono, J.S. Scardino, P. Cuzick, J. Cooper, C.S. and Transatlantic Prostate Group. 2008. Heterogeneity and clinical significance of ETV1 translocations in human prostate cancer. Br.J.Cancer 99:2:314-320.

Aveyard, J.S. Skilleter, A. Habuchi, T. and Knowles, M.A. 1999. Somatic mutation of PTEN in bladder carcinoma. Br.J.Cancer 80:5-6:904-908.

Ayala, G.E. Dai, H. Powell, M. Li, R. Ding, Y. Wheeler, T.M. Shine, D. Kadmon, D. Thompson, T. Miles, B.J. Ittmann, M.M. and Rowley, D. 2008. Cancer-related axonogenesis and neurogenesis in prostate cancer. Clin.Cancer Res. 14:23:7593-7603.

Babiarz, J.E. Ruby, J.G. Wang, Y. Bartel, D.P. and Blelloch, R. 2008. Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. Genes Dev. 22:20:2773-2785.

Babjuk, M. Oosterlinck, W. Sylvester, R. Kaasinen, E. Bohle, A. Palou-Redorta, J. Roupret, M. and European Association of Urology (EAU). 2011. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. Eur.Urol. 59:6:997-1008.

Baek, D. Villen, J. Shin, C. Camargo, F.D. Gygi, S.P. and Bartel, D.P. 2008. The impact of microRNAs on protein output. Nature 455:7209:64-71.

Baker, S.J. Preisinger, A.C. Jessup, J.M. Paraskeva, C. Markowitz, S. Willson, J.K. Hamilton, S. and Vogelstein, B. 1990. P53 Gene Mutations Occur in Combination with 17p Allelic Deletions as Late Events in Colorectal Tumorigenesis. Cancer Res. 50:23:7717-7722.

Bao, S. Wu, Q. McLendon, R.E. Hao, Y. Shi, Q. Hjelmeland, A.B. Dewhirst, M.W. Bigner, D.D. and Rich, J.N. 2006. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444:7120:756-760.

Barabe, F. Kennedy, J.A. Hope, K.J. and Dick, J.E. 2007. Modeling the initiation and progression of human acute leukemia in mice. Science 316:5824:600-604.

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. Nickerson, E. Chae, S.S. Boysen, G.

Auclair, D. Onofrio, R.C. Park, K. Kitabayashi, N. MacDonald, T.Y. Sheikh, K. Vuong, T. Guiducci, C. Cibulskis, K. Sivachenko, A. Carter, S.L. Saksena, G. Voet, D. Hussain, W.M. Ramos, A.H. Winckler, W. Redman, M.C. Ardlie, K. Tewari, A.K. Mosquera, J.M. Rupp, N. Wild, P.J. Moch, H. Morrissey, C. Nelson, P.S. Kantoff, P.W. Gabriel, S.B. Golub, T.R. Meyerson, M. Lander, E.S. Getz, G. Rubin, M.A. and Garraway, L.A. 2012. Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. Nat.Genet. 44:6:685-689.

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. Eur.Urol. 64:4:567-576.

Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:2:281-297.

Behm-Ansmant, I. Rehwinkel, J. Doerks, T. Stark, A. Bork, P. and Izaurralde, E. 2006. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev. 20:14:1885-1898.

Beltran, H. Rickman, D.S. Park, K. Chae, S.S. Sboner, A. MacDonald, T.Y. Wang, Y. Sheikh, K.L. Terry, S. Tagawa, S.T. Dhir, R. Nelson, J.B. de la Taille, A. Allory, Y. Gerstein, M.B. Perner, S. Pienta, K.J. Chinnaiyan, A.M. Wang, Y. Collins, C.C. Gleave, M.E. Demichelis, F. Nanus, D.M. and Rubin, M.A. 2011. Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. Cancer.Discov. 1:6:487-495.

Berger, M.F. Lawrence, M.S. Demichelis, F. Drier, Y. Cibulskis, K. Sivachenko, A.Y. Sboner, A. Esgueva, R. Pflueger, D. Sougnez, C. Onofrio, R. Carter, S.L. Park, K. Habegger, L. Ambrogio, L. Fennell, T. Parkin, M. Saksena, G. Voet, D. Ramos, A.H. Pugh, T.J. Wilkinson, J. Fisher, S. Winckler, W. Mahan, S. Ardlie, K. Baldwin, J. Simons, J.W. Kitabayashi, N. MacDonald, T.Y. Kantoff, P.W. Chin, L. Gabriel, S.B. Gerstein, M.B. Golub, T.R. Meyerson, M. Tewari, A. Lander, E.S. Getz, G. Rubin, M.A. and Garraway, L.A. 2011. The genomic complexity of primary human prostate cancer. Nature 470:7333:214-220.

Bertone, P. Stolc, V. Royce, T.E. Rozowsky, J.S. Urban, A.E. Zhu, X. Rinn, J.L. Tongprasit, W. Samanta, M. Weissman, S. Gerstein, M. and Snyder, M. 2004. Global identification of human transcribed sequences with genome tiling arrays. Science 306:5705:2242-2246.

Bhatia, K. Spangler, G. Gaidano, G. Hamdy, N. Dalla-Favera, R. and Magrath, I. 1994. Mutations in the coding region of c-myc occur frequently in acquired immunodeficiency syndrome-associated lymphomas. Blood 84:3:883-888.

Bhatia-Gaur, R. Donjacour, A.A. Sciavolino, P.J. Kim, M. Desai, N. Young, P. Norton, C.R. Gridley, T. Cardiff, R.D. Cunha, G.R. Abate-Shen, C. and Shen, M.M. 1999. Roles for Nkx3.1 in prostate development and cancer. Genes Dev. 13:8:966-977.

Birnie, R. Bryce, S.D. Roome, C. Dussupt, V. Droop, A. Lang, S.H. Berry, P.A. Hyde, C.F. Lewis, J.L. Stower, M.J. Maitland, N.J. and Collins, A.T. 2008. Gene expression profiling of human prostate cancer stem cells reveals a pro-inflammatory phenotype and the importance of extracellular matrix interactions. Genome Biol. 9:5:R83-2008-9-5-r83. Epub 2008 May 20.

Bismar, T.A. Yoshimoto, M. Vollmer, R.T. Duan, Q. Firszt, M. Corcos, J. and Squire, J.A. 2011. PTEN genomic deletion is an early event associated with ERG gene rearrangements in prostate cancer. BJU Int. 107:3:477-485.

Bissell, M.J. and Radisky, D. 2001. Putting tumours in context. Nat.Rev.Cancer. 1:1:46-54.

Biteau, B. Hochmuth, C.E. and Jasper, H. 2011. Maintaining tissue homeostasis: dynamic control of somatic stem cell activity. Cell.Stem Cell. 9:5:402-411.

Blair, A. Hogge, D.E. Ailles, L.E. Lansdorp, P.M. and Sutherland, H.J. 1997. Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. Blood 89:9:3104-3112.

Blair, A. and Sutherland, H.J. 2000. Primitive acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo lack surface expression of c-kit (CD117). Exp.Hematol. 28:6:660-671.

Blaveri, E. Brewer, J.L. Roydasgupta, R. Fridlyand, J. DeVries, S. Koppie, T. Pejavar, S. Mehta, K. Carroll, P. Simko, J.P. and Waldman, F.M. 2005. Bladder cancer stage and outcome by array-based comparative genomic hybridization. Clin.Cancer Res. 11:19 Pt 1:7012-7022.

Blow, M.J. Grocock, R.J. van Dongen, S. Enright, A.J. Dicks, E. Futreal, P.A. Wooster, R. and Stratton, M.R. 2006. RNA editing of human microRNAs. Genome Biol. 7:4:R27.

Bonci, D. Coppola, V. Musumeci, M. Addario, A. Giuffrida, R. Memeo, L. D'Urso, L. Pagliuca, A. Biffoni, M. Labbaye, C. Bartucci, M. Muto, G. Peschle, C. and De Maria, R. 2008. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat.Med. 14:11:1271-1277.

Bonkhoff, H. and Remberger, K. 1996. Differentiation pathways and histogenetic aspects of normal and abnormal prostatic growth: a stem cell model. Prostate 28:2:98-106.

Bonkhoff, H. Stein, U. and Remberger, K. 1994. Multidirectional differentiation in the normal, hyperplastic, and neoplastic human prostate: simultaneous demonstration of cell-specific epithelial markers. Hum.Pathol. 25:1:42-46.

Bonnet, D. and Dick, J.E. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat.Med. 3:7:730-737.

Borchert, G.M. Lanier, W. and Davidson, B.L. 2006. RNA polymerase III transcribes human microRNAs. Nat.Struct.Mol.Biol. 13:12:1097-1101.

Bos, J.L. 1989. Ras Oncogenes in Human Cancer: a Review. Cancer Res. 49:17:4682-4689.

Bostwick, D.G. Liu, L. Brawer, M.K. and Qian, J. 2004. High-grade prostatic intraepithelial neoplasia. Rev.Urol. 6:4:171-179.

Bostwick, D.G. and Qian, J. 2004. High-grade prostatic intraepithelial neoplasia. Mod.Pathol. 17:3:360-379.

Bowen, C. and Gelmann, E.P. 2010. NKX3.1 activates cellular response to DNA damage. Cancer Res. 70:8:3089-3097.

Boyer, L.A. Plath, K. Zeitlinger, J. Brambrink, T. Medeiros, L.A. Lee, T.I. Levine, S.S. Wernig, M. Tajonar, A. Ray, M.K. Bell, G.W. Otte, A.P. Vidal, M. Gifford, D.K. Young, R.A. and Jaenisch, R. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441:7091:349-353.

Brennecke, J. Stark, A. Russell, R.B. and Cohen, S.M. 2005. Principles of microRNA-target recognition. PLoS Biol. 3:3:e85.

Bringuier, P.P. Tamimi, Y. Schuuring, E. and Schalken, J. 1996. Expression of cyclin D1 and EMS1 in bladder tumours; relationship with chromosome 11q13 amplification. Oncogene 12:8:1747-1753.

Brooks, J.D. Weinstein, M. Lin, X. Sun, Y. Pin, S.S. Bova, G.S. Epstein, J.I. Isaacs, W.B. and Nelson, W.G. 1998. CG island methylation changes near the GSTP1 gene in prostatic intraepithelial neoplasia. Cancer Epidemiol.Biomarkers Prev. 7:6:531-536.

Bu, Y. and Cao, D. 2012. The origin of cancer stem cells. Front.Biosci.(Schol Ed) 4:819-830.

Burger, P.E. Xiong, X. Coetzee, S. Salm, S.N. Moscatelli, D. Goto, K. and Wilson, E.L. 2005. Sca-1 expression identifies stem cells in the proximal region of prostatic ducts with high capacity to reconstitute prostatic tissue. Proc.Natl.Acad.Sci.U.S.A. 102:20:7180-7185.

Cai, T. Nesi, G. Dal Canto, M. Tinacci, G. Mondaini, N. Piazzini, M. Geppetti, P. and Bartoletti, R. 2010. Loss of heterozygosis on IFN-alpha locus is a prognostic indicator of bacillus Calmette-Guerin response for nonmuscle invasive bladder cancer. J.Urol. 183:5:1738-1743.

Cairns, P. Evron, E. Okami, K. Halachmi, N. Esteller, M. Herman, J.G. Bose, S. Wang, S.I. Parsons, R. and Sidransky, D. 1998. Point mutation and homozygous deletion of PTEN/MMAC1 in primary bladder cancers. Oncogene 16:24:3215-3218.

Cairns, P. Proctor, A.J. and Knowles, M.A. 1991. Loss of heterozygosity at the RB locus is frequent and correlates with muscle invasion in bladder carcinoma. Oncogene 6:12:2305-2309.

Calin, G.A. and Croce, C.M. 2006. MicroRNA signatures in human cancers. Nat.Rev.Cancer. 6:11:857-866.

Calin, G.A. Dumitru, C.D. Shimizu, M. Bichi, R. Zupo, S. Noch, E. Aldler, H. Rattan, S. Keating, M. Rai, K. Rassenti, L. Kipps, T. Negrini, M. Bullrich, F. and Croce, C.M. 2002. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc.Natl.Acad.Sci.U.S.A. 99:24:15524-15529.

Calin, G.A. Sevignani, C. Dumitru, C.D. Hyslop, T. Noch, E. Yendamuri, S. Shimizu, M. Rattan, S. Bullrich, F. Negrini, M. and Croce, C.M. 2004. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc.Natl.Acad.Sci.U.S.A. 101:9:2999-3004.

Campbell, S.L. Khosravi-Far, R. Rossman, K.L. Clark, G.J. and Der, C.J. 1998. Increasing complexity of Ras signaling. Oncogene 17:11 Reviews:1395-1413.

Cang, Y. and Prelich, G. 2002. Direct stimulation of transcription by negative cofactor 2 (NC2) through TATA-binding protein (TBP). Proc.Natl.Acad.Sci.U.S.A. 99:20:12727-12732.

Carlsson, J. Helenius, G. Karlsson, M. Lubovac, Z. Andren, O. Olsson, B. and Klinga-Levan, K. 2010. Validation of suitable endogenous control genes for expression studies of miRNA in prostate cancer tissues. Cancer Genet.Cytogenet. 202:2:71-75.

Carpten, J. Nupponen, N. Isaacs, S. Sood, R. Robbins, C. Xu, J. Faruque, M. Moses, T. Ewing, C. Gillanders, E. Hu, P. Bujnovszky, P. Makalowska, I. Baffoe-Bonnie, A. Faith, D. Smith, J. Stephan, D. Wiley, K. Brownstein, M. Gildea, D. Kelly, B. Jenkins, R. Hostetter, G. Matikainen, M. Schleutker, J. Klinger, K. Connors, T. Xiang, Y. Wang, Z. De Marzo, A. Papadopoulos, N. Kallioniemi, O.P. Burk, R. Meyers, D. Gronberg, H. Meltzer, P. Silverman, R. Bailey-Wilson, J. Walsh, P. Isaacs, W. and Trent, J. 2002. Germline mutations in the ribonuclease L gene in families showing linkage with HPC1. Nat.Genet. 30:2:181-184.

Carter, B.S. Beaty, T.H. Steinberg, G.D. Childs, B. and Walsh, P.C. 1992. Mendelian inheritance of familial prostate cancer. Proc.Natl.Acad.Sci.U.S.A. 89:8:3367-3371.

Cavenee, W.K. Dryja, T.P. Phillips, R.A. Benedict, W.F. Godbout, R. Gallie, B.L. Murphree, A.L. Strong, L.C. and White, R.L. 1983. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature 305:5937:779-784.

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. Eur.Urol. 61:6:1079-1092.

Chalhoub, N. and Baker, S.J. 2009. PTEN and the PI3-kinase pathway in cancer. Annu.Rev.Pathol. 4:127-150.

Chang, M. Tsuchiya, K. Batchelor, R.H. Rabinovitch, P.S. Kulander, B.G. Haggitt, R.C. and Burmer, G.C. 1994. Deletion mapping of chromosome 8p in colorectal carcinoma and dysplasia arising in ulcerative colitis, prostatic carcinoma, and malignant fibrous histiocytomas. Am.J.Pathol. 144:1:1-6.

Chen, Y. Zaman, M.S. Deng, G. Majid, S. Saini, S. Liu, J. Tanaka, Y. and Dahiya, R. 2011. MicroRNAs 221/222 and genistein-mediated regulation of ARHI tumor suppressor gene in prostate cancer. Cancer.Prev.Res.(Phila) 4:1:76-86.

Chim, S.S. Shing, T.K. Hung, E.C. Leung, T.Y. Lau, T.K. Chiu, R.W. and Lo, Y.M. 2008. Detection and characterization of placental microRNAs in maternal plasma. Clin.Chem. 54:3:482-490.

Chiou, H.Y. Chiou, S.T. Hsu, Y.H. Chou, Y.L. Tseng, C.H. Wei, M.L. and Chen, C.J. 2001. Incidence of transitional cell carcinoma and arsenic in drinking water: a follow-up study of 8,102 residents in an arseniasis-endemic area in northeastern Taiwan. Am.J.Epidemiol. 153:5:411-418.

Chung, C.C. and Chanock, S.J. 2011. Current status of genome-wide association studies in cancer. Hum.Genet. 130:1:59-78.

Cimmino, A. Calin, G.A. Fabbri, M. Iorio, M.V. Ferracin, M. Shimizu, M. Wojcik, S.E. Aqeilan, R.I. Zupo, S. Dono, M. Rassenti, L. Alder, H. Volinia, S. Liu, C.G. Kipps, T.J. Negrini, M. and Croce, C.M. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc.Natl.Acad.Sci.U.S.A. 102:39:13944-13949.

Clark, J. Attard, G. Jhavar, S. Flohr, P. Reid, A. De-Bono, J. Eeles, R. Scardino, P. Cuzick, J. Fisher, G. Parker, M.D. Foster, C.S. Berney, D. Kovacs, G. and Cooper, C.S. 2008. Complex patterns of ETS gene alteration arise during cancer development in the human prostate. Oncogene 27:14:1993-2003.

Clark, J. Merson, S. Jhavar, S. Flohr, P. Edwards, S. Foster, C.S. Eeles, R. Martin, F.L. Phillips, D.H. Crundwell, M. Christmas, T. Thompson, A. Fisher, C. Kovacs, G. and Cooper, C.S. 2007. Diversity of TMPRSS2-ERG fusion transcripts in the human prostate. Oncogene 26:18:2667-2673.

Clarkson, B. Strife, A. Wisniewski, D. Lambek, C.L. and Liu, C. 2003. Chronic myelogenous leukemia as a paradigm of early cancer and possible curative strategies. Leukemia 17:7:1211-1262.

Cole, C. Sobala, A. Lu, C. Thatcher, S.R. Bowman, A. Brown, J.W. Green, P.J. Barton, G.J. and Hutvagner, G. 2009. Filtering of deep sequencing data reveals the existence of abundant Dicer-dependent small RNAs derived from tRNAs. RNA 15:12:2147-2160.

Collins, A.T. Berry, P.A. Hyde, C. Stower, M.J. and Maitland, N.J. 2005. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res. 65:23:10946-10951.

Collins, A.T. Habib, F.K. Maitland, N.J. and Neal, D.E. 2001. Identification and isolation of human prostate epithelial stem cells based on alpha(2)beta(1)-integrin expression. J.Cell.Sci. 114:Pt 21:3865-3872.

Cookson, M.S. and Sarosdy, M.F. 1992. Management of stage T1 superficial bladder cancer with intravesical bacillus Calmette-Guerin therapy. J.Urol. 148:3:797-801.

Coppola, V. De Maria, R. and Bonci, D. 2010. MicroRNAs and prostate cancer. Endocr.Relat.Cancer 17:1:F1-17.

Cordon-Cardo, C. 2008. Molecular alterations associated with bladder cancer initiation and progression. Scand.J.Urol.Nephrol.Suppl. (218):154-65. doi:218:154-165.

Crasta, K. Ganem, N.J. Dagher, R. Lantermann, A.B. Ivanova, E.V. Pan, Y. Nezi, L. Protopopov, A. Chowdhury, D. and Pellman, D. 2012. DNA breaks and chromosome pulverization from errors in mitosis. Nature 482:7383:53-58.

Crespi, B. and Summers, K. 2005. Evolutionary biology of cancer. Trends Ecol.Evol. 20:10:545-552.

Croce, C.M. 2008. Oncogenes and cancer. N.Engl.J.Med. 358:5:502-511.

Cunha, G.R. and Lung, B. 1978. The possible influence of temporal factors in androgenic responsiveness of urogenital tissue recombinants from wild-type and androgen-insensitive (Tfm) mice. J.Exp.Zool. 205:2:181-193.

Dalla-Favera, R. Bregni, M. Erikson, J. Patterson, D. Gallo, R.C. and Croce, C.M. 1982. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc.Natl.Acad.Sci.U.S.A. 79:24:7824-7827.

De Marzo, A.M. DeWeese, T.L. Platz, E.A. Meeker, A.K. Nakayama, M. Epstein, J.I. Isaacs, W.B. and Nelson, W.G. 2004. Pathological and molecular mechanisms of prostate carcinogenesis: implications for diagnosis, detection, prevention, and treatment. J.Cell.Biochem. 91:3:459-477.

De Marzo, A.M. Marchi, V.L. Epstein, J.I. and Nelson, W.G. 1999. Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. Am.J.Pathol. 155:6:1985-1992.

De Marzo, A.M. Platz, E.A. Sutcliffe, S. Xu, J. Gronberg, H. Drake, C.G. Nakai, Y. Isaacs, W.B. and Nelson, W.G. 2007. Inflammation in prostate carcinogenesis. Nat.Rev.Cancer. 7:4:256-269.

Demichelis, F. Fall, K. Perner, S. Andren, O. Schmidt, F. Setlur, S.R. Hoshida, Y. Mosquera, J.M. Pawitan, Y. Lee, C. Adami, H.O. Mucci, L.A. Kantoff, P.W. Andersson, S.O. Chinnaiyan, A.M. Johansson, J.E. and Rubin, M.A. 2007. TMPRSS2:ERG gene fusion associated with lethal prostate cancer in a watchful waiting cohort. Oncogene 26:31:4596-4599.

Denli, A.M. Tops, B.B. Plasterk, R.H. Ketting, R.F. and Hannon, G.J. 2004. Processing of primary microRNAs by the Microprocessor complex. Nature 432:7014:231-235.

Dick, J.E. 2003. Breast cancer stem cells revealed. Proc.Natl.Acad.Sci.U.S.A. 100:7:3547-3549.

Djavan, B. Susani, M. Bursa, B. Basharkhah, A. Simak, R. and Marberger, M. 1999. Predictability and significance of multifocal prostate cancer in the radical prostatectomy specimen. Tech.Urol. 5:3:139-142.

Dong, Q. Meng, P. Wang, T. Qin, W. Qin, W. Wang, F. Yuan, J. Chen, Z. Yang, A. and Wang, H. 2010. MicroRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. PLoS One 5:4:e10147.

Dudek, A.M. Grotenhuis, A.J. Vermeulen, S.H. Kiemeney, L.A. and Verhaegh, G.W. 2013. Urinary Bladder Cancer Susceptibility Markers. What Do We Know about Functional Mechanisms? Int.J.Mol.Sci. 14:6:12346-12366.

Dyson, N. 1998. The regulation of E2F by pRB-family proteins. Genes Dev. 12:15:2245-2262.

Eddy, S.R. 2001. Non-coding RNA genes and the modern RNA world. Nat.Rev.Genet. 2:12:919-929.

Edwards, S.M. Kote-Jarai, Z. Meitz, J. Hamoudi, R. Hope, Q. Osin, P. Jackson, R. Southgate, C. Singh, R. Falconer, A. Dearnaley, D.P. Ardern-Jones, A. Murkin, A. Dowe, A. Kelly, J. Williams, S. Oram, R. Stevens, M. Teare, D.M. Ponder, B.A. Gayther, S.A. Easton, D.F. Eeles, R.A. Cancer Research UK/Bristish Prostate Group UK Familial Prostate Cancer Study Collaborators and British Association of Urological Surgeons Section of Oncology. 2003. Two percent of men with early-onset prostate cancer harbor germline mutations in the BRCA2 gene. Am.J.Hum.Genet. 72:1:1-12.

Eeles, R. Goh, C. Castro, E. Bancroft, E. Guy, M. Al Olama, A.A. Easton, D. and Kote-Jarai, Z. 2014. The genetic epidemiology of prostate cancer and its clinical implications. Nat.Rev.Urol. 11:1:18-31.

Eguchi, S. Yamamoto, Y. Sakano, S. Chochi, Y. Nakao, M. Kawauchi, S. Furuya, T. Oga, A. Matsuyama, H. and Sasaki, K. 2010. The loss of 8p23.3 is a novel marker for predicting progression and recurrence of bladder tumors without muscle invasion. Cancer Genet.Cytogenet. 200:1:16-22.

Eis, P.S. Tam, W. Sun, L. Chadburn, A. Li, Z. Gomez, M.F. Lund, E. and Dahlberg, J.E. 2005. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc.Natl.Acad.Sci.U.S.A. 102:10:3627-3632.

Ellwood-Yen, K. Graeber, T.G. Wongvipat, J. Iruela-Arispe, M.L. Zhang, J. Matusik, R. Thomas, G.V. and Sawyers, C.L. 2003. Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer.Cell. 4:3:223-238.

Emara, M.M. Ivanov, P. Hickman, T. Dawra, N. Tisdale, S. Kedersha, N. Hu, G.F. and Anderson, P. 2010. Angiogenin-induced tRNA-derived stress-induced RNAs promote stress-induced stress granule assembly. J.Biol.Chem. 285:14:10959-10968.

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 Res. 55:14:2959-2962.

ENCODE Project Consortium Birney, E. Stamatoyannopoulos, J.A. Dutta, A. Guigo, R. Gingeras, T.R. Margulies, E.H. Weng, Z. Snyder, M. Dermitzakis, E.T. Thurman, R.E. Kuehn, M.S. Taylor, C.M. Neph, S. Koch, C.M. Asthana, S. Malhotra, A. Adzhubei, I. Greenbaum, J.A. Andrews, R.M. Flicek, P. Boyle, P.J. Cao, H. Carter, N.P. Clelland, G.K. Davis, S. Day, N. Dhami, P. Dillon, S.C. Dorschner, M.O. Fiegler, H. Giresi, P.G. Goldy, J. Hawrylycz, M. Haydock, A. Humbert, R. James, K.D. Johnson, B.E. Johnson, E.M. Frum, T.T. Rosenzweig, E.R. Karnani, N. Lee, K. Lefebvre, G.C. Navas, P.A. Neri, F. Parker, S.C. Sabo, P.J. Sandstrom, R. Shafer, A. Vetrie, D. Weaver, M. Wilcox, S. Yu, M. Collins, F.S. Dekker, J. Lieb, J.D. Tullius, T.D. Crawford, G.E.

Sunyaev, S. Noble, W.S. Dunham, I. Denoeud, F. Reymond, A. Kapranov, P. Rozowsky, J. Zheng, D. Castelo, R. Frankish, A. Harrow, J. Ghosh, S. Sandelin, A. Hofacker, I.L. Baertsch, R. Keefe, D. Dike, S. Cheng, J. Hirsch, H.A. Sekinger, E.A. Lagarde, J. Abril, J.F. Shahab, A. Flamm, C. Fried, C. Hackermuller, J. Hertel, J. Lindemeyer, M. Missal, K. Tanzer, A. Washietl, S. Korbel, J. Emanuelsson, O. Pedersen, J.S. Holroyd, N. Taylor, R. Swarbreck, D. Matthews, N. Dickson, M.C. Thomas, D.J. Weirauch, M.T. Gilbert, J. Drenkow, J. Bell, I. Zhao, X. Srinivasan, K.G. Sung, W.K. Ooi, H.S. Chiu, K.P. Foissac, S. Alioto, T. Brent, M. Pachter, L. Tress, M.L. Valencia, A. Choo, S.W. Choo, C.Y. Ucla, C. Manzano, C. Wyss, C. Cheung, E. Clark, T.G. Brown, J.B. Ganesh, M. Patel, S. Tammana, H. Chrast, J. Henrichsen, C.N. Kai, C. Kawai, J. Nagalakshmi, U. Wu, J. Lian, Z. Lian, J. Newburger, P. Zhang, X. Bickel, P. Mattick, J.S. Carninci, P. Hayashizaki, Y. Weissman, S. Hubbard, T. Myers, R.M. Rogers, J. Stadler, P.F. Lowe, T.M. Wei, C.L. Ruan, Y. Struhl, K. Gerstein, M. Antonarakis, S.E. Fu, Y. Green, E.D. Karaoz, U. Siepel, A. Taylor, J. Liefer, L.A. Wetterstrand, K.A. Good, P.J. Feingold, E.A. Guyer, M.S. Cooper, G.M. Asimenos, G. Dewey, C.N. Hou, M. Nikolaev, S. Montoya-Burgos, J.I. Loytynoja, A. Whelan, S. Pardi, F. Massingham, T. Huang, H. Zhang, N.R. Holmes, I. Mullikin, J.C. Ureta-Vidal, A. Paten, B. Seringhaus, M. Church, D. Rosenbloom, K. Kent, W.J. Stone, E.A. NISC Comparative Sequencing Program Baylor College of Medicine Human Genome Sequencing Center Washington University Genome Sequencing Center Broad Institute Children's Hospital Oakland Research Institute Batzoglou, S. Goldman, N. Hardison, R.C. Haussler, D. Miller, W. Sidow, A. Trinklein, N.D. Zhang, Z.D. Barrera, L. Stuart, R. King, D.C. Ameur, A. Enroth, S. Bieda, M.C. Kim, J. Bhinge, A.A. Jiang, N. Liu, J. Yao, F. Vega, V.B. Lee, C.W. Ng, P. Shahab, A. Yang, A. Moqtaderi, Z. Zhu, Z. Xu, X. Squazzo, S. Oberley, M.J. Inman, D. Singer, M.A. Richmond, T.A. Munn, K.J. Rada-Iglesias, A. Wallerman, O. Komorowski, J. Fowler, J.C. Couttet, P. Bruce, A.W. Dovey, O.M. Ellis, P.D. Langford, C.F. Nix, D.A. Euskirchen, G. Hartman, S. Urban, A.E. Kraus, P. Van Calcar, S. Heintzman, N. Kim, T.H. Wang, K. Qu, C. Hon, G. Luna, R. Glass, C.K. Rosenfeld, M.G. Aldred, S.F. Cooper, S.J. Halees, A. Lin, J.M. Shulha, H.P. Zhang, X. Xu, M. Haidar, J.N. Yu, Y. Ruan, Y. Iyer, V.R. Green, R.D. Wadelius, C. Farnham, P.J. Ren, B. Harte, R.A. Hinrichs, A.S. Trumbower, H. Clawson, H. Hillman-Jackson, J. Zweig, A.S. Smith, K. Thakkapallayil, A. Barber, G. Kuhn, R.M. Karolchik, D. Armengol, L. Bird, C.P. de Bakker, P.I. Kern, A.D. Lopez-Bigas, N. Martin, J.D. Stranger, B.E. Woodroffe, A. Davydov, E. Dimas, A. Eyras, E. Hallgrimsdottir, I.B. Huppert, J. Zody, M.C. Abecasis, G.R. Estivill, X. Bouffard, G.G. Guan, X. Hansen, N.F. Idol, J.R. Maduro, V.V. Maskeri, B. McDowell, J.C. Park, M. Thomas, P.J. Young, A.C. Blakesley, R.W. Muzny, D.M. Sodergren, E. Wheeler, D.A. Worley, K.C. Jiang, H. Weinstock, G.M. Gibbs, R.A. Graves, T. Fulton, R. Mardis, E.R. Wilson, R.K. Clamp, M. Cuff, J. Gnerre, S. Jaffe, D.B. Chang, J.L. Lindblad-Toh, K. Lander, E.S. Koriabine, M. Nefedov, M. Osoegawa, K. Yoshinaga, Y. Zhu, B. and de Jong, P.J. 2007. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 447:7146:799-816.

English, H.F. Santen, R.J. and Isaacs, J.T. 1987. Response of glandular versus basal rat ventral prostatic epithelial cells to androgen withdrawal and replacement. Prostate 11:3:229-242.

Epstein, J.I. 2010. An update of the Gleason grading system. J.Urol. 183:2:433-440.

Esteller, M. 2011. Non-coding RNAs in human disease. Nat.Rev.Genet. 12:12:861-874.

Eulalio, A. Huntzinger, E. Nishihara, T. Rehwinkel, J. Fauser, M. and Izaurralde, E. 2009. Deadenylation is a widespread effect of miRNA regulation. RNA 15:1:21-32.

Evans, G.S. and Chandler, J.A. 1987. Cell proliferation studies in the rat prostate: II. The effects of castration and androgen-induced regeneration upon basal and secretory cell proliferation. Prostate 11:4:339-351.

Ewing, C.M. Ray, A.M. Lange, E.M. Zuhlke, K.A. Robbins, C.M. Tembe, W.D. Wiley, K.E. Isaacs, S.D. Johng, D. Wang, Y. Bizon, C. Yan, G. Gielzak, M. Partin, A.W. Shanmugam, V. Izatt, T. Sinari, S. Craig, D.W. Zheng, S.L. Walsh, P.C. Montie, J.E. Xu, J. Carpten, J.D. Isaacs, W.B. and Cooney, K.A. 2012. Germline mutations in HOXB13 and prostate-cancer risk. N.Engl.J.Med. 366:2:141-149.

Fadl-Elmula, I. 2005. Chromosomal changes in uroepithelial carcinomas. Cell.Chromosome 4:1.

Feilotter, H.E. Nagai, M.A. Boag, A.H. Eng, C. and Mulligan, L.M. 1998. Analysis of PTEN and the 10q23 region in primary prostate carcinomas. Oncogene 16:13:1743-1748.

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. Ostrander, E.A. Stanford, J.L. and Huntsman, D.G. 2008. Association of TMPRSS2-ERG gene fusion with clinical characteristics and outcomes: results from a population-based study of prostate cancer. BMC Cancer 8:230-2407-8-230.

Freedman, M.L. Monteiro, A.N. Gayther, S.A. Coetzee, G.A. Risch, A. Plass, C. Casey, G. De Biasi, M. Carlson, C. Duggan, D. James, M. Liu, P. Tichelaar, J.W. Vikis, H.G. You, M. and Mills, I.G. 2011. Principles for the post-GWAS functional characterization of cancer risk loci. Nat.Genet. 43:6:513-518.

Friend, S.H. Bernards, R. Rogelj, S. Weinberg, R.A. Rapaport, J.M. Albert, D.M. and Dryja, T.P. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323:6089:643-646.

Fritsche, M. Haessler, C. and Brandner, G. 1993. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene 8:2:307-318.

Fu, H. Feng, J. Liu, Q. Sun, F. Tie, Y. Zhu, J. Xing, R. Sun, Z. and Zheng, X. 2009. Stress induces tRNA cleavage by angiogenin in mammalian cells. FEBS Lett. 583:2:437-442.

Fujita, S. Ito, T. Mizutani, T. Minoguchi, S. Yamamichi, N. Sakurai, K. and Iba, H. 2008. miR-21 Gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. J.Mol.Biol. 378:3:492-504.

Fujita, Y. Kojima, K. Ohhashi, R. Hamada, N. Nozawa, Y. Kitamoto, A. Sato, A. Kondo, S. Kojima, T. Deguchi, T. and Ito, M. 2010. MiR-148a attenuates paclitaxel resistance of hormone-refractory, drug-resistant prostate cancer PC3 cells by regulating MSK1 expression. J.Biol.Chem. 285:25:19076-19084.

Fuse, M. Nohata, N. Kojima, S. Sakamoto, S. Chiyomaru, T. Kawakami, K. Enokida, H. Nakagawa, M. Naya, Y. Ichikawa, T. and Seki, N. 2011. Restoration of miR-145 expression suppresses cell proliferation, migration and invasion in prostate cancer by targeting FSCN1. Int.J.Oncol. 38:4:1093-1101.

Gaddipati, J.P. McLeod, D.G. Heidenberg, H.B. Sesterhenn, I.A. Finger, M.J. Moul, J.W. and Srivastava, S. 1994. Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. Cancer Res. 54:11:2861-2864.

Galardi, S. Mercatelli, N. Giorda, E. Massalini, S. Frajese, G.V. Ciafre, S.A. and Farace, M.G. 2007. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J.Biol.Chem. 282:32:23716-23724.

Gandellini, P. Folini, M. Longoni, N. Pennati, M. Binda, M. Colecchia, M. Salvioni, R. Supino, R. Moretti, R. Limonta, P. Valdagni, R. Daidone, M.G. and Zaffaroni, N. 2009. miR-205 Exerts tumor-suppressive functions in human prostate through down-regulation of protein kinase Cepsilon. Cancer Res. 69:6:2287-2295.

Gee, H.E. Buffa, F.M. Camps, C. Ramachandran, A. Leek, R. Taylor, M. Patil, M. Sheldon, H. Betts, G. Homer, J. West, C. Ragoussis, J. and Harris, A.L. 2011. The small-nucleolar RNAs commonly used for microRNA normalisation correlate with tumour pathology and prognosis. Br.J.Cancer 104:7:1168-1177.

Geisberg, J.V. Holstege, F.C. Young, R.A. and Struhl, K. 2001. Yeast NC2 associates with the RNA polymerase II preinitiation complex and selectively affects transcription in vivo. Mol.Cell.Biol. 21:8:2736-2742.

Gelmann, E.P. 2003. Searching for the gatekeeper oncogene of prostate cancer. Crit.Rev.Oncol.Hematol. 46 Suppl:S11-20.

George, R.E. Attiyeh, E.F. Li, S. Moreau, L.A. Neuberg, D. Li, C. Fox, E.A. Meyerson, M. Diller, L. Fortina, P. Look, A.T. and Maris, J.M. 2007. Genome-wide analysis of neuroblastomas using high-density single nucleotide polymorphism arrays. PLoS One 2:2:e255.

Gil, J. Kerai, P. Lleonart, M. Bernard, D. Cigudosa, J.C. Peters, G. Carnero, A. and Beach, D. 2005. Immortalization of primary human prostate epithelial cells by c-Myc. Cancer Res. 65:6:2179-2185.

Gironella, M. Seux, M. Xie, M.J. Cano, C. Tomasini, R. Gommeaux, J. Garcia, S. Nowak, J. Yeung, M.L. Jeang, K.T. Chaix, A. Fazli, L. Motoo, Y. Wang, Q. Rocchi, P. Russo, A. Gleave, M. Dagorn, J.C. Iovanna, J.L. Carrier, A. Pebusque, M.J. and Dusetti, N.J. 2007. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. Proc.Natl.Acad.Sci.U.S.A. 104:41:16170-16175.

Goldstein, A.S. Huang, J. Guo, C. Garraway, I.P. and Witte, O.N. 2010. Identification of a cell of origin for human prostate cancer. Science 329:5991:568-571.

Goldstein, A.S. Lawson, D.A. Cheng, D. Sun, W. Garraway, I.P. and Witte, O.N. 2008. Trop2 identifies a subpopulation of murine and human prostate basal cells with stem cell characteristics. Proc.Natl.Acad.Sci.U.S.A. 105:52:20882-20887.

Golka, K. Wiese, A. Assennato, G. and Bolt, H.M. 2004. Occupational exposure and urological cancer. World J.Urol. 21:6:382-391.

Goodenbour, J.M. and Pan, T. 2006. Diversity of tRNA genes in eukaryotes. Nucleic Acids Res. 34:21:6137-6146.

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. Reuter, V.E. and Gerald, W.L. 2009. TMPRSS2-ERG gene fusion is not associated with outcome in patients treated by prostatectomy. Cancer Res. 69:4:1400-1406.

Gottlieb, B. Beitel, L.K. Wu, J.H. and Trifiro, M. 2004. The androgen receptor gene mutations database (ARDB): 2004 update. Hum.Mutat. 23:6:527-533.

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. Asangani, I.A. Ateeq, B. Chun, S.Y. Siddiqui, J. Sam, L. Anstett, M. Mehra, R. Prensner, J.R. Palanisamy, N. Ryslik, G.A. Vandin, F. Raphael, B.J. Kunju, L.P. Rhodes, D.R. Pienta, K.J. Chinnaiyan, A.M. and Tomlins, S.A. 2012. The mutational landscape of lethal castration-resistant prostate cancer. Nature 487:7406:239-243.

Gregory, R.I. Chendrimada, T.P. Cooch, N. and Shiekhattar, R. 2005. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell 123:4:631-640.

Greither, T. Grochola, L.F. Udelnow, A. Lautenschlager, C. Wurl, P. and Taubert, H. 2010. Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. Int.J.Cancer 126:1:73-80.

Guan, Y. Gerhard, B. and Hogge, D.E. 2003. Detection, isolation, and stimulation of quiescent primitive leukemic progenitor cells from patients with acute myeloid leukemia (AML). Blood 101:8:3142-3149.

Gumy-Pause, F. Wacker, P. and Sappino, A.P. 2004. ATM gene and lymphoid malignancies. Leukemia 18:2:238-242.

Guo, Q. Xie, J. Dang, C.V. Liu, E.T. and Bishop, J.M. 1998. Identification of a large Myc-binding protein that contains RCC1-like repeats. Proc.Natl.Acad.Sci.U.S.A. 95:16:9172-9177.

Gurel, B. Iwata, T. Koh, C.M. Jenkins, R.B. Lan, F. Van Dang, C. Hicks, J.L. Morgan, J. Cornish, T.C. Sutcliffe, S. Isaacs, W.B. Luo, J. and De Marzo, A.M. 2008. Nuclear MYC protein overexpression is an early alteration in human prostate carcinogenesis. Mod.Pathol. 21:9:1156-1167.

Guzman, M.L. Neering, S.J. Upchurch, D. Grimes, B. Howard, D.S. Rizzieri, D.A. Luger, S.M. and Jordan, C.T. 2001. Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. Blood 98:8:2301-2307.

Guzman, M.L. Rossi, R.M. Karnischky, L. Li, X. Peterson, D.R. Howard, D.S. and Jordan, C.T. 2005. The sesquiterpene lactone parthenolide induces apoptosis of human acute myelogenous leukemia stem and progenitor cells. Blood 105:11:4163-4169.

Habuchi, T. Devlin, J. Elder, P.A. and Knowles, M.A. 1995. Detailed deletion mapping of chromosome 9q in bladder cancer: evidence for two tumour suppressor loci. Oncogene 11:8:1671-1674.

Habuchi, T. Luscombe, M. Elder, P.A. and Knowles, M.A. 1998. Structure and methylation-based silencing of a gene (DBCCR1) within a candidate bladder cancer tumor suppressor region at 9q32-q33. Genomics 48:3:277-288.

Han, S. Witt, R.M. Santos, T.M. Polizzano, C. Sabatini, B.L. and Ramesh, V. 2008. Pam (Protein associated with Myc) functions as an E3 ubiquitin ligase and regulates TSC/mTOR signaling. Cell.Signal. 20:6:1084-1091.

Hanahan, D. and Weinberg, R.A. 2011. Hallmarks of cancer: the next generation. Cell 144:5:646-674.

Hausser, J. and Zavolan, M. 2014. Identification and consequences of miRNA-target interactions--beyond repression of gene expression. Nat.Rev.Genet. 15:9:599-612.

He, L. Thomson, J.M. Hemann, M.T. Hernando-Monge, E. Mu, D. Goodson, S. Powers, S. Cordon-Cardo, C. Lowe, S.W. Hannon, G.J. and Hammond, S.M. 2005. A microRNA polycistron as a potential human oncogene. Nature 435:7043:828-833.

He, W.W. Sciavolino, P.J. Wing, J. Augustus, M. Hudson, P. Meissner, P.S. Curtis, R.T. Shell, B.K. Bostwick, D.G. Tindall, D.J. Gelmann, E.P. Abate-Shen, C. and Carter, K.C. 1997. A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics 43:1:69-77.

Heinlein, C.A. and Chang, C. 2004. Androgen receptor in prostate cancer. Endocr.Rev. 25:2:276-308.

Helgeson, B.E. Tomlins, S.A. Shah, N. Laxman, B. Cao, Q. Prensner, J.R. Cao, X. Singla, N. Montie, J.E. Varambally, S. Mehra, R. and Chinnaiyan, A.M. 2008. Characterization of TMPRSS2:ETV5 and SLC45A3:ETV5 gene fusions in prostate cancer. Cancer Res. 68:1:73-80.

Hendriksen, P.J. Dits, N.F. Kokame, K. Veldhoven, A. van Weerden, W.M. Bangma, C.H. Trapman, J. and Jenster, G. 2006. Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res. 66:10:5012-5020.

Hermans, K.G. Bressers, A.A. van der Korput, H.A. Dits, N.F. Jenster, G. and Trapman, J. 2008a. Two unique novel prostate-specific and androgen-regulated fusion partners of ETV4 in prostate cancer. Cancer Res. 68:9:3094-3098.

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. Korsten, H. Kraaij, R. Jenster, G. and Trapman, J. 2008b. Truncated ETV1, fused to novel tissue-specific genes, and full-length ETV1 in prostate cancer. Cancer Res. 68:18:7541-7549.

Hill, R.P. 2006. Identifying cancer stem cells in solid tumors: case not proven. Cancer Res. 66:4:1891-5; discussion 1890.

Hillion, J. Wood, L.J. Mukherjee, M. Bhattacharya, R. Di Cello, F. Kowalski, J. Elbahloul, O. Segal, J. Poirier, J. Rudin, C.M. Dhara, S. Belton, A. Joseph, B. Zucker, S. and Resar, L.M. 2009. Upregulation of MMP-2 by HMGA1 promotes transformation in undifferentiated, large-cell lung cancer. Mol.Cancer.Res. 7:11:1803-1812.

Hjelmborg, J.B. Scheike, T. Holst, K. Skytthe, A. Penney, K.L. Graff, R.E. Pukkala, E. Christensen, K. Adami, H.O. Holm, N.V. Nuttall, E. Hansen, S. Hartman, M. Czene, K. Harris, J.R. Kaprio, J. and Mucci, L.A. 2014. The heritability of prostate cancer in the Nordic Twin Study of Cancer. Cancer Epidemiol.Biomarkers Prev. 23:11:2303-2310.

Hofseth, L.J. Hussain, S.P. and Harris, C.C. 2004. P53: 25 Years After its Discovery. Trends Pharmacol.Sci. 25:4:177-181.

Hoglund, M. 2012. The bladder cancer genome; chromosomal changes as prognostic makers, opportunities, and obstacles. Urol.Oncol. 30:4:533-540.

Hollenhorst, P.C. McIntosh, L.P. and Graves, B.J. 2011. Genomic and biochemical insights into the specificity of ETS transcription factors. Annu.Rev.Biochem. 80:437-471.

Hollstein, M. Sidransky, D. Vogelstein, B. and Harris, C.C. 1991. P53 Mutations in Human Cancers. Science 253:5015:49-53.

Holmang, S. Hedelin, H. Anderstrom, C. and Johansson, S.L. 1995. The relationship among multiple recurrences, progression and prognosis of patients with stages Ta and T1 transitional cell cancer of the bladder followed for at least 20 years. J.Urol. 153:6:1823-6; discussion 1826-7.

Hombach-Klonisch, S. Panigrahi, S. Rashedi, I. Seifert, A. Alberti, E. Pocar, P. Kurpisz, M. Schulze-Osthoff, K. Mackiewicz, A. and Los, M. 2008. Adult stem cells and their trans-differentiation potential--perspectives and therapeutic applications. J.Mol.Med.(Berl) 86:12:1301-1314.

Hoogstrate, Y. Jenster, G. and Martens-Uzunova, E.S. 2015. FlaiMapper: computational annotation of small ncRNA-derived fragments using RNA-seq high-throughput data. Bioinformatics 31:5:665-673.

Hotte, S.J. and Saad, F. 2010. Current management of castrate-resistant prostate cancer. Curr.Oncol. 17 Suppl 2:S72-9.

Hovey, R.M. Chu, L. Balazs, M. DeVries, S. Moore, D. Sauter, G. Carroll, P.R. and Waldman, F.M. 1998. Genetic alterations in primary bladder cancers and their metastases. Cancer Res. 58:16:3555-3560.

Hu, R. Dunn, T.A. Wei, S. Isharwal, S. Veltri, R.W. Humphreys, E. Han, M. Partin, A.W. Vessella, R.L. Isaacs, W.B. Bova, G.S. and Luo, J. 2009. Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer. Cancer Res. 69:1:16-22.

Hu, R. Lu, C. Mostaghel, E.A. Yegnasubramanian, S. Gurel, M. Tannahill, C. Edwards, J. Isaacs, W.B. Nelson, P.S. Bluemn, E. Plymate, S.R. and Luo, J. 2012. Distinct transcriptional programs mediated by the ligand-dependent full-length androgen receptor and its splice variants in castration-resistant prostate cancer. Cancer Res. 72:14:3457-3462.

Huang, S. Guo, W. Tang, Y. Ren, D. Zou, X. and Peng, X. 2012. miR-143 and miR-145 inhibit stem cell characteristics of PC-3 prostate cancer cells. Oncol.Rep. 28:5:1831-1837.

Huggins, C. and Hodges, C.V. 2002. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. J.Urol. 168:1:9-12.

Hurst, C.D. Fiegler, H. Carr, P. Williams, S. Carter, N.P. and Knowles, M.A. 2004. High-resolution analysis of genomic copy number alterations in bladder cancer by microarray-based comparative genomic hybridization. Oncogene 23:12:2250-2263.

Hurt, E.M. Kawasaki, B.T. Klarmann, G.J. Thomas, S.B. and Farrar, W.L. 2008. CD44+ CD24(-) prostate cells are early cancer progenitor/stem cells that provide a model for patients with poor prognosis. Br.J.Cancer 98:4:756-765.

Hyytinen, E.R. Frierson, H.F.,Jr Boyd, J.C. Chung, L.W. and Dong, J.T. 1999. Three distinct regions of allelic loss at 13q14, 13q21-22, and 13q33 in prostate cancer. Genes Chromosomes Cancer 25:2:108-114.

Iliopoulos, D. Jaeger, S.A. Hirsch, H.A. Bulyk, M.L. and Struhl, K. 2010. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. Mol.Cell 39:4:493-506.

Imperato-McGinley, J. Binienda, Z. Arthur, A. Mininberg, D.T. Vaughan, E.D.,Jr and Quimby, F.W. 1985. The development of a male pseudohermaphroditic rat using an inhibitor of the enzyme 5 alpha-reductase. Endocrinology 116:2:807-812.

Iorio, M.V. Ferracin, M. Liu, C.G. Veronese, A. Spizzo, R. Sabbioni, S. Magri, E. Pedriali, M. Fabbri, M. Campiglio, M. Menard, S. Palazzo, J.P. Rosenberg, A. Musiani, P. Volinia, S. Nenci, I. Calin, G.A. Querzoli, P. Negrini, M. and Croce, C.M. 2005. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 65:16:7065-7070.

Isaacs, J.T. and Coffey, D.S. 1989. Etiology and disease process of benign prostatic hyperplasia. Prostate Suppl. 2:33-50.

Ishikawa, F. Yoshida, S. Saito, Y. Hijikata, A. Kitamura, H. Tanaka, S. Nakamura, R. Tanaka, T. Tomiyama, H. Saito, N. Fukata, M. Miyamoto, T. Lyons, B. Ohshima, K. Uchida, N. Taniguchi, S. Ohara, O. Akashi, K. Harada, M. and Shultz, L.D. 2007. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. Nat.Biotechnol. 25:11:1315-1321.

Ito, K. Bernardi, R. Morotti, A. Matsuoka, S. Saglio, G. Ikeda, Y. Rosenblatt, J. Avigan, D.E. Teruya-Feldstein, J. and Pandolfi, P.P. 2008. PML targeting eradicates quiescent leukaemia-initiating cells. Nature 453:7198:1072-1078.

Ivanov, P. Emara, M.M. Villen, J. Gygi, S.P. and Anderson, P. 2011. Angiogenin-induced tRNA fragments inhibit translation initiation. Mol.Cell 43:4:613-623.

Jalava, S.E. Urbanucci, A. Latonen, L. Waltering, K.K. Sahu, B. Janne, O.A. Seppala, J. Lahdesmaki, H. Tammela, T.L. and Visakorpi, T. 2012. Androgen-regulated miR-32 targets BTG2 and is overexpressed in castration-resistant prostate cancer. Oncogene 31:41:4460-4471.

Jemal, A. Bray, F. Center, M.M. Ferlay, J. Ward, E. and Forman, D. 2011. Global cancer statistics. CA Cancer.J.Clin. 61:2:69-90.

Jenkins, R.B. Qian, J. Lieber, M.M. and Bostwick, D.G. 1997. Detection of c-myc oncogene amplification and chromosomal anomalies in metastatic prostatic carcinoma by fluorescence in situ hybridization. Cancer Res. 57:3:524-531.

Jeronimo, C. Henrique, R. Hoque, M.O. Mambo, E. Ribeiro, F.R. Varzim, G. Oliveira, J. Teixeira, M.R. Lopes, C. and Sidransky, D. 2004. A quantitative promoter methylation profile of prostate cancer. Clin.Cancer Res. 10:24:8472-8478.

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. J.Natl.Cancer Inst. 93:22:1747-1752.

Jia, L. Landan, G. Pomerantz, M. Jaschek, R. Herman, P. Reich, D. Yan, C. Khalid, O. Kantoff, P. Oh, W. Manak, J.R. Berman, B.P. Henderson, B.E. Frenkel, B. Haiman, C.A. Freedman, M. Tanay, A. and Coetzee, G.A. 2009. Functional enhancers at the gene-poor 8q24 cancer-linked locus. PLoS Genet. 5:8:e1000597.

Jiang, S. Zhang, H.W. Lu, M.H. He, X.H. Li, Y. Gu, H. Liu, M.F. and Wang, E.D. 2010. MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene. Cancer Res. 70:8:3119-3127.

Jin, Y. Stewenius, Y. Lindgren, D. Frigyesi, A. Calcagnile, O. Jonson, T. Edqvist, A. Larsson, N. Lundberg, L.M. Chebil, G. Liedberg, F. Gudjonsson, S. Mansson, W. Hoglund, M. and Gisselsson, D. 2007. Distinct mitotic segregation errors mediate chromosomal instability in aggressive urothelial cancers. Clin.Cancer Res. 13:6:1703-1712.

Jochl, C. Rederstorff, M. Hertel, J. Stadler, P.F. Hofacker, I.L. Schrettl, M. Haas, H. and Huttenhofer, A. 2008. Small ncRNA transcriptome analysis from Aspergillus fumigatus suggests a novel mechanism for regulation of protein synthesis. Nucleic Acids Res. 36:8:2677-2689.

Johnson, S.M. Grosshans, H. Shingara, J. Byrom, M. Jarvis, R. Cheng, A. Labourier, E. Reinert, K.L. Brown, D. and Slack, F.J. 2005. RAS is regulated by the let-7 microRNA family. Cell 120:5:635-647.

Jordan, C.T. Guzman, M.L. and Noble, M. 2006. Cancer stem cells. N.Engl.J.Med. 355:12:1253-1261.

Kajanne, R. Miettinen, P. Tenhunen, M. and Leppa, S. 2009. Transcription factor AP-1 promotes growth and radioresistance in prostate cancer cells. Int.J.Oncol. 35:5:1175-1182.

Kallioniemi, A. Kallioniemi, O.P. Citro, G. Sauter, G. DeVries, S. Kerschmann, R. Caroll, P. and Waldman, F. 1995. Identification of gains and losses of DNA sequences in primary bladder cancer by comparative genomic hybridization. Genes Chromosomes Cancer 12:3:213-219.

Kastan, M.B. and Lim, D.S. 2000. The many substrates and functions of ATM. Nat.Rev.Mol.Cell Biol. 1:3:179-186.

Kastendieck, H. 1980. Correlations between atypical primary hyperplasia and carcinoma of the prostate. A histological study of 180 total prostatectomies. Pathol.Res.Pract. 169:3-4:366-387.

Kaukoniemi, K.M. Rauhala, H.E. Scaravilli, M. Latonen, L. Annala, M. Vessella, R.L. Nykter, M. Tammela, T.L. and Visakorpi, T. 2015. Epigenetically altered miR-193b targets cyclin D1 in prostate cancer. Cancer.Med. 4:9:1417-1425.

Kawaji, H. Nakamura, M. Takahashi, Y. Sandelin, A. Katayama, S. Fukuda, S. Daub, C.O. Kai, C. Kawai, J. Yasuda, J. Carninci, P. and Hayashizaki, Y. 2008. Hidden layers of human small RNAs. BMC Genomics 9:157-2164-9-157.

Kelly, P.N. Dakic, A. Adams, J.M. Nutt, S.L. and Strasser, A. 2007. Tumor growth need not be driven by rare cancer stem cells. Science 317:5836:337.

Kennedy, J.A. Barabe, F. Poeppl, A.G. Wang, J.C. and Dick, J.E. 2007. Comment on "Tumor growth need not be driven by rare cancer stem cells". Science 318:5857:1722; author reply 1722.

Kern, S.E. and Shibata, D. 2007. The fuzzy math of solid tumor stem cells: a perspective. Cancer Res. 67:19:8985-8988.

Kinzler, K.W. and Vogelstein, B. 1997. Cancer-susceptibility genes. Gatekeepers and caretakers. Nature 386:6627:761, 763.

Kirchner, S. and Ignatova, Z. 2015. Emerging roles of tRNA in adaptive translation, signalling dynamics and disease. Nat.Rev.Genet. 16:2:98-112.

Kiss, T. 2002. Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. Cell 109:2:145-148.

Kleinerman, R.A. Tucker, M.A. Tarone, R.E. Abramson, D.H. Seddon, J.M. Stovall, M. Li, F.P. and Fraumeni, J.F.,Jr. 2005. Risk of new cancers after radiotherapy in long-term survivors of retinoblastoma: an extended follow-up. J.Clin.Oncol. 23:10:2272-2279.

Klezovitch, O. Risk, M. Coleman, I. Lucas, J.M. Null, M. True, L.D. Nelson, P.S. and Vasioukhin, V. 2008. A causal role for ERG in neoplastic transformation of prostate epithelium. Proc.Natl.Acad.Sci.U.S.A. 105:6:2105-2110.

Knudson, A.G.,Jr. 1971. Mutation and cancer: statistical study of retinoblastoma. Proc.Natl.Acad.Sci.U.S.A. 68:4:820-823.

Koh, C.M. Bieberich, C.J. Dang, C.V. Nelson, W.G. Yegnasubramanian, S. and De Marzo, A.M. 2010. MYC and Prostate Cancer. Genes Cancer. 1:6:617-628.

Koivisto, P. Kononen, J. Palmberg, C. Tammela, T. Hyytinen, E. Isola, J. Trapman, J. Cleutjens, K. Noordzij, A. Visakorpi, T. and Kallioniemi, O.P. 1997. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res. 57:2:314-319.

Kong, D. Heath, E. Chen, W. Cher, M. Powell, I. Heilbrun, L. Li, Y. Ali, S. Sethi, S. Hassan, O. Hwang, C. Gupta, N. Chitale, D. Sakr, W.A. Menon, M. and Sarkar, F.H. 2012. Epigenetic silencing of miR-34a in human prostate cancer cells and tumor tissue specimens can be reversed by BR-DIM treatment. Am.J.Transl.Res. 4:1:14-23.

Kong, W. He, L. Coppola, M. Guo, J. Esposito, N.N. Coppola, D. and Cheng, J.Q. 2010. MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. J.Biol.Chem. 285:23:17869-17879.

Kotliarov, Y. Steed, M.E. Christopher, N. Walling, J. Su, Q. Center, A. Heiss, J. Rosenblum, M. Mikkelsen, T. Zenklusen, J.C. and Fine, H.A. 2006. High-resolution global genomic survey of 178 gliomas reveals novel regions of copy number alteration and allelic imbalances. Cancer Res. 66:19:9428-9436.

Kozomara, A. and Griffiths-Jones, S. 2014. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 42:Database issue:D68-73.

Kurashina, K. Yamashita, Y. Ueno, T. Koinuma, K. Ohashi, J. Horie, H. Miyakura, Y. Hamada, T. Haruta, H. Hatanaka, H. Soda, M. Choi, Y.L. Takada, S. Yasuda, Y. Nagai, H. and Mano, H. 2008. Chromosome copy number analysis in screening for prognosis-related genomic regions in colorectal carcinoma. Cancer.Sci. 99:9:1835-1840.

Kurita, T. Medina, R.T. Mills, A.A. and Cunha, G.R. 2004. Role of p63 and basal cells in the prostate. Development 131:20:4955-4964.

Lane, D.P. 1992. Cancer. p53, guardian of the genome. Nature 358:6381:15-16.

Lapidot, T. Sirard, C. Vormoor, J. Murdoch, B. Hoang, T. Caceres-Cortes, J. Minden, M. Paterson, B. Caligiuri, M.A. and Dick, J.E. 1994. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 367:6464:645-648.

Lapointe, J. Li, C. Higgins, J.P. van de Rijn, M. Bair, E. Montgomery, K. Ferrari, M. Egevad, L. Rayford, W. Bergerheim, U. Ekman, P. DeMarzo, A.M. Tibshirani, R. Botstein, D. Brown, P.O. Brooks, J.D. and Pollack, J.R. 2004. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc.Natl.Acad.Sci.U.S.A. 101:3:811-816.

Lasorella, A. Benezra, R. and Iavarone, A. 2014. The ID proteins: master regulators of cancer stem cells and tumour aggressiveness. Nat.Rev.Cancer. 14:2:77-91.

Lawson, D.A. Xin, L. Lukacs, R.U. Cheng, D. and Witte, O.N. 2007. Isolation and functional characterization of murine prostate stem cells. Proc.Natl.Acad.Sci.U.S.A. 104:1:181-186.

Lawson, D.A. Zong, Y. Memarzadeh, S. Xin, L. Huang, J. and Witte, O.N. 2010. Basal epithelial stem cells are efficient targets for prostate cancer initiation. Proc.Natl.Acad.Sci.U.S.A. 107:6:2610-2615.

le Sage, C. Nagel, R. Egan, D.A. Schrier, M. Mesman, E. Mangiola, A. Anile, C. Maira, G. Mercatelli, N. Ciafre, S.A. Farace, M.G. and Agami, R. 2007. Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. EMBO J. 26:15:3699-3708.

Lee, R. and Droller, M.J. 2000. The natural history of bladder cancer. Implications for therapy. Urol.Clin.North Am. 27:1:1-13, vii.

Lee, R.C. Feinbaum, R.L. and Ambros, V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:5:843-854.

Lee, S.R. and Collins, K. 2005. Starvation-induced cleavage of the tRNA anticodon loop in Tetrahymena thermophila. J.Biol.Chem. 280:52:42744-42749.

Lee, W.H. Morton, R.A. Epstein, J.I. Brooks, J.D. Campbell, P.A. Bova, G.S. Hsieh, W.S. Isaacs, W.B. and Nelson, W.G. 1994. Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. Proc.Natl.Acad.Sci.U.S.A. 91:24:11733-11737.

Lee, Y. Kim, M. Han, J. Yeom, K.H. Lee, S. Baek, S.H. and Kim, V.N. 2004. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 23:20:4051-4060.

Lee, Y.S. Kim, H.K. Chung, S. Kim, K.S. and Dutta, A. 2005. Depletion of human micro-RNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. J.Biol.Chem. 280:17:16635-16641.

Lee, Y.S. Shibata, Y. Malhotra, A. and Dutta, A. 2009. A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). Genes Dev. 23:22:2639-2649.

Leevers, S.J. Paterson, H.F. and Marshall, C.J. 1994. Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature 369:6479:411-414.

Leonardo, T.R. Schultheisz, H.L. Loring, J.F. and Laurent, L.C. 2012. The functions of microRNAs in pluripotency and reprogramming. Nat.Cell Biol. 14:11:1114-1121.

Leone, G. DeGregori, J. Yan, Z. Jakoi, L. Ishida, S. Williams, R.S. and Nevins, J.R. 1998. E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. Genes Dev. 12:14:2120-2130.

Leong, K.G. Wang, B.E. Johnson, L. and Gao, W.Q. 2008. Generation of a prostate from a single adult stem cell. Nature 456:7223:804-808.

Lessard, J. and Sauvageau, G. 2003. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature 423:6937:255-260.

Levine, A.J. 1997. P53, the Cellular Gatekeeper for Growth and Division. Cell 88:3:323-331.

Levine, A.J. Momand, J. and Finlay, C.A. 1991. The p53 tumour suppressor gene. Nature 351:6326:453-456.

Levitt, N.C. and Hickson, I.D. 2002. Caretaker tumour suppressor genes that defend genome integrity. Trends Mol.Med. 8:4:179-186.

Levitz, R. Chapman, D. Amitsur, M. Green, R. Snyder, L. and Kaufmann, G. 1990. The optional E. coli prr locus encodes a latent form of phage T4-induced anticodon nuclease. EMBO J. 9:5:1383-1389.

Li, C. Heidt, D.G. Dalerba, P. Burant, C.F. Zhang, L. Adsay, V. Wicha, M. Clarke, M.F. and Simeone, D.M. 2007. Identification of pancreatic cancer stem cells. Cancer Res. 67:3:1030-1037.

Li, J. Yen, C. Liaw, D. Podsypanina, K. Bose, S. Wang, S.I. Puc, J. Miliaresis, C. Rodgers, L. McCombie, R. Bigner, S.H. Giovanella, B.C. Ittmann, M. Tycko, B. Hibshoosh, H. Wigler, M.H. and Parsons, R. 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275:5308:1943-1947.

Li, T. Li, D. Sha, J. Sun, P. and Huang, Y. 2009. MicroRNA-21 directly targets MARCKS and promotes apoptosis resistance and invasion in prostate cancer cells. Biochem.Biophys.Res.Commun. 383:3:280-285.

Lichtenstein, P. Holm, N.V. Verkasalo, P.K. Iliadou, A. Kaprio, J. Koskenvuo, M. Pukkala, E. Skytthe, A. and Hemminki, K. 2000. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. N.Engl.J.Med. 343:2:78-85.

Lichter, P. Joos, S. Bentz, M. and Lampel, S. 2000. Comparative genomic hybridization: uses and limitations. Semin.Hematol. 37:4:348-357.

Lilja, H. 1985. A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. J.Clin.Invest. 76:5:1899-1903.

Lilja, H. Ulmert, D. and Vickers, A.J. 2008. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nat.Rev.Cancer. 8:4:268-278.

Lin, S. and Gregory, R.I. 2015. MicroRNA biogenesis pathways in cancer. Nat.Rev.Cancer. 15:6:321-333.

Linja, M.J. Savinainen, K.J. Saramaki, O.R. Tammela, T.L. Vessella, R.L. and Visakorpi, T. 2001. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res. 61:9:3550-3555.

Linja, M.J. and Visakorpi, T. 2004. Alterations of androgen receptor in prostate cancer. J.Steroid Biochem.Mol.Biol. 92:4:255-264.

Liu, C. Kelnar, K. Liu, B. Chen, X. Calhoun-Davis, T. Li, H. Patrawala, L. Yan, H. Jeter, C. Honorio, S. Wiggins, J.F. Bader, A.G. Fagin, R. Brown, D. and Tang, D.G. 2011. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat.Med. 17:2:211-215.

Lodygin, D. Tarasov, V. Epanchintsev, A. Berking, C. Knyazeva, T. Korner, H. Knyazev, P. Diebold, J. and Hermeking, H. 2008. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. Cell.Cycle 7:16:2591-2600.

Lowy, D.R. and Willumsen, B.M. 1993. Function and regulation of ras. Annu.Rev.Biochem. 62:851-891.

Lu, J. Getz, G. Miska, E.A. Alvarez-Saavedra, E. Lamb, J. Peck, D. Sweet-Cordero, A. Ebert, B.L. Mak, R.H. Ferrando, A.A. Downing, J.R. Jacks, T. Horvitz, H.R. and Golub, T.R. 2005. MicroRNA expression profiles classify human cancers. Nature 435:7043:834-838.

Ma, R. Jiang, T. and Kang, X. 2012. Circulating microRNAs in cancer: origin, function and application. J.Exp.Clin.Cancer Res. 31:38-9966-31-38.

Marais, R. Light, Y. Paterson, H.F. and Marshall, C.J. 1995. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J. 14:13:3136-3145.

Marcelli, M. Ittmann, M. Mariani, S. Sutherland, R. Nigam, R. Murthy, L. Zhao, Y. DiConcini, D. Puxeddu, E. Esen, A. Eastham, J. Weigel, N.L. and Lamb, D.J. 2000. Androgen receptor mutations in prostate cancer. Cancer Res. 60:4:944-949.

Marotta, L.L. and Polyak, K. 2009. Cancer stem cells: a model in the making. Curr.Opin.Genet.Dev. 19:1:44-50.

Martens-Uzunova, E.S. Hoogstrate, Y. Kalsbeek, A. Pigmans, B. Vredenbregt-van den Berg, M. Dits, N. Nielsen, S.J. Baker, A. Visakorpi, T. Bangma, C. and Jenster, G. 2015. C/D-box snoRNA-derived RNA production is associated with malignant transformation and metastatic progression in prostate cancer. Oncotarget 6:19:17430-17444.

Martens-Uzunova, E.S. Jalava, S.E. Dits, N.F. van Leenders, G.J. Moller, S. Trapman, J. Bangma, C.H. Litman, T. Visakorpi, T. and Jenster, G. 2012. Diagnostic and prognostic signatures from the small non-coding RNA transcriptome in prostate cancer. Oncogene 31:8:978-991.

Martens-Uzunova, E.S. Olvedy, M. and Jenster, G. 2013. Beyond microRNA--novel RNAs derived from small non-coding RNA and their implication in cancer. Cancer Lett. 340:2:201-211.

Martinez-Sanchez, A. and Murphy, C.L. 2013. miR-1247 functions by targeting cartilage transcription factor SOX9. J.Biol.Chem. 288:43:30802-30814.

Maser, R.S. Choudhury, B. Campbell, P.J. Feng, B. Wong, K.K. Protopopov, A. O'Neil, J. Gutierrez, A. Ivanova, E. Perna, I. Lin, E. Mani, V. Jiang, S. McNamara, K. Zaghlul, S. Edkins, S. Stevens, C. Brennan, C. Martin, E.S. Wiedemeyer, R. Kabbarah, O. Nogueira, C. Histen, G. Aster, J. Mansour, M. Duke, V. Foroni, L. Fielding, A.K. Goldstone, A.H. Rowe, J.M. Wang, Y.A. Look, A.T. Stratton, M.R. Chin, L. Futreal, P.A. and DePinho, R.A. 2007. Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. Nature 447:7147:966-971.

Mathonnet, G. Fabian, M.R. Svitkin, Y.V. Parsyan, A. Huck, L. Murata, T. Biffo, S. Merrick, W.C. Darzynkiewicz, E. Pillai, R.S. Filipowicz, W. Duchaine, T.F. and Sonenberg, N. 2007. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science 317:5845:1764-1767.

Maute, R.L. Schneider, C. Sumazin, P. Holmes, A. Califano, A. Basso, K. and Dalla-Favera, R. 2013. tRNA-derived microRNA modulates proliferation and the DNA damage

response and is down-regulated in B cell lymphoma. Proc.Natl.Acad.Sci.U.S.A. 110:4:1404-1409.

Mayr, C. Hemann, M.T. and Bartel, D.P. 2007. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science 315:5818:1576-1579.

McCabe, B.D. Hom, S. Aberle, H. Fetter, R.D. Marques, G. Haerry, T.E. Wan, H. O'Connor, M.B. Goodman, C.S. and Haghighi, A.P. 2004. Highwire regulates presynaptic BMP signaling essential for synaptic growth. Neuron 41:6:891-905.

McNeal, J.E. and Bostwick, D.G. 1986. Intraductal dysplasia: a premalignant lesion of the prostate. Hum.Pathol. 17:1:64-71.

Mehra, R. Han, B. Tomlins, S.A. Wang, L. Menon, A. Wasco, M.J. Shen, R. Montie, J.E. Chinnaiyan, A.M. and Shah, R.B. 2007a. Heterogeneity of TMPRSS2 gene rearrangements in multifocal prostate adenocarcinoma: molecular evidence for an independent group of diseases. Cancer Res. 67:17:7991-7995.

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. and Shah, R.B. 2007b. Comprehensive assessment of TMPRSS2 and ETS family gene aberrations in clinically localized prostate cancer. Mod.Pathol. 20:5:538-544.

Mellinger, G.T. Gleason, D. and Bailar, J.,3rd. 1967. The histology and prognosis of prostatic cancer. J.Urol. 97:2:331-337.

Meng, F. Henson, R. Wehbe-Janek, H. Ghoshal, K. Jacob, S.T. and Patel, T. 2007. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 133:2:647-658.

Mermelstein, F. Yeung, K. Cao, J. Inostroza, J.A. Erdjument-Bromage, H. Eagelson, K. Landsman, D. Levitt, P. Tempst, P. and Reinberg, D. 1996. Requirement of a corepressor for Dr1-mediated repression of transcription. Genes Dev. 10:8:1033-1048.

Mertens, D. Philippen, A. Ruppel, M. Allegra, D. Bhattacharya, N. Tschuch, C. Wolf, S. Idler, I. Zenz, T. and Stilgenbauer, S. 2009. Chronic lymphocytic leukemia and 13q14: miRs and more. Leuk.Lymphoma 50:3:502-505.

Michaud, D.S. Augustsson, K. Rimm, E.B. Stampfer, M.J. Willet, W.C. and Giovannucci, E. 2001. A prospective study on intake of animal products and risk of prostate cancer. Cancer Causes Control 12:6:557-567.

Michor, F. Iwasa, Y. and Nowak, M.A. 2004. Dynamics of cancer progression. Nat.Rev.Cancer. 4:3:197-205.

Miller, G.J. and Cygan, J.M. 1994. Morphology of prostate cancer: the effects of multifocality on histological grade, tumor volume and capsule penetration. J.Urol. 152:5 Pt 2:1709-1713.

Mitsiades, N. Sung, C.C. Schultz, N. Danila, D.C. He, B. Eedunuri, V.K. Fleisher, M. Sander, C. Sawyers, C.L. and Scher, H.I. 2012. Distinct patterns of dysregulated expression of enzymes involved in androgen synthesis and metabolism in metastatic prostate cancer tumors. Cancer Res. 72:23:6142-6152.

Montgomery, R.B. Mostaghel, E.A. Vessella, R. Hess, D.L. Kalhorn, T.F. Higano, C.S. True, L.D. and Nelson, P.S. 2008. Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. Cancer Res. 68:11:4447-4454.

Montironi, R. Mazzucchelli, R. Algaba, F. and Lopez-Beltran, A. 2000. Morphological identification of the patterns of prostatic intraepithelial neoplasia and their importance. J.Clin.Pathol. 53:9:655-665.

Montironi, R. Mazzucchelli, R. Lopez-Beltran, A. Cheng, L. and Scarpelli, M. 2007. Mechanisms of disease: high-grade prostatic intraepithelial neoplasia and other proposed preneoplastic lesions in the prostate. Nat.Clin.Pract.Urol. 4:6:321-332.

Mosquera, J.M. Perner, S. Genega, E.M. Sanda, M. Hofer, M.D. Mertz, K.D. Paris, P.L. Simko, J. Bismar, T.A. Ayala, G. Shah, R.B. Loda, M. and Rubin, M.A. 2008. Characterization of TMPRSS2-ERG fusion high-grade prostatic intraepithelial neoplasia and potential clinical implications. Clin.Cancer Res. 14:11:3380-3385.

Mostafa, M.H. Sheweita, S.A. and O'Connor, P.J. 1999. Relationship between schistosomiasis and bladder cancer. Clin.Microbiol.Rev. 12:1:97-111.

Mourtada-Maarabouni, M. Pickard, M.R. Hedge, V.L. Farzaneh, F. and Williams, G.T. 2009. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. Oncogene 28:2:195-208.

Mukherji, S. Ebert, M.S. Zheng, G.X. Tsang, J.S. Sharp, P.A. and van Oudenaarden, A. 2011. MicroRNAs can generate thresholds in target gene expression. Nat.Genet. 43:9:854-859.

Muller, M. Rink, K. Krause, H. and Miller, K. 2000. PTEN/MMAC1 mutations in prostate cancer. Prostate Cancer.Prostatic Dis. 3:S1:S32.

Murata, T. Takayama, K. Katayama, S. Urano, T. Horie-Inoue, K. Ikeda, K. Takahashi, S. Kawazu, C. Hasegawa, A. Ouchi, Y. Homma, Y. Hayashizaki, Y. and Inoue, S. 2010. miR-148a is an androgen-responsive microRNA that promotes LNCaP prostate cell growth by repressing its target CAND1 expression. Prostate Cancer.Prostatic Dis. 13:4:356-361.

Murphree, A.L. and Benedict, W.F. 1984. Retinoblastoma: clues to human oncogenesis. Science 223:4640:1028-1033.

Musumeci, M. Coppola, V. Addario, A. Patrizii, M. Maugeri-Sacca, M. Memeo, L. Colarossi, C. Francescangeli, F. Biffoni, M. Collura, D. Giacobbe, A. D'Urso, L. Falchi, M. Venneri, M.A. Muto, G. De Maria, R. and Bonci, D. 2011. Control of tumor and microenvironment cross-talk by miR-15a and miR-16 in prostate cancer. Oncogene 30:41:4231-4242.

Nadiminty, N. Tummala, R. Lou, W. Zhu, Y. Shi, X.B. Zou, J.X. Chen, H. Zhang, J. Chen, X. Luo, J. deVere White, R.W. Kung, H.J. Evans, C.P. and Gao, A.C. 2012. MicroRNA let-7c is downregulated in prostate cancer and suppresses prostate cancer growth. PLoS One 7:3:e32832.

Nadler, R.B. Humphrey, P.A. Smith, D.S. Catalona, W.J. and Ratliff, T.L. 1995. Effect of inflammation and benign prostatic hyperplasia on elevated serum prostate specific antigen levels. J.Urol. 154:2 Pt 1:407-413.

Nagai, M.A. Yamamoto, L. Salaorni, S. Pacheco, M.M. Brentani, M.M. Barbosa, E.M. Brentani, R.R. Mazoyer, S. Smith, S.A. and Ponder, B.A. 1994. Detailed deletion mapping of chromosome segment 17q12-21 in sporadic breast tumours. Genes Chromosomes Cancer 11:1:58-62.

Nakata, K. Abrams, B. Grill, B. Goncharov, A. Huang, X. Chisholm, A.D. and Jin, Y. 2005. Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. Cell 120:3:407-420.

Nakayama, M. Bennett, C.J. Hicks, J.L. Epstein, J.I. Platz, E.A. Nelson, W.G. and De Marzo, A.M. 2003. Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. Am.J.Pathol. 163:3:923-933.

Nakayama, M. Gonzalgo, M.L. Yegnasubramanian, S. Lin, X. De Marzo, A.M. and Nelson, W.G. 2004. GSTP1 CpG island hypermethylation as a molecular biomarker for prostate cancer. J.Cell.Biochem. 91:3:540-552.

Nam, R.K. Sugar, L. Yang, W. Srivastava, S. Klotz, L.H. Yang, L.Y. Stanimirovic, A. Encioiu, E. Neill, M. Loblaw, D.A. Trachtenberg, J. Narod, S.A. and Seth, A. 2007. Expression of the TMPRSS2:ERG fusion gene predicts cancer recurrence after surgery for localised prostate cancer. Br.J.Cancer 97:12:1690-1695.

Navon, J.D. Soliman, H. Khonsari, F. and Ahlering, T. 1997. Screening cystoscopy and survival of spinal cord injured patients with squamous cell cancer of the bladder. J.Urol. 157:6:2109-2111.

Nelson, C.P. Kidd, L.C. Sauvageot, J. Isaacs, W.B. De Marzo, A.M. Groopman, J.D. Nelson, W.G. and Kensler, T.W. 2001. Protection against 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine cytotoxicity and DNA adduct formation in human prostate by glutathione S-transferase P1. Cancer Res. 61:1:103-109.

Nevins, J.R. 1998. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ. 9:8:585-593.

Nguyen, D.P. Li, J. and Tewari, A.K. 2014. Inflammation and prostate cancer: the role of interleukin 6 (IL-6). BJU Int. 113:6:986-992.

Nguyen, H.C. Xie, W. Yang, M. Hsieh, C.L. Drouin, S. Lee, G.S. and Kantoff, P.W. 2013. Expression differences of circulating microRNAs in metastatic castration resistant prostate cancer and low-risk, localized prostate cancer. Prostate 73:4:346-354.

Nguyen, L.V. Vanner, R. Dirks, P. and Eaves, C.J. 2012. Cancer stem cells: an evolving concept. Nat.Rev.Cancer. 12:2:133-143.

Northcott, P.A. Nakahara, Y. Wu, X. Feuk, L. Ellison, D.W. Croul, S. Mack, S. Kongkham, P.N. Peacock, J. Dubuc, A. Ra, Y.S. Zilberberg, K. McLeod, J. Scherer, S.W. Sunil Rao, J. Eberhart, C.G. Grajkowska, W. Gillespie, Y. Lach, B. Grundy, R. Pollack, I.F. Hamilton, R.L. Van Meter, T. Carlotti, C.G. Boop, F. Bigner, D. Gilbertson, R.J. Rutka, J.T. and Taylor, M.D. 2009. Multiple recurrent genetic events converge on control of histone lysine methylation in medulloblastoma. Nat.Genet. 41:4:465-472.

Nupponen, N.N. Isola, J. and Visakorpi, T. 2000. Mapping the amplification of EIF3S3 in breast and prostate cancer. Genes Chromosomes Cancer 28:2:203-210.

Nupponen, N.N. Kakkola, L. Koivisto, P. and Visakorpi, T. 1998. Genetic alterations in hormone-refractory recurrent prostate carcinomas. Am.J.Pathol. 153:1:141-148.

O'Brien, C.A. Pollett, A. Gallinger, S. and Dick, J.E. 2007. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445:7123:106-110.

O'Donnell, K.A. Wentzel, E.A. Zeller, K.I. Dang, C.V. and Mendell, J.T. 2005. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435:7043:839-843.

Okada, H. Tsubura, A. Okamura, A. Senzaki, H. Naka, Y. Komatz, Y. and Morii, S. 1992. Keratin profiles in normal/hyperplastic prostates and prostate carcinoma. Virchows Arch.A Pathol.Anat.Histopathol. 421:2:157-161.

Olive, V. Jiang, I. and He, L. 2010. mir-17-92, a cluster of miRNAs in the midst of the cancer network. Int.J.Biochem.Cell Biol. 42:8:1348-1354.

Oostlander, A.E. Meijer, G.A. and Ylstra, B. 2004. Microarray-based comparative genomic hybridization and its applications in human genetics. Clin.Genet. 66:6:488-495.

Oren, M. and Rotter, V. 1999. Introduction: p53--the first twenty years. Cell Mol.Life Sci. 55:1:9-11.

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. J.Urol. 165:4:1329-1334.

Ota, A. Tagawa, H. Karnan, S. Tsuzuki, S. Karpas, A. Kira, S. Yoshida, Y. and Seto, M. 2004. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res. 64:9:3087-3095.

Ouyang, X. DeWeese, T.L. Nelson, W.G. and Abate-Shen, C. 2005. Loss-of-function of Nkx3.1 promotes increased oxidative damage in prostate carcinogenesis. Cancer Res. 65:15:6773-6779.

Owens, M.A. Horten, B.C. and Da Silva, M.M. 2004. HER2 amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. Clin.Breast Cancer. 5:1:63-69.

Ozen, M. Creighton, C.J. Ozdemir, M. and Ittmann, M. 2008. Widespread deregulation of microRNA expression in human prostate cancer. Oncogene 27:12:1788-1793.

Packenham, J.P. Taylor, J.A. Anna, C.H. White, C.M. and Devereux, T.R. 1995. Homozygous deletions but no sequence mutations in coding regions of p15 or p16 in human primary bladder tumors. Mol.Carcinog. 14:3:147-151.

Palanisamy, N. Ateeq, B. Kalyana-Sundaram, S. Pflueger, D. Ramnarayanan, K. Shankar, S. Han, B. Cao, Q. Cao, X. Suleman, K. Kumar-Sinha, C. Dhanasekaran, S.M. Chen, Y.B. Esgueva, R. Banerjee, S. LaFargue, C.J. Siddiqui, J. Demichelis, F. Moeller, P. Bismar, T.A. Kuefer, R. Fullen, D.R. Johnson, T.M. Greenson, J.K. Giordano, T.J. Tan, P. Tomlins, S.A. Varambally, S. Rubin, M.A. Maher, C.A. and Chinnaiyan, A.M. 2010. Rearrangements of the RAF kinase pathway in prostate cancer, gastric cancer and melanoma. Nat.Med. 16:7:793-798.

Pampalona, J. Soler, D. Genesca, A. and Tusell, L. 2010. Telomere dysfunction and chromosome structure modulate the contribution of individual chromosomes in abnormal nuclear morphologies. Mutat.Res. 683:1-2:16-22.

Park, K. Tomlins, S.A. Mudaliar, K.M. Chiu, Y.L. Esgueva, R. Mehra, R. Suleman, K. Varambally, S. Brenner, J.C. MacDonald, T. Srivastava, A. Tewari, A.K. Sathyanarayana, U. Nagy, D. Pestano, G. Kunju, L.P. Demichelis, F. Chinnaiyan, A.M.

and Rubin, M.A. 2010. Antibody-based detection of ERG rearrangement-positive prostate cancer. Neoplasia 12:7:590-598.

Parsons, J.K. Gage, W.R. Nelson, W.G. and De Marzo, A.M. 2001. P63 Protein Expression is Rare in Prostate Adenocarcinoma: Implications for Cancer Diagnosis and Carcinogenesis. Urology 58:4:619-624.

Patrawala, L. Calhoun-Davis, T. Schneider-Broussard, R. and Tang, D.G. 2007. Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+alpha2beta1+ cell population is enriched in tumor-initiating cells. Cancer Res. 67:14:6796-6805.

Pearson, P.L. and Van der Luijt, R.B. 1998. The genetic analysis of cancer. J.Intern.Med. 243:6:413-417.

Peltier, H.J. and Latham, G.J. 2008. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA 14:5:844-852.

Petersen, C.P. Bordeleau, M.E. Pelletier, J. and Sharp, P.A. 2006. Short RNAs repress translation after initiation in mammalian cells. Mol.Cell 21:4:533-542.

Petrovics, G. Liu, A. Shaheduzzaman, S. Furusato, B. Sun, C. Chen, Y. Nau, M. Ravindranath, L. Chen, Y. Dobi, A. Srikantan, V. Sesterhenn, I.A. McLeod, D.G. Vahey, M. Moul, J.W. and Srivastava, S. 2005. Frequent overexpression of ETS-related gene-1 (ERG1) in prostate cancer transcriptome. Oncogene 24:23:3847-3852.

Piccirillo, S.G. Reynolds, B.A. Zanetti, N. Lamorte, G. Binda, E. Broggi, G. Brem, H. Olivi, A. Dimeco, F. and Vescovi, A.L. 2006. Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. Nature 444:7120:761-765.

Pinkel, D. Segraves, R. Sudar, D. Clark, S. Poole, I. Kowbel, D. Collins, C. Kuo, W.L. Chen, C. Zhai, Y. Dairkee, S.H. Ljung, B.M. Gray, J.W. and Albertson, D.G. 1998. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat.Genet. 20:2:207-211.

Pomerantz, M.M. Beckwith, C.A. Regan, M.M. Wyman, S.K. Petrovics, G. Chen, Y. Hawksworth, D.J. Schumacher, F.R. Mucci, L. Penney, K.L. Stampfer, M.J. Chan, J.A. Ardlie, K.G. Fritz, B.R. Parkin, R.K. Lin, D.W. Dyke, M. Herman, P. Lee, S. Oh, W.K. Kantoff, P.W. Tewari, M. McLeod, D.G. Srivastava, S. and Freedman, M.L. 2009. Evaluation of the 8q24 prostate cancer risk locus and MYC expression. Cancer Res. 69:13:5568-5574.

Porkka, K.P. Ogg, E.L. Saramaki, O.R. Vessella, R.L. Pukkila, H. Lahdesmaki, H. van Weerden, W.M. Wolf, M. Kallioniemi, O.P. Jenster, G. and Visakorpi, T. 2011. The

miR-15a-miR-16-1 locus is homozygously deleted in a subset of prostate cancers. Genes Chromosomes Cancer 50:7:499-509.

Porkka, K.P. Pfeiffer, M.J. Waltering, K.K. Vessella, R.L. Tammela, T.L. and Visakorpi, T. 2007. MicroRNA expression profiling in prostate cancer. Cancer Res. 67:13:6130-6135.

Porkka, K.P. Tammela, T.L. Vessella, R.L. and Visakorpi, T. 2004. RAD21 and KIAA0196 at 8q24 are amplified and overexpressed in prostate cancer. Genes Chromosomes Cancer 39:1:1-10.

Pruitt, K. and Der, C.J. 2001. Ras and Rho regulation of the cell cycle and oncogenesis. Cancer Lett. 171:1:1-10.

Qian, J. Bostwick, D.G. Takahashi, S. Borell, T.J. Herath, J.F. Lieber, M.M. and Jenkins, R.B. 1995. Chromosomal anomalies in prostatic intraepithelial neoplasia and carcinoma detected by fluorescence in situ hybridization. Cancer Res. 55:22:5408-5414.

Rahman, M.M. Qian, Z.R. Wang, E.L. Sultana, R. Kudo, E. Nakasono, M. Hayashi, T. Kakiuchi, S. and Sano, T. 2009. Frequent overexpression of HMGA1 and 2 in gastroenteropancreatic neuroendocrine tumours and its relationship to let-7 downregulation. Br.J.Cancer 100:3:501-510.

Rajewsky, N. 2006. microRNA target predictions in animals. Nat.Genet. 38 Suppl:S8-13.

Ramos, A.H. Dutt, A. Mermel, C. Perner, S. Cho, J. Lafargue, C.J. Johnson, L.A. Stiedl, A.C. Tanaka, K.E. Bass, A.J. Barretina, J. Weir, B.A. Beroukhim, R. Thomas, R.K. Minna, J.D. Chirieac, L.R. Lindeman, N.I. Giordano, T. Beer, D.G. Wagner, P. Wistuba, I.I. Rubin, M.A. and Meyerson, M. 2009. Amplification of chromosomal segment 4q12 in non-small cell lung cancer. Cancer.Biol.Ther. 8:21:2042-2050.

Ramos-Montoya, A. Lamb, A.D. Russell, R. Carroll, T. Jurmeister, S. Galeano-Dalmau, N. Massie, C.E. Boren, J. Bon, H. Theodorou, V. Vias, M. Shaw, G.L. Sharma, N.L. Ross-Adams, H. Scott, H.E. Vowler, S.L. Howat, W.J. Warren, A.Y. Wooster, R.F. Mills, I.G. and Neal, D.E. 2014. HES6 drives a critical AR transcriptional programme to induce castration-resistant prostate cancer through activation of an E2F1-mediated cell cycle network. EMBO Mol.Med. 6:5:651-661.

Rane, J.K. Droop, A.P. Pellacani, D. Polson, E.S. Simms, M.S. Collins, A.T. Caves, L.S. and Maitland, N.J. 2014. Conserved two-step regulatory mechanism of human epithelial differentiation. Stem Cell.Reports 2:2:180-188.

Rauhala, H.E. Jalava, S.E. Isotalo, J. Bracken, H. Lehmusvaara, S. Tammela, T.L. Oja, H. and Visakorpi, T. 2010. miR-193b is an epigenetically regulated putative tumor suppressor in prostate cancer. Int.J.Cancer 127:6:1363-1372.

Reeves, R. Edberg, D.D. and Li, Y. 2001. Architectural transcription factor HMGI(Y) promotes tumor progression and mesenchymal transition of human epithelial cells. Mol.Cell.Biol. 21:2:575-594.

Reinhart, B.J. Slack, F.J. Basson, M. Pasquinelli, A.E. Bettinger, J.C. Rougvie, A.E. Horvitz, H.R. and Ruvkun, G. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403:6772:901-906.

Reuter, V.E. 2006. The pathology of bladder cancer. Urology 67:3 Suppl 1:11-7; discussion 17-8.

Reuther, G.W. and Der, C.J. 2000. The Ras branch of small GTPases: Ras family members don't fall far from the tree. Curr.Opin.Cell Biol. 12:2:157-165.

Reya, T. Morrison, S.J. Clarke, M.F. and Weissman, I.L. 2001. Stem cells, cancer, and cancer stem cells. Nature 414:6859:105-111.

Ribas, J. and Lupold, S.E. 2010. The transcriptional regulation of miR-21, its multiple transcripts, and their implication in prostate cancer. Cell.Cycle 9:5:923-929.

Ribas, J. Ni, X. Haffner, M. Wentzel, E.A. Salmasi, A.H. Chowdhury, W.H. Kudrolli, T.A. Yegnasubramanian, S. Luo, J. Rodriguez, R. Mendell, J.T. and Lupold, S.E. 2009. miR-21: an androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. Cancer Res. 69:18:7165-7169.

Ricci-Vitiani, L. Lombardi, D.G. Pilozzi, E. Biffoni, M. Todaro, M. Peschle, C. and De Maria, R. 2007. Identification and expansion of human colon-cancer-initiating cells. Nature 445:7123:111-115.

Richardson, G.D. Robson, C.N. Lang, S.H. Neal, D.E. Maitland, N.J. and Collins, A.T. 2004. CD133, a novel marker for human prostatic epithelial stem cells. J.Cell.Sci. 117:Pt 16:3539-3545.

Richter, J. Beffa, L. Wagner, U. Schraml, P. Gasser, T.C. Moch, H. Mihatsch, M.J. and Sauter, G. 1998. Patterns of chromosomal imbalances in advanced urinary bladder cancer detected by comparative genomic hybridization. Am.J.Pathol. 153:5:1615-1621.

Richter, J. Jiang, F. Gorog, J.P. Sartorius, G. Egenter, C. Gasser, T.C. Moch, H. Mihatsch, M.J. and Sauter, G. 1997. Marked genetic differences between stage pTa and stage pT1 papillary bladder cancer detected by comparative genomic hybridization. Cancer Res. 57:14:2860-2864.

Ringner, M. 2008. What is principal component analysis? Nat.Biotechnol. 26:3:303-304.

Roush, S. and Slack, F.J. 2008. The let-7 family of microRNAs. Trends Cell Biol. 18:10:505-516.

Rubin, I. and Yarden, Y. 2001. The basic biology of HER2. Ann.Oncol. 12 Suppl 1:S3-8.

Rubin, M.A. Maher, C.A. and Chinnaiyan, A.M. 2011. Common gene rearrangements in prostate cancer. J.Clin.Oncol. 29:27:3659-3668.

Ryan, C.J. and Tindall, D.J. 2011. Androgen receptor rediscovered: the new biology and targeting the androgen receptor therapeutically. J.Clin.Oncol. 29:27:3651-3658.

Rybak, A.P. and Tang, D. 2013. SOX2 plays a critical role in EGFR-mediated self-renewal of human prostate cancer stem-like cells. Cell.Signal. 25:12:2734-2742.

Saini, S. Majid, S. Yamamura, S. Tabatabai, L. Suh, S.O. Shahryari, V. Chen, Y. Deng, G. Tanaka, Y. and Dahiya, R. 2011. Regulatory Role of mir-203 in Prostate Cancer Progression and Metastasis. Clin.Cancer Res. 17:16:5287-5298.

Sampson, V.B. Rong, N.H. Han, J. Yang, Q. Aris, V. Soteropoulos, P. Petrelli, N.J. Dunn, S.P. and Krueger, L.J. 2007. MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. Cancer Res. 67:20:9762-9770.

Sanchez-Carbayo, M. Socci, N.D. Lozano, J. Saint, F. and Cordon-Cardo, C. 2006. Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. J.Clin.Oncol. 24:5:778-789.

Sandberg, A.A. 2002. Cytogenetics and molecular genetics of bladder cancer: a personal view. Am.J.Med.Genet. 115:3:173-182.

Sandberg, A.A. 1992. Chromosome changes in early bladder neoplasms. J.Cell.Biochem.Suppl. 16I:76-79.

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. Clin.Cancer Res. 14:11:3395-3400.

Saramaki, O.R. Porkka, K.P. Vessella, R.L. and Visakorpi, T. 2006. Genetic aberrations in prostate cancer by microarray analysis. Int.J.Cancer 119:6:1322-1329.

Savinainen, K.J. Helenius, M.A. Lehtonen, H.J. and Visakorpi, T. 2006. Overexpression of EIF3S3 promotes cancer cell growth. Prostate 66:11:1144-1150.

Savitsky, K. Bar-Shira, A. Gilad, S. Rotman, G. Ziv, Y. Vanagaite, L. Tagle, D.A. Smith, S. Uziel, T. Sfez, S. Ashkenazi, M. Pecker, I. Frydman, M. Harnik, R. Patanjali, S.R. Simmons, A. Clines, G.A. Sartiel, A. Gatti, R.A. Chessa, L. Sanal, O. Lavin, M.F.

Jaspers, N.G. Taylor, A.M. Arlett, C.F. Miki, T. Weissman, S.M. Lovett, M. Collins, F.S. and Shiloh, Y. 1995. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science 268:5218:1749-1753.

Sawhney, R. Bourgeois, D. and Chaudhary, U.B. 2006. Neo-adjuvant chemotherapy for muscle-invasive bladder cancer: a look ahead. Ann.Oncol. 17:9:1360-1369.

Saxena, S. Jonsson, Z.O. and Dutta, A. 2003. Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. J.Biol.Chem. 278:45:44312-44319.

Scher, H.I. Buchanan, G. Gerald, W. Butler, L.M. and Tilley, W.D. 2004. Targeting the androgen receptor: improving outcomes for castration-resistant prostate cancer. Endocr.Relat.Cancer 11:3:459-476.

Scher, H.I. and Sawyers, C.L. 2005. Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. J.Clin.Oncol. 23:32:8253-8261.

Schmidt, E.V. 1999. The role of c-myc in cellular growth control. Oncogene 18:19:2988-2996.

Scholich, K. Pierre, S. and Patel, T.B. 2001. Protein associated with Myc (PAM) is a potent inhibitor of adenylyl cyclases. J.Biol.Chem. 276:50:47583-47589.

Schroder, F.H. Hugosson, J. Roobol, M.J. Tammela, T.L. Ciatto, S. Nelen, V. Kwiatkowski, M. Lujan, M. Lilja, H. Zappa, M. Denis, L.J. Recker, F. Berenguer, A. Maattanen, L. Bangma, C.H. Aus, G. Villers, A. Rebillard, X. van der Kwast, T. Blijenberg, B.G. Moss, S.M. de Koning, H.J. Auvinen, A. and ERSPC Investigators. 2009. Screening and prostate-cancer mortality in a randomized European study. N.Engl.J.Med. 360:13:1320-1328.

Seggerson, K. Tang, L. and Moss, E.G. 2002. Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Dev.Biol. 243:2:215-225.

Sempere, L.F. Freemantle, S. Pitha-Rowe, I. Moss, E. Dmitrovsky, E. and Ambros, V. 2004. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol. 5:3:R13.

Sgarra, R. Rustighi, A. Tessari, M.A. Di Bernardo, J. Altamura, S. Fusco, A. Manfioletti, G. and Giancotti, V. 2004. Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer. FEBS Lett. 574:1-3:1-8.

Shackleton, M. Quintana, E. Fearon, E.R. and Morrison, S.J. 2009. Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell 138:5:822-829.

Shelley, M.D. Kynaston, H. Court, J. Wilt, T.J. Coles, B. Burgon, K. and Mason, M.D. 2001. A systematic review of intravesical bacillus Calmette-Guerin plus transurethral resection vs transurethral resection alone in Ta and T1 bladder cancer. BJU Int. 88:3:209-216.

Shi, L. Chen, J. Yang, J. Pan, T. Zhang, S. and Wang, Z. 2010. MiR-21 protected human glioblastoma U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase-3 activity. Brain Res. 1352:255-264.

Shi, S. Lu, Y. Qin, Y. Li, W. Cheng, H. Xu, Y. Xu, J. Long, J. Liu, L. Liu, C. and Yu, X. 2014. miR-1247 is correlated with prognosis of pancreatic cancer and inhibits cell proliferation by targeting neuropilins. Curr.Mol.Med. 14:3:316-327.

Shi, X.B. Xue, L. Ma, A.H. Tepper, C.G. Kung, H.J. and White, R.W. 2011. miR-125b promotes growth of prostate cancer xenograft tumor through targeting pro-apoptotic genes. Prostate 71:5:538-549.

Shi, X.B. Xue, L. Yang, J. Ma, A.H. Zhao, J. Xu, M. Tepper, C.G. Evans, C.P. Kung, H.J. and deVere White, R.W. 2007. An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth of prostate cancer cells. Proc.Natl.Acad.Sci.U.S.A. 104:50:19983-19988.

Shiloh, Y. 2003. ATM and related protein kinases: safeguarding genome integrity. Nat.Rev.Cancer. 3:3:155-168.

Shokeir, A.A. 2004. Squamous cell carcinoma of the bladder: pathology, diagnosis and treatment. BJU Int. 93:2:216-220.

Shore, N. 2014. Management of early-stage prostate cancer. Am.J.Manag.Care 20:12 Suppl:S260-72.

Sidransky, D. Von Eschenbach, A. Tsai, Y.C. Jones, P. Summerhayes, I. Marshall, F. Paul, M. Green, P. Hamilton, S.R. and Frost, P. 1991. Identification of p53 gene mutations in bladder cancers and urine samples. Science 252:5006:706-709.

Signoretti, S. and Loda, M. 2007. Prostate stem cells: from development to cancer. Semin.Cancer Biol. 17:3:219-224.

Signoretti, S. Waltregny, D. Dilks, J. Isaac, B. Lin, D. Garraway, L. Yang, A. Montironi, R. McKeon, F. and Loda, M. 2000. P63 is a Prostate Basal Cell Marker and is Required for Prostate Development. Am.J.Pathol. 157:6:1769-1775.

Simon, R. Burger, H. Brinkschmidt, C. Bocker, W. Hertle, L. and Terpe, H.J. 1998. Chromosomal aberrations associated with invasion in papillary superficial bladder cancer. J.Pathol. 185:4:345-351.

Singh, S.K. Hawkins, C. Clarke, I.D. Squire, J.A. Bayani, J. Hide, T. Henkelman, R.M. Cusimano, M.D. and Dirks, P.B. 2004. Identification of human brain tumour initiating cells. Nature 432:7015:396-401.

Sinha, R. Park, Y. Graubard, B.I. Leitzmann, M.F. Hollenbeck, A. Schatzkin, A. and Cross, A.J. 2009. Meat and meat-related compounds and risk of prostate cancer in a large prospective cohort study in the United States. Am.J.Epidemiol. 170:9:1165-1177.

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. Saad, F. Bismar, T.A. and Squire, J.A. 2009. PTEN genomic deletion is associated with p-Akt and AR signalling in poorer outcome, hormone refractory prostate cancer. J.Pathol. 218:4:505-513.

Slamon, D.J. Clark, G.M. Wong, S.G. Levin, W.J. Ullrich, A. and McGuire, W.L. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:4785:177-182.

Smeets, W. Pauwels, R. Laarakkers, L. Debruyne, F. and Geraedts, J. 1987. Chromosomal analysis of bladder cancer. III. Nonrandom alterations. Cancer Genet.Cytogenet. 29:1:29-41.

Sobala, A. and Hutvagner, G. 2013. Small RNAs derived from the 5' end of tRNA can inhibit protein translation in human cells. RNA Biol. 10:4:553-563.

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 45:7:717-719.

Sotelo, J. Esposito, D. Duhagon, M.A. Banfield, K. Mehalko, J. Liao, H. Stephens, R.M. Harris, T.J. Munroe, D.J. and Wu, X. 2010. Long-range enhancers on 8q24 regulate c-Myc. Proc.Natl.Acad.Sci.U.S.A. 107:7:3001-3005.

Squire, J.A. 2009. TMPRSS2-ERG and PTEN loss in prostate cancer. Nat.Genet. 41:5:509-510.

Srikantan, S. Abdelmohsen, K. Lee, E.K. Tominaga, K. Subaran, S.S. Kuwano, Y. Kulshrestha, R. Panchakshari, R. Kim, H.H. Yang, X. Martindale, J.L. Marasa, B.S. Kim, M.M. Wersto, R.P. Indig, F.E. Chowdhury, D. and Gorospe, M. 2011. Translational control of TOP2A influences doxorubicin efficacy. Mol.Cell.Biol. 31:18:3790-3801.

Starczynski, J. Atkey, N. Connelly, Y. O'Grady, T. Campbell, F.M. di Palma, S. Wencyk, P. Jasani, B. Gandy, M. Bartlett, J.M. and UKNEQAS. 2012. HER2 gene amplification in breast cancer: a rogues' gallery of challenging diagnostic cases: UKNEQAS interpretation guidelines and research recommendations. Am.J.Clin.Pathol. 137:4:595-605.

Steinberg, G.D. Carter, B.S. Beaty, T.H. Childs, B. and Walsh, P.C. 1990. Family history and the risk of prostate cancer. Prostate 17:4:337-347.

Stephens, P.J. Greenman, C.D. Fu, B. Yang, F. Bignell, G.R. Mudie, L.J. Pleasance, E.D. Lau, K.W. Beare, D. Stebbings, L.A. McLaren, S. Lin, M.L. McBride, D.J. Varela, I. Nik-Zainal, S. Leroy, C. Jia, M. Menzies, A. Butler, A.P. Teague, J.W. Quail, M.A. Burton, J. Swerdlow, H. Carter, N.P. Morsberger, L.A. Iacobuzio-Donahue, C. Follows, G.A. Green, A.R. Flanagan, A.M. Stratton, M.R. Futreal, P.A. and Campbell, P.J. 2011. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell 144:1:27-40.

Stern, D.F. 2000. Tyrosine kinase signalling in breast cancer: ErbB family receptor tyrosine kinases. Breast Cancer Res. 2:3:176-183.

Stewart, S.L. Cardinez, C.J. Richardson, L.C. Norman, L. Kaufmann, R. Pechacek, T.F. Thompson, T.D. Weir, H.K. Sabatino, S.A. and Centers for Disease Control and Prevention (CDC). 2008. Surveillance for cancers associated with tobacco use--United States, 1999-2004. MMWR Surveill.Summ. 57:8:1-33.

Stratton, M.R. Campbell, P.J. and Futreal, P.A. 2009. The cancer genome. Nature 458:7239:719-724.

Stroschein, S.L. Wang, W. Zhou, S. Zhou, Q. and Luo, K. 1999. Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein. Science 286:5440:771-774.

Suh, S.O. Chen, Y. Zaman, M.S. Hirata, H. Yamamura, S. Shahryari, V. Liu, J. Tabatabai, Z.L. Kakar, S. Deng, G. Tanaka, Y. and Dahiya, R. 2011. MicroRNA-145 is regulated by DNA methylation and p53 gene mutation in prostate cancer. Carcinogenesis 32:5:772-778.

Sun, J. Liu, W. Adams, T.S. Sun, J. Li, X. Turner, A.R. Chang, B. Kim, J.W. Zheng, S.L. Isaacs, W.B. and Xu, J. 2007. DNA copy number alterations in prostate cancers: a combined analysis of published CGH studies. Prostate 67:7:692-700.

Sung, S.Y. Liao, C.H. Wu, H.P. Hsiao, W.C. Wu, I.H. Jinpu Yu Lin, S.H. and Hsieh, C.L. 2013. Loss of let-7 microRNA upregulates IL-6 in bone marrow-derived mesenchymal stem cells triggering a reactive stromal response to prostate cancer. PLoS One 8:8:e71637.

Suzuki, H. Sato, N. Watabe, Y. Masai, M. Seino, S. and Shimazaki, J. 1993. Androgen receptor gene mutations in human prostate cancer. J.Steroid Biochem.Mol.Biol. 46:6:759-765.

Tam, W. Ben-Yehuda, D. and Hayward, W.S. 1997. bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. Mol.Cell.Biol. 17:3:1490-1502.

Tanzer, A. and Stadler, P.F. 2004. Molecular evolution of a microRNA cluster. J.Mol.Biol. 339:2:327-335.

Taplin, M.E. Bubley, G.J. Ko, Y.J. Small, E.J. Upton, M. Rajeshkumar, B. and Balk, S.P. 1999. Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res. 59:11:2511-2515.

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. N.Engl.J.Med. 332:21:1393-1398.

Tavtigian, S.V. Simard, J. Teng, D.H. Abtin, V. Baumgard, M. Beck, A. Camp, N.J. Carillo, A.R. Chen, Y. Dayananth, P. Desrochers, M. Dumont, M. Farnham, J.M. Frank, D. Frye, C. Ghaffari, S. Gupte, J.S. Hu, R. Iliev, D. Janecki, T. Kort, E.N. Laity, K.E. Leavitt, A. Leblanc, G. McArthur-Morrison, J. Pederson, A. Penn, B. Peterson, K.T. Reid, J.E. Richards, S. Schroeder, M. Smith, R. Snyder, S.C. Swedlund, B. Swensen, J. Thomas, A. Tranchant, M. Woodland, A.M. Labrie, F. Skolnick, M.H. Neuhausen, S. Rommens, J. and Cannon-Albright, L.A. 2001. A candidate prostate cancer susceptibility gene at chromosome 17p. Nat.Genet. 27:2:172-180.

Taylor, B.S. Schultz, N. Hieronymus, H. Gopalan, A. Xiao, Y. Carver, B.S. Arora, V.K. Kaushik, P. Cerami, E. Reva, B. Antipin, Y. Mitsiades, N. Landers, T. Dolgalev, I. Major, J.E. Wilson, M. Socci, N.D. Lash, A.E. Heguy, A. Eastham, J.A. Scher, H.I. Reuter, V.E. Scardino, P.T. Sander, C. Sawyers, C.L. and Gerald, W.L. 2010. Integrative genomic profiling of human prostate cancer. Cancer.Cell. 18:1:11-22.

Thompson, D.M. Lu, C. Green, P.J. and Parker, R. 2008. tRNA cleavage is a conserved response to oxidative stress in eukaryotes. RNA 14:10:2095-2103.

Thompson, D.M. and Parker, R. 2009. Stressing out over tRNA cleavage. Cell 138:2:215-219.

Thomsen, M.K. Ambroisine, L. Wynn, S. Cheah, K.S. Foster, C.S. Fisher, G. Berney, D.M. Moller, H. Reuter, V.E. Scardino, P. Cuzick, J. Ragavan, N. Singh, P.B. Martin, F.L. Butler, C.M. Cooper, C.S. Swain, A. and Transatlantic Prostate Group. 2010. SOX9 elevation in the prostate promotes proliferation and cooperates with PTEN loss to drive tumor formation. Cancer Res. 70:3:979-987.

Thorstenson, Y.R. Roxas, A. Kroiss, R. Jenkins, M.A. Yu, K.M. Bachrich, T. Muhr, D. Wayne, T.L. Chu, G. Davis, R.W. Wagner, T.M. and Oefner, P.J. 2003. Contributions of ATM mutations to familial breast and ovarian cancer. Cancer Res. 63:12:3325-3333.

Tilley, W.D. Buchanan, G. Hickey, T.E. and Bentel, J.M. 1996. Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin.Cancer Res. 2:2:277-285.

Titen, S.W. and Golic, K.G. 2008. Telomere loss provokes multiple pathways to apoptosis and produces genomic instability in Drosophila melanogaster. Genetics 180:4:1821-1832.

Toffoli, G. Viel, A. Tumiotto, L. Giannini, F. Volpe, R. Quaia, M. and Boiocchi, M. 1992. Expression of glutathione-S-transferase-pi in human tumours. Eur.J.Cancer 28A:8-9:1441-1446.

Tomasetti, C. and Vogelstein, B. 2015. Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. Science 347:6217:78-81.

Tomlins, S.A. Mehra, R. Rhodes, D.R. Cao, X. Wang, L. Dhanasekaran, S.M. Kalyana-Sundaram, S. Wei, J.T. Rubin, M.A. Pienta, K.J. Shah, R.B. and Chinnaiyan, A.M. 2007. Integrative molecular concept modeling of prostate cancer progression. Nat.Genet. 39:1:41-51.

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. and Chinnaiyan, A.M. 2006a. TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. Cancer Res. 66:7:3396-3400.

Tomlins, S.A. Rhodes, D.R. Perner, S. Dhanasekaran, S.M. Mehra, R. Sun, X.W. Varambally, S. Cao, X. Tchinda, J. Kuefer, R. Lee, C. Montie, J.E. Shah, R.B. Pienta, K.J. Rubin, M.A. and Chinnaiyan, A.M. 2005. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:5748:644-648.

Tomlins, S.A. Rubin, M.A. and Chinnaiyan, A.M. 2006b. Integrative biology of prostate cancer progression. Annu.Rev.Pathol. 1:243-271.

Torre, L.A. Bray, F. Siegel, R.L. Ferlay, J. Lortet-Tieulent, J. and Jemal, A. 2015. Global cancer statistics, 2012. CA Cancer.J.Clin. 65:2:87-108.

Tsujimoto, Y. Cossman, J. Jaffe, E. and Croce, C.M. 1985. Involvement of the bcl-2 gene in human follicular lymphoma. Science 228:4706:1440-1443.

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. Mod.Pathol. 20:9:921-928.

Tubio, J.M. and Estivill, X. 2011. Cancer: When catastrophe strikes a cell. Nature 470:7335:476-477.

Tucci, P. Agostini, M. Grespi, F. Markert, E.K. Terrinoni, A. Vousden, K.H. Muller, P.A. Dotsch, V. Kehrloesser, S. Sayan, B.S. Giaccone, G. Lowe, S.W. Takahashi, N. Vandenabeele, P. Knight, R.A. Levine, A.J. and Melino, G. 2012. Loss of p63 and its microRNA-205 target results in enhanced cell migration and metastasis in prostate cancer. Proc.Natl.Acad.Sci.U.S.A. 109:38:15312-15317.

Tzai, T.S. Chen, H.H. Chan, S.H. Ho, C.L. Tsai, Y.S. Cheng, H.L. Dai, Y.C. Lin, J.S. Yang, W.H. and Chow, N.H. 2003. Clinical significance of allelotype profiling for urothelial carcinoma. Urology 62:2:378-384.

Uzgare, A.R. Xu, Y. and Isaacs, J.T. 2004. In vitro culturing and characteristics of transit amplifying epithelial cells from human prostate tissue. J.Cell.Biochem. 91:1:196-205.

Van Den Berg, C. Guan, X.Y. Von Hoff, D. Jenkins, R. Bittner Griffin, C. Kallioniemi, O. Visakorpi McGill and Herath, J. 1995. DNA sequence amplification in human prostate cancer identified by chromosome microdissection: potential prognostic implications. Clin.Cancer Res. 1:1:11-18.

van den Bosch, S. and Alfred Witjes, J. 2011. Long-term cancer-specific survival in patients with high-risk, non-muscle-invasive bladder cancer and tumour progression: a systematic review. Eur.Urol. 60:3:493-500.

van der Meijden, A.P. 1998. Bladder cancer. BMJ 317:7169:1366-1369.

van Leenders, G.J. and Schalken, J.A. 2003. Epithelial cell differentiation in the human prostate epithelium: implications for the pathogenesis and therapy of prostate cancer. Crit.Rev.Oncol.Hematol. 46 Suppl:S3-10.

Varambally, S. Cao, Q. Mani, R.S. Shankar, S. Wang, X. Ateeq, B. Laxman, B. Cao, X. Jing, X. Ramnarayanan, K. Brenner, J.C. Yu, J. Kim, J.H. Han, B. Tan, P. Kumar-Sinha, C. Lonigro, R.J. Palanisamy, N. Maher, C.A. and Chinnaiyan, A.M. 2008. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science 322:5908:1695-1699.

Varambally, S. Dhanasekaran, S.M. Zhou, M. Barrette, T.R. Kumar-Sinha, C. Sanda, M.G. Ghosh, D. Pienta, K.J. Sewalt, R.G. Otte, A.P. Rubin, M.A. and Chinnaiyan, A.M. 2002. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419:6907:624-629.

Veltman, J.A. Fridlyand, J. Pejavar, S. Olshen, A.B. Korkola, J.E. DeVries, S. Carroll, P. Kuo, W.L. Pinkel, D. Albertson, D. Cordon-Cardo, C. Jain, A.N. and Waldman, F.M. 2003. Array-based comparative genomic hybridization for genome-wide screening of DNA copy number in bladder tumors. Cancer Res. 63:11:2872-2880.

Veltman, J.A. Schoenmakers, E.F. Eussen, B.H. Janssen, I. Merkx, G. van Cleef, B. van Ravenswaaij, C.M. Brunner, H.G. Smeets, D. and van Kessel, A.G. 2002. High-throughput analysis of subtelomeric chromosome rearrangements by use of array-based comparative genomic hybridization. Am.J.Hum.Genet. 70:5:1269-1276.

Verdoodt, B. Neid, M. Vogt, M. Kuhn, V. Liffers, S.T. Palisaar, R.J. Noldus, J. Tannapfel, A. and Mirmohammadsadegh, A. 2013. MicroRNA-205, a novel regulator of the anti-apoptotic protein Bcl2, is downregulated in prostate cancer. Int.J.Oncol. 43:1:307-314.

Villanueva, C.M. Fernandez, F. Malats, N. Grimalt, J.O. and Kogevinas, M. 2003. Meta-analysis of studies on individual consumption of chlorinated drinking water and bladder cancer. J.Epidemiol.Community Health 57:3:166-173.

Villers, A. McNeal, J.E. Freiha, F.S. and Stamey, T.A. 1992. Multiple cancers in the prostate. Morphologic features of clinically recognized versus incidental tumors. Cancer 70:9:2313-2318.

Visakorpi, T. Hyytinen, E. Koivisto, P. Tanner, M. Keinanen, R. Palmberg, C. Palotie, A. Tammela, T. Isola, J. and Kallioniemi, O.P. 1995a. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat.Genet. 9:4:401-406.

Visakorpi, T. Kallioniemi, A.H. Syvanen, A.C. Hyytinen, E.R. Karhu, R. Tammela, T. Isola, J.J. and Kallioniemi, O.P. 1995b. Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. Cancer Res. 55:2:342-347.

Visvader, J.E. 2011. Cells of origin in cancer. Nature 469:7330:314-322.

Visvader, J.E. and Lindeman, G.J. 2008. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat.Rev.Cancer. 8:10:755-768.

Vita, M. and Henriksson, M. 2006. The Myc oncoprotein as a therapeutic target for human cancer. Semin.Cancer Biol. 16:4:318-330.

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 Res. 56:10:2411-2416.

Vogelstein, B. Lane, D. and Levine, A.J. 2000. Surfing the p53 network. Nature 408:6810:307-310.

Volinia, S. Calin, G.A. Liu, C.G. Ambs, S. Cimmino, A. Petrocca, F. Visone, R. Iorio, M. Roldo, C. Ferracin, M. Prueitt, R.L. Yanaihara, N. Lanza, G. Scarpa, A. Vecchione, A. Negrini, M. Harris, C.C. and Croce, C.M. 2006. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc.Natl.Acad.Sci.U.S.A. 103:7:2257-2261.

Voorter, C. Joos, S. Bringuier, P.P. Vallinga, M. Poddighe, P. Schalken, J. du Manoir, S. Ramaekers, F. Lichter, P. and Hopman, A. 1995. Detection of chromosomal imbalances in transitional cell carcinoma of the bladder by comparative genomic hybridization. Am.J.Pathol. 146:6:1341-1354.

Vousden, K.H. and Lu, X. 2002. Live or let die: the cell's response to p53. Nat.Rev.Cancer. 2:8:594-604.

Walter, B.A. Valera, V.A. Pinto, P.A. and Merino, M.J. 2013. Comprehensive microRNA Profiling of Prostate Cancer. J.Cancer. 4:5:350-357.

Wang, G. Lunardi, A. Zhang, J. Chen, Z. Ala, U. Webster, K.A. Tay, Y. Gonzalez-Billalabeitia, E. Egia, A. Shaffer, D.R. Carver, B. Liu, X.S. Taulli, R. Kuo, W.P. Nardella, C. Signoretti, S. Cordon-Cardo, C. Gerald, W.L. and Pandolfi, P.P. 2013. Zbtb7a suppresses prostate cancer through repression of a Sox9-dependent pathway for cellular senescence bypass and tumor invasion. Nat.Genet. 45:7:739-746.

Wang, H. Leav, I. Ibaragi, S. Wegner, M. Hu, G.F. Lu, M.L. Balk, S.P. and Yuan, X. 2008. SOX9 is expressed in human fetal prostate epithelium and enhances prostate cancer invasion. Cancer Res. 68:6:1625-1630.

Wang, J.C. and Dick, J.E. 2005. Cancer stem cells: lessons from leukemia. Trends Cell Biol. 15:9:494-501.

Wang, M.R. Perissel, B. Taillandier, J. Kemeny, J.L. Fonck, Y. Lautier, A. Benkhalifa, M. and Malet, P. 1994. Nonrandom changes of chromosome 10 in bladder cancer. Detection by FISH to interphase nuclei. Cancer Genet.Cytogenet. 73:1:8-10.

Wang, P. Kim, Y. Pollack, J. Narasimhan, B. and Tibshirani, R. 2005. A method for calling gains and losses in array CGH data. Biostatistics 6:1:45-58.

Wang, X. Cao, L. Wang, Y. Wang, X. Liu, N. and You, Y. 2012. Regulation of let-7 and its target oncogenes (Review). Oncol.Lett. 3:5:955-960.

Wang, X. Kruithof-de Julio, M. Economides, K.D. Walker, D. Yu, H. Halili, M.V. Hu, Y.P. Price, S.M. Abate-Shen, C. and Shen, M.M. 2009. A luminal epithelial stem cell that is a cell of origin for prostate cancer. Nature 461:7263:495-500.

Wang, Y. Hayward, S. Cao, M. Thayer, K. and Cunha, G. 2001. Cell differentiation lineage in the prostate. Differentiation 68:4-5:270-279.

Wang, Z.A. and Shen, M.M. 2011. Revisiting the concept of cancer stem cells in prostate cancer. Oncogene 30:11:1261-1271.

Wei, C. Guomin, W. Yujun, L. and Ruizhe, Q. 2007. Cancer stem-like cells in human prostate carcinoma cells DU145: the seeds of the cell line? Cancer.Biol.Ther. 6:5:763-768.

Weiss, M.M. Hermsen, M.A. Meijer, G.A. van Grieken, N.C. Baak, J.P. Kuipers, E.J. and van Diest, P.J. 1999. Comparative genomic hybridisation. Mol.Pathol. 52:5:243-251.

Whang, Y.E. Wu, X. Suzuki, H. Reiter, R.E. Tran, C. Vessella, R.L. Said, J.W. Isaacs, W.B. and Sawyers, C.L. 1998. Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. Proc.Natl.Acad.Sci.U.S.A. 95:9:5246-5250.

Williamson, M.P. Elder, P.A. and Knowles, M.A. 1994. The spectrum of TP53 mutations in bladder carcinoma. Genes Chromosomes Cancer 9:2:108-118.

Williamson, M.P. Elder, P.A. Shaw, M.E. Devlin, J. and Knowles, M.A. 1995. p16 (CDKN2) is a major deletion target at 9p21 in bladder cancer. Hum.Mol.Genet. 4:9:1569-1577.

Wolf, A.M. Wender, R.C. Etzioni, R.B. Thompson, I.M. D'Amico, A.V. Volk, R.J. Brooks, D.D. Dash, C. Guessous, I. Andrews, K. DeSantis, C. Smith, R.A. and American Cancer Society Prostate Cancer Advisory Committee. 2010. American Cancer Society guideline for the early detection of prostate cancer: update 2010. CA Cancer.J.Clin. 60:2:70-98.

Wolff, D.J. 2007. The genetics of bladder cancer: a cytogeneticist's perspective. Cytogenet.Genome Res. 118:2-4:177-181.

Xin, L. Ide, H. Kim, Y. Dubey, P. and Witte, O.N. 2003. In vivo regeneration of murine prostate from dissociated cell populations of postnatal epithelia and urogenital sinus mesenchyme. Proc.Natl.Acad.Sci.U.S.A. 100 Suppl 1:11896-11903.

Xu, J. Lange, E.M. Lu, L. Zheng, S.L. Wang, Z. Thibodeau, S.N. Cannon-Albright, L.A. Teerlink, C.C. Camp, N.J. Johnson, A.M. Zuhlke, K.A. Stanford, J.L. Ostrander, E.A. Wiley, K.E. Isaacs, S.D. Walsh, P.C. Maier, C. Luedeke, M. Vogel, W. Schleutker, J. Wahlfors, T. Tammela, T. Schaid, D. McDonnell, S.K. DeRycke, M.S. Cancel-Tassin, G. Cussenot, O. Wiklund, F. Gronberg, H. Eeles, R. Easton, D. Kote-Jarai, Z.

Whittemore, A.S. Hsieh, C.L. Giles, G.G. Hopper, J.L. Severi, G. Catalona, W.J. Mandal, D. Ledet, E. Foulkes, W.D. Hamel, N. Mahle, L. Moller, P. Powell, I. Bailey-Wilson, J.E. Carpten, J.D. Seminara, D. Cooney, K.A. Isaacs, W.B. and International Consortium for Prostate Cancer Genetics. 2013. HOXB13 is a susceptibility gene for prostate cancer: results from the International Consortium for Prostate Cancer Genetics (ICPCG). Hum.Genet. 132:1:5-14.

Xu, J. Zheng, S.L. Komiya, A. Mychaleckyj, J.C. Isaacs, S.D. Hu, J.J. Sterling, D. Lange, E.M. Hawkins, G.A. Turner, A. Ewing, C.M. Faith, D.A. Johnson, J.R. Suzuki, H. Bujnovszky, P. Wiley, K.E. DeMarzo, A.M. Bova, G.S. Chang, B. Hall, M.C. McCullough, D.L. Partin, A.W. Kassabian, V.S. Carpten, J.D. Bailey-Wilson, J.E. Trent, J.M. Ohar, J. Bleecker, E.R. Walsh, P.C. Isaacs, W.B. and Meyers, D.A. 2002. Germline mutations and sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. Nat.Genet. 32:2:321-325.

Xu, K. Wu, Z.J. Groner, A.C. He, H.H. Cai, C. Lis, R.T. Wu, X. Stack, E.C. Loda, M. Liu, T. Xu, H. Cato, L. Thornton, J.E. Gregory, R.I. Morrissey, C. Vessella, R.L. Montironi, R. Magi-Galluzzi, C. Kantoff, P.W. Balk, S.P. Liu, X.S. and Brown, M. 2012. EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycomb-independent. Science 338:6113:1465-1469.

Yadav, S.S. Li, J. Lavery, H.J. Yadav, K.K. and Tewari, A.K. 2015. Next-generation sequencing technology in prostate cancer diagnosis, prognosis, and personalized treatment. Urol.Oncol. 33:6:267.e1-267.e13.

Yamakuchi, M. Ferlito, M. and Lowenstein, C.J. 2008. miR-34a repression of SIRT1 regulates apoptosis. Proc.Natl.Acad.Sci.U.S.A. 105:36:13421-13426.

Yamamoto, Y. Matsuyama, H. Furuya, T. Oga, A. Yoshihiro, S. Okuda, M. Kawauchi, S. Sasaki, K. and Naito, K. 2004. Centrosome hyperamplification predicts progression and tumor recurrence in bladder cancer. Clin.Cancer Res. 10:19:6449-6455.

Yamamura, S. Saini, S. Majid, S. Hirata, H. Ueno, K. Deng, G. and Dahiya, R. 2012. MicroRNA-34a modulates c-Myc transcriptional complexes to suppress malignancy in human prostate cancer cells. PLoS One 7:1:e29722.

Yamasaki, S. Ivanov, P. Hu, G.F. and Anderson, P. 2009. Angiogenin cleaves tRNA and promotes stress-induced translational repression. J.Cell Biol. 185:1:35-42.

Yan, H. Choi, A.J. Lee, B.H. and Ting, A.H. 2011. Identification and functional analysis of epigenetically silenced microRNAs in colorectal cancer cells. PLoS One 6:6:e20628.

Yanaihara, N. Caplen, N. Bowman, E. Seike, M. Kumamoto, K. Yi, M. Stephens, R.M. Okamoto, A. Yokota, J. Tanaka, T. Calin, G.A. Liu, C.G. Croce, C.M. and Harris, C.C. 2006. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer.Cell. 9:3:189-198.

Yang, H. Scholich, K. Poser, S. Storm, D.R. Patel, T.B. and Goldowitz, D. 2002. Developmental expression of PAM (protein associated with MYC) in the rodent brain. Brain Res.Dev.Brain Res. 136:1:35-42.

Yang, Z.F. Ho, D.W. Ng, M.N. Lau, C.K. Yu, W.C. Ngai, P. Chu, P.W. Lam, C.T. Poon, R.T. and Fan, S.T. 2008. Significance of CD90+ cancer stem cells in human liver cancer. Cancer.Cell. 13:2:153-166.

Yeager, M. Orr, N. Hayes, R.B. Jacobs, K.B. Kraft, P. Wacholder, S. Minichiello, M.J. Fearnhead, P. Yu, K. Chatterjee, N. Wang, Z. Welch, R. Staats, B.J. Calle, E.E. Feigelson, H.S. Thun, M.J. Rodriguez, C. Albanes, D. Virtamo, J. Weinstein, S. Schumacher, F.R. Giovannucci, E. Willett, W.C. Cancel-Tassin, G. Cussenot, O. Valeri, A. Andriole, G.L. Gelmann, E.P. Tucker, M. Gerhard, D.S. Fraumeni, J.F.,Jr Hoover, R. Hunter, D.J. Chanock, S.J. and Thomas, G. 2007. Genome-wide association study of prostate cancer identifies a second risk locus at 8q24. Nat.Genet. 39:5:645-649.

Yeh, S. Tsai, M.Y. Xu, Q. Mu, X.M. Lardy, H. Huang, K.E. Lin, H. Yeh, S.D. Altuwaijri, S. Zhou, X. Xing, L. Boyce, B.F. Hung, M.C. Zhang, S. Gan, L. and Chang, C. 2002. Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. Proc.Natl.Acad.Sci.U.S.A. 99:21:13498-13503.

Yekta, S. Shih, I.H. and Bartel, D.P. 2004. MicroRNA-directed cleavage of HOXB8 mRNA. Science 304:5670:594-596.

Yeung, M.L. Bennasser, Y. Watashi, K. Le, S.Y. Houzet, L. and Jeang, K.T. 2009. Pyrosequencing of small non-coding RNAs in HIV-1 infected cells: evidence for the processing of a viral-cellular double-stranded RNA hybrid. Nucleic Acids Res. 37:19:6575-6586.

Yi, R. Qin, Y. Macara, I.G. and Cullen, B.R. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 17:24:3011-3016.

Yip, K.W. and Reed, J.C. 2008. Bcl-2 family proteins and cancer. Oncogene 27:50:6398-6406.

Ylipaa, A. Kivinummi, K. Kohvakka, A. Annala, M. Latonen, L. Scaravilli, M. Kartasalo, K. Leppanen, S.P. Karakurt, S. Seppala, J. Yli-Harja, O. Tammela, T.L. Zhang, W. Visakorpi, T. and Nykter, M. 2015. Transcriptome sequencing reveals PCAT5 as a novel ERG-regulated long non-coding RNA in prostate cancer. Cancer Res.

Yoshimoto, M. Cunha, I.W. Coudry, R.A. Fonseca, F.P. Torres, C.H. Soares, F.A. and Squire, J.A. 2007. FISH analysis of 107 prostate cancers shows that PTEN genomic deletion is associated with poor clinical outcome. Br.J.Cancer 97:5:678-685.

Yoshimoto, M. Cutz, J.C. Nuin, P.A. Joshua, A.M. Bayani, J. Evans, A.J. Zielenska, M. and Squire, J.A. 2006a. Interphase FISH analysis of PTEN in histologic sections shows genomic deletions in 68% of primary prostate cancer and 23% of high-grade prostatic intra-epithelial neoplasias. Cancer Genet.Cytogenet. 169:2:128-137.

Yoshimoto, M. Joshua, A.M. Chilton-Macneill, S. Bayani, J. Selvarajah, S. Evans, A.J. Zielenska, M. and Squire, J.A. 2006b. Three-color FISH analysis of TMPRSS2/ERG fusions in prostate cancer indicates that genomic microdeletion of chromosome 21 is associated with rearrangement. Neoplasia 8:6:465-469.

Yu, F. Yao, H. Zhu, P. Zhang, X. Pan, Q. Gong, C. Huang, Y. Hu, X. Su, F. Lieberman, J. and Song, E. 2007. Let-7 Regulates Self Renewal and Tumorigenicity of Breast Cancer Cells. Cell 131:6:1109-1123.

Yu, J. Yu, J. Mani, R.S. Cao, Q. Brenner, C.J. Cao, X. Wang, X. Wu, L. Li, J. Hu, M. Gong, Y. Cheng, H. Laxman, B. Vellaichamy, A. Shankar, S. Li, Y. Dhanasekaran, S.M. Morey, R. Barrette, T. Lonigro, R.J. Tomlins, S.A. Varambally, S. Qin, Z.S. and Chinnaiyan, A.M. 2010. An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. Cancer.Cell. 17:5:443-454.

Yu, Y.P. Landsittel, D. Jing, L. Nelson, J. Ren, B. Liu, L. McDonald, C. Thomas, R. Dhir, R. Finkelstein, S. Michalopoulos, G. Becich, M. and Luo, J.H. 2004. Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. J.Clin.Oncol. 22:14:2790-2799.

Zhang, B. Pan, X. Cobb, G.P. and Anderson, T.A. 2007. microRNAs as oncogenes and tumor suppressors. Dev.Biol. 302:1:1-12.

Zhang, H.L. Yang, L.F. Zhu, Y. Yao, X.D. Zhang, S.L. Dai, B. Zhu, Y.P. Shen, Y.J. Shi, G.H. and Ye, D.W. 2011. Serum miRNA-21: elevated levels in patients with metastatic hormone-refractory prostate cancer and potential predictive factor for the efficacy of docetaxel-based chemotherapy. Prostate 71:3:326-331.

Zhao, J. Richter, J. Wagner, U. Roth, B. Schraml, P. Zellweger, T. Ackermann, D. Schmid, U. Moch, H. Mihatsch, M.J. Gasser, T.C. and Sauter, G. 1999. Chromosomal imbalances in noninvasive papillary bladder neoplasms (pTa). Cancer Res. 59:18:4658-4661.

Zhu, C. Li, J. Cheng, G. Zhou, H. Tao, L. Cai, H. Li, P. Cao, Q. Ju, X. Meng, X. Wang, M. Zhang, Z. Qin, C. Hua, L. Yin, C. and Shao, P. 2013. miR-154 inhibits EMT by targeting HMGA2 in prostate cancer cells. Mol.Cell.Biochem. 379:1-2:69-75.

Zhu, H. and Garcia, J.A. 2013. Targeting the adrenal gland in castration-resistant prostate cancer: a case for orteronel, a selective CYP-17 17,20-lyase inhibitor. Curr.Oncol.Rep. 15:2:105-112.

Zhu, S. Wu, H. Wu, F. Nie, D. Sheng, S. and Mo, Y.Y. 2008. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res. 18:3:350-359.

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Mapping of the chromosomal amplification 1p21-22 in bladder cancer

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Abstract

Background: The aim of the study was to characterize a recurrent amplification at chromosomal region 1p21-22 in bladder cancer.

Methods: ArrayCGH (aCGH) was performed to identify DNA copy number variations in 7 clinical samples and 6 bladder cancer cell lines. FISH was used to map the amplicon at 1p21-22 in the cell lines. Gene expression microarrays and qRT-PCR were used to study the expression of putative target genes in the region.

Results: aCGH identified an amplification at 1p21-22 in 10/13 (77%) samples. The minimal region of the amplification was mapped to a region of about 1 Mb in size, containing a total of 11 known genes. The highest amplification was found in SCaBER squamous cell carcinoma cell line. Four genes, *TMED5*, *DR1*, *RPL5* and *EVI5*, showed significant overexpression in the SCaBER cell line compared to all the other samples tested. Oncomine database analysis revealed upregulation of *DR1* in superficial and infiltrating bladder cancer samples, compared to normal bladder.

Conclusions: In conclusions, we have identified and mapped chromosomal amplification at 1p21-22 in bladder cancer as well as studied the expression of the genes in the region. *DR1* was found to be significantly overexpressed in the SCaBER, which is a model of squamous cell carcinoma. However, the overexpression was found also in a published clinical sample cohort of superficial and infiltrating bladder cancers. Further studies with more clinical material are needed to investigate the role of the amplification at 1p21-22.

Keywords: Gene amplification, Bladder cancer, DR1, aCGH

Background

Bladder cancer is the fourth most common cancer in men in developed countries and the second most common malignancy of the urinary tract [1]. The majority of bladder cancer cases arise from the urothelium, the epithelium lining the inside of the bladder and these cases are thus called urothelial carcinomas. Squamous cell carcinoma of the urinary bladder is a rarer malignant neoplasm and it accounts for 3–5% of bladder cancer in Western populations [2].

Several studies have investigated the chromosomal alterations associated with development and progression of bladder cancer. Different methods to detect copy number changes, such as classical cytogenetics, interphase fluorescence *in situ* hybridization (FISH), Southern blot analysis, quantitative polymerase chain reaction (PCR)-based assays and comparative genomic hybridization (CGH) have been used [3].

Several CGH studies providing information about typical losses, gains and amplifications in bladder cancer have been published [4-8]. However, the resolution of conventional CGH is generally limited to regions greater than 10 Mb. The development of array-based technologies for CGH [9,10] led to > 10-fold increase of the resolution and consequently to the analysis of copy number alterations at single gene level. A few array-CGH (aCGH) genome-wide studies have been performed on both clinical bladder cancers [11,12] as well as cell lines [13]. They have highlighted copy-number alterations in smaller scale, with high accuracy of localization. Some of these genetic changes have been associated with known oncogenes or tumor suppressor genes. Loss of genetic material on chromosome 9 is one of the most frequent alteration in TCC, with 9p and 9q, often both, lost entirely or in part

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Table 1 FISH mapping of 1p21-22 amplicon

Clones	Chromosome location	Cell lines							
		SCaBER	HT-1376	UM-UC-3	TCCSUP	RT4	J82	T24	5637
RP11-82E1	91,116,728–91,294,152	3/4 (0.9)							3/3 (1.00)
RP5-865 M20	92,068,692–92,181,253	2/4 (0.54)	10/6 (1,53)		3/4 (0.86)	4/4 (0.98)	3/3 (1.00)	3/3 (1.17)	3/3 (1.00)
RP4-621B10	92,517,154–92,659,879	2/4 (0.54)	10/6 (1.89)		3/4 (0.86)	4/4 (1.00)	3/3 (1.00)	3/3 (1.12)	3/3 (1.00)
RP5-1014C4	92,854,755–93,007,879	7/4 **(2.02)**	11/6 (1.91)	4/4 (1.02)					3/3 (1.00)
RP11-977E2	93,042,494–93,249,510	8/4 **(2.35)**	10/6 (1.65)	3/4 (0.75)	3/4 (0.73)	4/4 (0.93)	3/3 (0.86)	4/3 (1.24)	3/3 (1.00)
RP5-976O13	93,529,940–93,632,330	10/4 **(3.07)**	10/6 (1.64)		3/4 (0.78)		3/3 (1.06)		3/3 (1.00)
RP4-713B5	93,760,493–93,865,044	11/4 **(3.02)**	10/6 (1.83)						3/3 (1.00)
RP11-272P3	94,980,681–95,180,686	3/4 (0.99)	11/6 (1.91)						3/3 (1.00)
RP11-146P11	95,983,612–96,156,674	4/4 (1.04)	10/6 (1.85)	4/4 (1.05)	3/4 (0.82)	4/4 (1.05)	3/3 (0.93)		3/3 (1.00)
RP11-122C9	97,095,507–97,282,884	3/4 (1.07)	10/6 (1.91)						3/3 (1.00)

The first value represents the median of signals from the locus-specific probe indicated under 'clones'; the second value represents the median number of signal from the chromosome 1 centromeric probe. The ratio between the two values is bracketed. SCaBER cell line shows a high level amplification between the positions 92,854,755 and 93,865,044 (GRCh37/h19), whereas HT-1376 cell line shows a copy-number gain.

[14,15]. Candidate target genes include *CDKN2A* [16], *DBCCR1* [17], and *TSC1* [18]. Deletion of 10q has been associated with *PTEN* locus [19,20], 13q with *RB1* [21] and 17p with *TP53* [22]. Common DNA amplifications contain known or candidate oncogenes as well, including cyclin D1 (*CCND1*) at 11q13 [23,24], *ERBB2* at 17q21 [25,26], *E2F3* at 6p22 [27,28], *MDM2* at 12q14 [29], and *MYC* at 8q24 [30]. Recurrent amplifications have also been found at 1q, 3p, 3q, 8p, 8q, and 12q [5,6,8]. Furthermore, activating mutations of oncogenes *HRAS* [31] and *FGFR3* [32] seem to be common. Gain-of-function mutations affecting *RAS* and *FGFR3* and loss-of-function mutation affecting *RB*, *PTEN* and *TP53* have also been associated with the pathological stage and/or outcome of bladder cancer [33,34].

In this study, we report the characterization of a common amplification at chromosomal region 1p21-22. The amplicon was identified by aCGH analysis of clinical specimens obtained from bladder cancer patients and in bladder cancer cell lines.

Methods

Clinical samples

Freshly frozen samples from 7 bladder cancer tissues were used for this study. The samples were obtained from Tampere University Hospital and include five urothelial carcinomas, one lymphoepithelial carcinoma and one undifferentiated carcinoma. DNA was extracted using DNAzol reagent (Molecular Research Center, Inc. Cincinnati, OH), according to manufacturer's protocol. The use of the clinical samples was approved by the ethical committee of the Tampere University Hospital.

Cell lines

The bladder cancer cell lines UM-UC-3, TCCSUP, RT4, T24, HT-1376, J82, SCaBER, 5637, HT-1197 and SW780 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured according to the recommended conditions.

Array comparative genomic hybridization

16 K cDNA microarray-slides were obtained from the Finnish Microarray DNA Centre (http://www.btk.fi/microarray-and-sequencing/) (Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland). The poly-L-lysine coated slides contain approximately 16000 annotated clones from sequence verified I.M.A.G.E. Consortium cDNA library in duplicate. Comparative genomic hybridization to microarray (aCGH) was done as described previously [35]. Briefly, 2 to 10 μg RsaI-digested (Fermentas UAB, Vilnius, Lithuania) DNA was labeled with Cy5-dCTP, and normal male reference DNA with Cy3-dCTP (Amersham Biosciences UK Ltd., Little Chalfont, United Kingdom), using a BioPrime Labeling Kit

Table 2 PCR primers

Gene	Forward primer	Reverse primer
DR1	TGCAAGAGTGTAAAAAGTAGCATT	TGCTGCATTTGAAGCCATT
EVI5	AGCAGAGTGATGAGGCCAGT	CTTCACTCAGTCGGGCTTG
RPL5	TGGAAGAAGATGAAGATGCTTAC	GACGACATACCTCTTCTTTTTAACTTC
TMED5	TCACACCTTCCCTCGATAGC	AAGGTTTTGCCTTCTGGAGAG
TBP	GAATATAATCCCAAGCGGTTTG	ACTTCACATCACAGCTCCCC

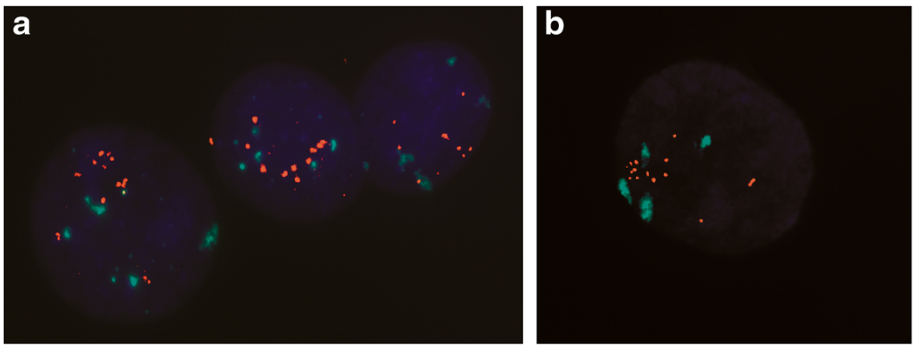

Figure 1 Fluorescence *in situ* hybridization. (a) HT-1376 cell line nuclei hybridized with the BAC clone RP11-122C9 showing copy number gain (RED: RP11-122C9, GREEN: pericentromeric chr.1), and **(b)** nuclei of SCaBER squamous cell carcinoma cell line model hybridized with the PAC clone RP4-713B5, showing a high level amplification (colors as in **a**).

(Invitrogen). The sample and reference DNAs were co-hybridized overnight at +65°C, under cover slips, to microarray slides, in a final volume of 38.5 μl of hybridization mix containing 3.4 × SSC, 0.3% SDS, 1.3 × Denhardt's (Sigma-Aldrich, St. Louis, MO), and 0.5 × DIG Blocking Buffer (Roche Diagnostics, Mannheim, Germany). After stringent washes, the slides were scanned with ScanArray4000 confocal laser scanner (Perkin Elmer, Boston, MA). Signal volumes were quantified using the QuantArray software program (Packard Bioscience, Bio- Chip Technology LCC, Billerica, MA). Data were analyzed using the cluster along chromosomes (CLAC) algorithm, as previously described and visualized using the software CGH-Miner [36].

Fluorescence *in situ* hybridization

Human genome PAC/BAC clones were purchased from Invitrogen™ Corporation. The list of clones is shown in Table 1 and the chromosome positions are indicated according to UCSC (University of California Santa Cruz) Genome Browser, February 2009 assembly (GRCh37/h19). The clones were labeled with digoxigenin-dUTP (Roche Diagnostics) or Alexa Fluor®-dUTP (Invitrogen™) by nick translation. A pericentromeric probe for chromosome 1 labeled with FITC-dUTP was obtained from Roche. The metaphase slides from the bladder cancer cell lines were prepared using standard techniques. The slides were denatured in 70% formamide/2xSSC at 70°C for 2 min and dehydrated in an ascending ethanol series. Hybridization was performed over night at 37°C. After stringent washes, the slides were stained with antidigoxigenin-rhodamine (Roche Diagnostics) for the digoxigenin-labeled probes and embedded in an antifade solution (Vectashied, Vector Laboratories, Burlingame, CA, USA) containing 4,6-diamidino-2-phenylindole (DAPI) as counter stain. Stained slides were analyzed on an epifluorescence microscope (Olympus) and acquired images were processed using Image-Pro® image-processing software (Media Cybernetics). A total of 50 nuclei were considered for statistical analysis of the FISH signals in each experiment. An amplification was defined as a locus-specific probe/centromere ratio >2. In each experiment the hybridization efficiency of the locus-specific and centromeric probes was evaluated using 5637 bladder cancer cell line as a triploid control.

Table 3 Known human genes at chromosome 1 position 92,940,318 - 93,828,148 (GRCh37/h19)

NAME	DESCRIPTION	LOCATION	GENOMIC SIZE (bp)
GFI1	Growth factor independent 1 transcription repressor (GFI1)	chr1:92,940,318 – 92,952,433	12116
EVI5	Ecotropic viral integration site 5 (EVI5)	chr1:92,974,253 – 93,257,961	283709
RPL5	Ribosomal protein L5 (RPL5)	chr1:93,297,594 – 93,307,481	9887
SNORD21	Small nucleolar RNA, C/D box 21 (SNORD21), small nucleolar RNA	chr1:93,302,846 – 93,302,940	95
SNORA66	Small nucleolar RNA, H/ACA box 66 (SNORA66), small nucleolar RNA	chr1:93,306,276 – 93,306,408	133
FAM69A	Family with sequence similarity 69, member A (FAM69A)	chr1:93,307,717 – 93,427,079	128794
MTF2	Metal response element binding transcription factor 2 (MTF2)	chr1:93,544,792 – 93,604,638	59847
TMED5	Transmembrane emp24 protein transport domain containing 5 (TMED5)	chr1:93,615,299 – 93,646,246	30948
CCDC18	Coiled-coil domain containing 18 (CCDC18)	chr1:93,646,281 – 93,744,287	98007
LOC100131564	Uncharacterized LOC100131564 (LOC100131564), non-coding RNA	chr1:93,775,666 – 93,811,368	35703
DR1	Down-regulator of transcription 1, TBP-binding (negative cofactor 2) (DR1)	chr1:93,811,478 – 93,828,148	16671

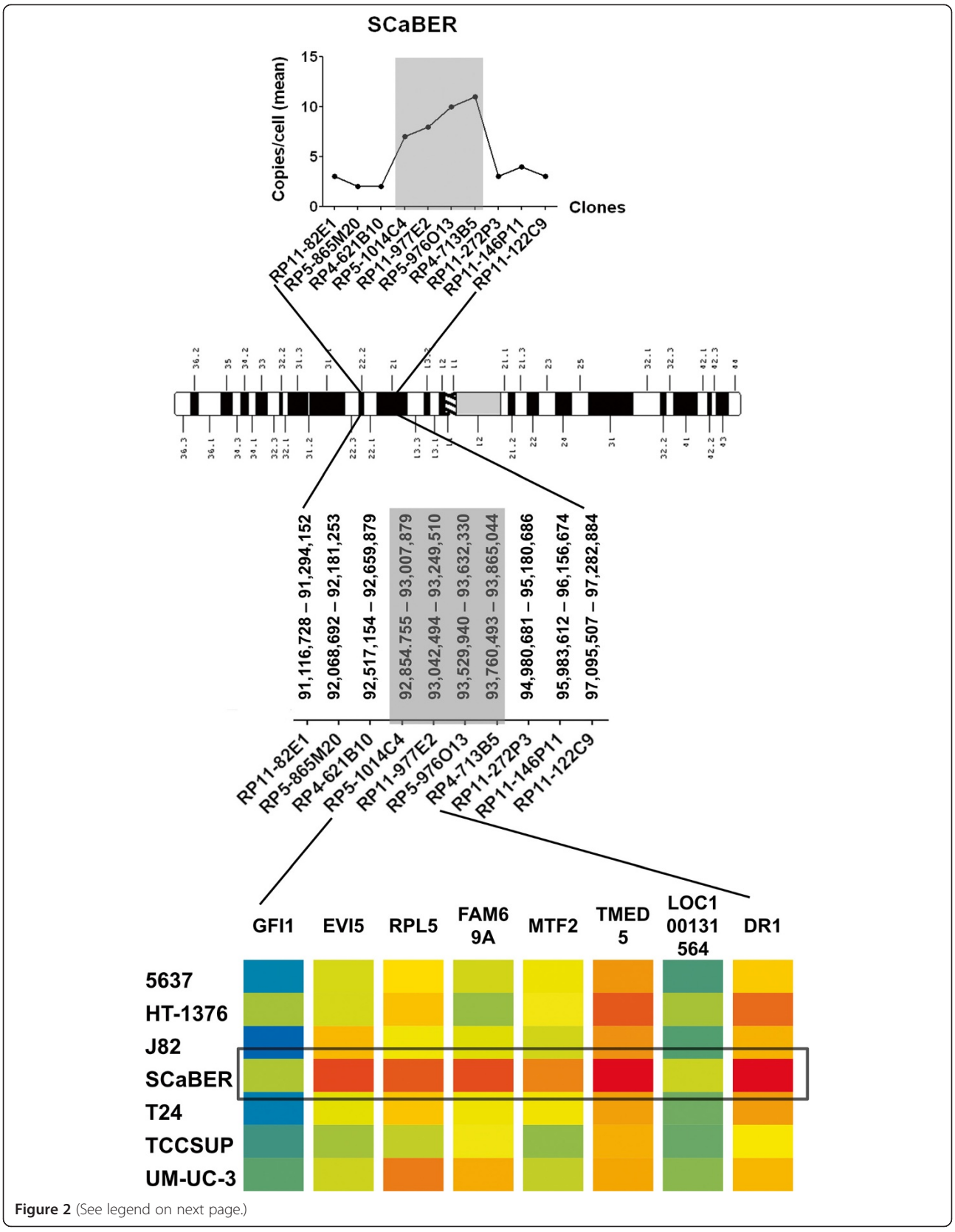

Figure 2 (See legend on next page.)

(See figure on previous page.)
Figure 2 Fine mapping the region of amplification. Chromosome 1 ideogram showing the region of amplification according to aCGH (above), the FISH scoring data on SCaBER cell lines indicating the minimal region of amplicon (in gray), and (below) an expression heatmap of the genes at chromosome 1, position 92,940,318 – 93,828,148 (red: overexpression, blue: underexpression), showing significant relative overexpression of *TMED5*, *DR1*, *EVI5* and *RPL5* in the SCaBER cell line.

RNA extraction and gene expression microarray

Total RNA from bladder cancer cell lines was collected and extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The samples were then amplified and hybridized using the Agilent whole genome oligo microarray platform (Agilent Technologies, Palo Alto, CA, USA) and Xpress Ref ™ Human Universal Reference Total RNA (SuperArray Bioscience Corporation) was used as a reference. The resulting data files from Agilent Feature Extraction Software (version 9.5.1.1) were imported into the Agilent GeneSpring GX software (version 11.0) for further analysis. A fold-change cutoff of 2 was used to determine differential gene expression.

Real time quantitative polymerase chain reaction (qRT-PCR)

Total RNA from bladder cancer cell lines, extracted as described above, was reverse transcribed using random hexamere primers and AMV reverse transcriptase (Thermo Scientific). Quantitative Real Time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) and a BioRad CFX96 ™ Real-Time PCR Detection System. Each sample was run in duplicate and expression values were normalized against TATA-binding protein (TBP). The primer sequences are shown in Table 2.

Results

Identification of the common amplicon at 1p21-22

The CLAC-analysis of the aCGH data from clinical samples and bladder cancer cell lines showed a region of increased copy number at chromosome 1p21-22 in 5 of 7 total clinical samples as well as in bladder cancer cell lines, 5637, RT4, T24, SW780 and SCaBER (data not shown). According to aCGH, the common region of gain comprised of 2 Mb.

Fine mapping of the 1p21-22 region

The region 1p21-22 was studied in bladder cancer cell lines by FISH analysis on interphase nuclei (Figure 1). All cell lines showed increased copy number of 1p21-22 region, and SCaBER cells where the only one which showed high-level amplification of the region (Figure 1b). We extensively analyzed cell lines with the PAC/BAC clones spanning a total of 6 Mb and were able to identify a minimal region of amplification between the chromosome positions 92,940,318 and 93,828,148 (Table 2). According to UCSC Genes Feb. 2009 GRCh37/hg19, a total of 11 human genes are located within the amplicon. Nine of them are known protein-coding genes (Table 3).

Microarray and qRT-PCR validation

The analysis of gene expression by microarray showed significant overexpression of 4 genes, namely *DR1*, *EVI5*, *RPL5* and *TMED5* only in the SCaBER, which harbors the highest level of amplification of the region (Figure 2). The results were validated by qRT-PCR and confirmed the overexpression of the genes in SCaBER, as compared to all the other cell lines (Figure 3). In addition, Oncomine database analysis for *DR1* expression in bladder cancer revealed a statistically significant ($P < 0.0001$) upregulation of the gene in clinical samples of both superficial and infiltrating bladder cancer, when compared to normal bladder [37] (Figure 4). *TMED5* showed significant upregulation in superficial bladder cancer, when compared to normal, whereas *RPL5* and *EVI5* did not show significant changes of expression levels in the same dataset.

Discussion

In this study, aCGH technology was utilized to identify new regions of amplifications in bladder cancer. Recurrent amplification was found in chromosomal locus 1p21-22. Subsequently, the locus was fine-mapped and characterized in the bladder cancer cell lines. Of the cell lines SCaBER showed the highest amplification of the region, thus it was used for mapping the amplicon. Fine mapping with the SCaBER model, the region was defined to ~1 Mb of size, containing 11 genes.

cDNA microarray and qRT-PCR analyses were used to measure the expression of these genes in bladder cancer cell lines. *DR1*, *EVI5*, *RPL5*, and *TMED5* showed overexpression in SCaBER compared to the other cell lines. *DR1* was found to be the most significantly overexpressed of the examined genes. Since SCaBER is a squamous cell carcinoma cell line, we wished to interrogate whether *DR1* is overexpressed also in the urothelial carcinoma. We utilized Oncomine database of clinical samples, which showed overexpression of *DR1* also in superficial and infiltrating bladder cancer.

DR1 is also known as *NC2beta* and has been shown to bind *DRAP1* to repress RNA polymerase II gene transcription [38]. Despite targeting the general transcription

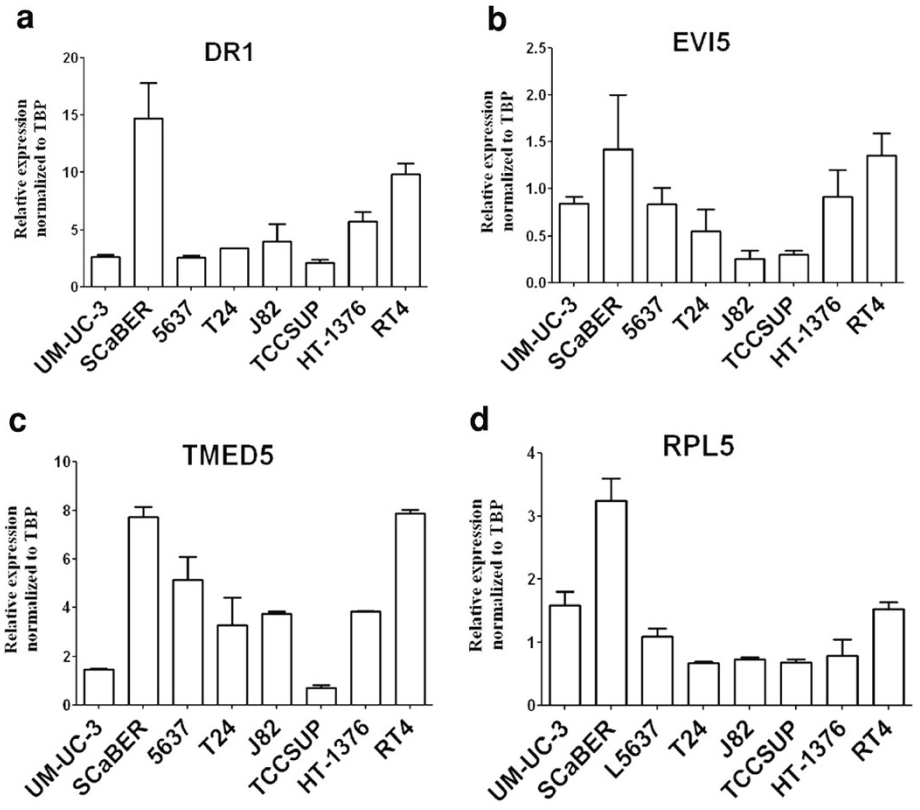

Figure 3 qRT-PCR validation of microarray expression data. *DR1* **(a)**, *EVI5* **(b)**, *RPL5* **(c)** and *TMED5* **(d)**, showing the highest level of expression in the SCaBER model, when compared to the other cell lines tested. The expression values of the genes were normalized against *TBP*.

machinery, only a subset of mRNAs has been shown to respond to the *DR1/DRAP1* inhibition [39] and the opposite transcription inducing effect of DR1/DRAP1 has also been shown for some mRNAs, suggesting the possibility of a specific regulatory effect [40].

According to Oncomine database *DR1*, *EVI5*, *TMED5* and *RPL5* are co-amplified also in brain [41-43], colon [44], lung cancer [45] and melanoma [46], indicating that amplification of 1p21-22 may be a recurrent alteration in several different types of cancers.

Conclusions

We have identified and mapped a common chromosomal amplification at 1p21-22 in bladder cancer. Squamous cell carcinoma cell line SCaBER, which had the highest level of amplification of the region, showed

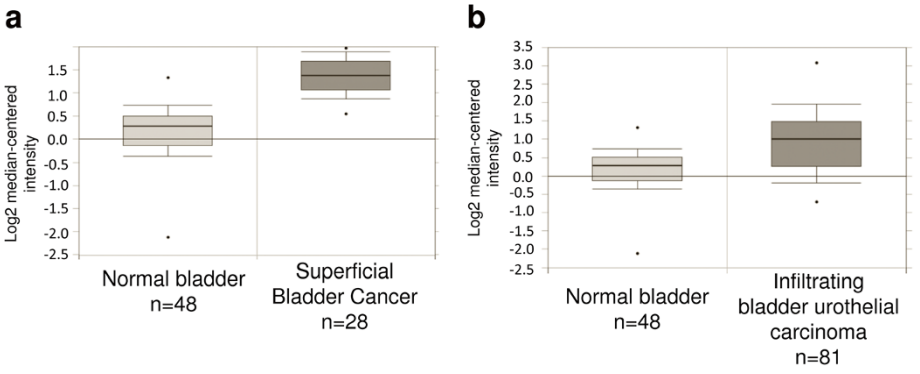

Figure 4 DR1 expression in bladder cancer according to Oncomine. Statistically significant ($p < 0.0001$) upregulation of *DR1* expression was found in superficial **(a)** and infiltrating **(b)** bladder cancer, when compared to normal bladder. A total of 157 samples were used in the Sanchez-Carbayo study (Sanchez-Carbayo et al., 2006).

overexpression of *DR1*. In a published data set, *DR1* was also overexpressed in clinical samples of superficial and infiltrating bladder cancers, suggesting that *DR1* is a putative target for the amplification. Further studies are needed to assess the role of the amplification at 1p21-22 in bladder cancer.

Competing interests
The authors declare that they have no competing interest.

Authors'contributions
OS and TV designed research, MS, PA, OS and TV planned experiments, TT provided clinical material, MS and PA performed the experiments, MS, PA, OS and TV analyzed the data, MS, OS and TV wrote the paper. All authors read and approved the final manuscript.

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References
1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: **Global cancer statistics.** *CA Cancer J Clin* 2011, **61:**69–902.
2. Lagwinski N, Thomas A, Stephenson AJ, Campbell S, Hoschar AP, El-Gabry E, Dreicer R, Hansel DE: **Squamous cell carcinoma of the bladder: a clinicopathologic analysis of 45 cases.** *Am J Surg Pathol* 2007, **31:**1777–1787.
3. Höglund M: **The bladder cancer genome; chromosomal changes as prognostic makers, opportunities, and obstacles.** *Urol Oncol* 2012, **30:**533–540.
4. Kallioniemi A, Kallioniemi OP, Citro G, Sauter G, DeVries S, Kerschmann R, Caroll P, Waldman F: **Identification of gains and losses of DNA sequences in primary bladder cancer by comparative genomic hybridization.** *Genes Chromosomes Cancer* 1995, **12:**213–219.
5. Voorter C, Joos S, Bringuier PP, Vallinga M, Poddighe P, Schalken J, du Manoir S, Ramaekers F, Lichter P, Hopman A: **Detection of chromosomal imbalances in transitional cell carcinoma of the bladder by comparative genomic hybridization.** *Am J Pathol* 1995, **146:**1341–1354.
6. Richter J, Jiang F, Gorog JP, Sartorius G, Egenter C, Gasser TC, Moch H, Mihatsch MJ, Sauter G: **Marked genetic differences between stage pTa and stage pT1 in papillary bladder cancer detected by comparative genomic hybridization.** *Cancer Res* 1997, **57:**2860–2864.
7. Hovey RM, Chu L, Balazs M, DeVries S, Moore D, Sauter G, Carroll PR, Waldman FM: **Genetic alterations in primary bladder cancer and their metastasis.** *Cancer Res* 1998, **58:**3555–3560.
8. Richter J, Wagner U, Schraml P, Maurer R, Alund G, Knonagel H, Moch H, Mihatsch MJ, Gasser TC, Sauter G: **Chromosomal imbalances are associated with high risk of progression in early invasive (pT1) urinary bladder cancer.** *Cancer Res* 1999, **59:**5687–5691.
9. Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y, Dairkee SH, Ljung BM, Gray JW, Albertson DG: **High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays.** *Nat Genet* 1998, **20:**207–211.
10. Hodgson G, Hager JH, Volik S, Hariono S, Wernick M, Moore D, Nowak N, Albertson DG, Pinkel D, Collins C, Hanahan D, Gray JW: **Genome scanning with array CGH delineates regional alterations in mouse islet carcinomas.** *Nat Genet* 2001, **29:**459–464.
11. Veltman JA, Fridlyand J, Pejavar S, Olshen AB, Korkola JE, DeVries S, Carroll P, Kuo WL, Pinkel D, Albertson D, Cordon-Cardo C, Jain AN, Waldman FM: **Array-based comparative genomic hybridization for genome-wide screening of DNA copy number in bladder tumors.** *Cancer Res* 2003, **63**(11):2872–2880.
12. Blaveri E, Brewer JL, Roydasgupta R, Fridlyand J, DeVries S, Koppie T, Pejavar S, Mehta K, Carroll P, Simko JP, Waldman FM: **Bladder cancer stage and outcome by array-based comparative genomic hybridization.** *Clin Cancer Res* 2005, **11**(19):7012–7022.
13. Hurst CD, Fiegler H, Carr P, Williams S, Carter NP, Knowles MA: **High-resolution analysis of genomic copy number alterations in bladder cancer by microarray-based comparative genomic hybridization.** *Oncogene* 2004, **23**(12):2250–2263.
14. Habuchi T, Devlin J, Elder PA, Knowles MA: **Detailed deletion mapping of chromosome 9q in bladder cancer: evidence for two tumour suppressor loci.** *Oncogene* 1995, **11:**1671–1674.
15. Kimura F, Florl AR, Seifert HH, Louhelainen J, Maas S, Knowles MA, Schulz WA: **Destabilization of chromosome 9 in transitional cell carcinoma of the urinary bladder.** *Br J Cancer* 2001, **85**(12):887–893.
16. Aveyard JS, Knowles MA: **Measurement of relative copy number of CDKN2A/ARF and CDKN2B in bladder cancer by real-time quantitative PCR and multiplex ligation-dependent probe amplification.** *J Mol Diagn* 2004, **6**(4):356–365.
17. Wright KO, Messing EM, Reeder JE: **DBCCR1 mediates death in cultured bladder tumor cells.** *Oncogene* 2004, **23**(1):82–90.
18. Hornigold N, Devlin J, Davies AM, Aveyard JS, Habuchi T, Knowles MA: **Mutation of the 9q34 gene *TSC1* in sporadic bladder cancer.** *Oncogene* 1999, **18**(16):2657–2661.
19. Aveyard JS, Skilleter A, Habuchi T, Knowles MA: **Somatic mutation of PTEN in bladder carcinoma.** *Br J Cancer* 1999, **80**(5–6):904–908.
20. Knowles MA: **Tumor suppressor loci in bladder cancer.** *Front Biosci* 2007, **12:**2233–2251.
21. Knowles MA: **The genetics of transitional cell carcinoma: progress and potential clinical application.** *BJU Int* 1999, **84**(4):412–427.
22. Erill N, Colomer A, Verdú M, Román R, Condom E, Hannaoui N, Banús JM, Cordon-Cardo C, Puig X: **Genetic and immunophenotype analyses of *TP53* in bladder cancer: *TP53* alterations are associated with tumor progression.** *Diagn Mol Pathol* 2004, **13**(4):217–223.
23. Proctor AJ, Coombs LM, Cairns JP, Knowles MA: **Amplification at chromosome 11q13 in transitional cell tumours of the bladder.** *Oncogene* 1991, **6**(5):789–795.
24. Bringuier PP, Tamimi Y, Schuuring E, Schalken J: **Expression of cyclin D1 and EMS1 in bladder tumors: relationship with chromosome 11q13 amplification.** *Oncogene* 1996, **12**(8):1747–1753.
25. Coombs LM, Pigott DA, Sweeney E, Proctor AJ, Eydmann ME, Parkinson C, Knowles MA: **Amplification and over-expression of c-erbB-2 in transitional cell carcinoma of the urinary bladder.** *Br J Cancer* 1991, **63**(4):601–608.
26. Ohta JI, Miyoshi Y, Uemura H, Fujinami K, Mikata K, Hosaka M, Tokita Y, Kubota Y: **Fluorescence in situ hybridization evaluation of c-erbB-2 gene amplification and chromosomal anomalies in bladder cancer.** *Clin Cancer Res* 2001, **7**(8):2463–2467.
27. Feber A, Clark J, Goodwin G, Dodson AR, Smith PH, Fletcher A, Edwards S, Flohr P, Falconer A, Roe T, Kovacs G, Dennis N, Fisher C, Wooster R, Huddart R, Foster CS, Cooper CS: **Amplification and overexpression of E2F3 in human bladder cancer.** *Oncogene* 2004, **23:**1627–1630.
28. Oeggerli M, Tomovska S, Schraml P, Calvano-Forte D, Schafroth S, Simon R, Gasser T, Mihatsch MJ, Sauter G: **E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer.** *Oncogene* 2004, **23:**5616–5623.
29. Simon R, Struckmann K, Schraml P, Wagner U, Forster T, Moch H, Fijan A, Bruderer J, Wilber K, Mihatsch MJ, Gasser T, Sauter G: **Amplification pattern of 12q13-q15 genes (MDM2, CDK4, GLI) in urinary bladder cancer.** *Oncogene* 2002, **21**(16):2476–2483.
30. Sauter G, Carroll P, Moch H, Kallioniemi A, Kerschmann R, Narayan P, Mihatsch MJ, Waldman FM: **c-myc copy number gains in bladder cancer detected by fluorescence in situ hybridization.** *Am J Pathol* 1995, **146**(5):1131–1139.
31. Jebar AH, Hurst CD, Tomlinson DC, Johnston C, Taylor CF, Knowles MA: **FGFR3 and Ras gene mutations are mutually exclusive genetic events in urothelial cell carcinoma.** *Oncogene* 2005, **24**(33):5218–5225.
32. Tomlinson DC, Baldo O, Harnden P, Knowles MA: **FGFR3 protein expression and its relationship to mutation status and prognostic variables in bladder cancer.** *J Pathol* 2007, **213**(1):91–98.
33. Cordon-Cardo C: **Molecular alterations associated with bladder cancer initiation and progression.** *Scand J Urol Nephrol* 2008, **42:**154–165.

34. Puzio-Kuter AM, Castillo-Martin M, Kinkade CW, Wang X, Shen TH, Matos T, Shen MM, Cordon-Cardo C, Abate-Shen C: **Inactivation of p53 and Pten promotes invasive bladder cancer.** *Genes Dev* 2009, **23**(6):675–680.
35. Saramäki OR, Porkka KP, Vessella RL, Visakorpi T: **Genetic aberrations in prostate cancer by microarray analysis.** *Int J Cancer* 2006, **119**(6):1322–1329.
36. Wang P, Kim Y, Pollack J, Narasimhan B, Tibshirani R: **A method for calling gains and losses in array CGH data.** *Biostatistics* 2005, **6**(1):45–58.
37. Sanchez-Carbayo M, Socci ND, Lozano J, Saint F, Cordon-Cardo C: **Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays.** *J Clin Oncol* 2006, **24**(5):778–789.
38. Mermelstein F, Yeung K, Cao J, Inostroza JA, Erdjument-Bromage H, Eagelson K, Landsman D, Levitt P, Tempst P, Reinberg D: **Requirement of a corepressor for Dr1-mediated repression of transcription.** *Genes Dev* 1996, **10:**1033–1048.
39. Geisberg JV, Holstege FC, Young RA, Struhl K: **Yeast NC2 associates with the RNA polymerase II preinitiation complex and selectively affects transcription in vivo.** *Mol Cell Biol* 2001, **21:**2736–2742.
40. Cang Y, Prelich G: **Direct stimulation of transcription by negative cofactor 2 (NC2) through TATA-binding protein (TBP).** *Proc Natl Acad Sci U S A* 2002, **99:**12727–12732.
41. Kurashina K, Yamashita Y, Ueno T, Koinuma K, Ohashi J, Horie H, Miyakura Y, Hamada T, Haruta H, Hatanaka H, Soda M, Choi YL, Takada S, Yasuda Y, Nagai H, Mano H: **Chromosome copy number analysis in screening for prognosis-related genomic regions in colorectal carcinoma.** *Cancer Sci* 2008, **99**(9):1835–1840.
42. Ramos AH, Dutt A, Mermel C, Perner S, Cho J, Lafargue CJ, Johnson LA, Stiedl AC, Tanaka KE, Bass AJ, Barretina J, Weir BA, Beroukhim R, Thomas RK, Minna JD, Chirieac LR, Lindeman NI, Giordano T, Beer DG, Wagner P, Wistuba II, Rubin MA, Meyerson M: **Amplification of chromosomal segment 4q12 in non-small cell lung cancer.** *Cancer Biol Ther* 2009, **8**(21):2042–2050.
43. Maser RS, Choudhury B, Campbell PJ, Feng B, Wong KK, Protopopov A, O'Neil J, Gutierrez A, Ivanova E, Perna I, Lin E, Mani V, Jiang S, McNamara K, Zaghlul S, Edkins S, Stevens C, Brennan C, Martin ES, Wiedemeyer R, Kabbarah O, Nogueira C, Histen G, Aster J, Mansour M, Duke V, Foroni L, Fielding AK, Goldstone AH, Rowe JM *et al.* **Chromosomally unstable mouse tumors have genomic alterations similar to diverse human cancers.** *Nature* 2007, **447**(7147):966–971.
44. George RE, Attiyeh EF, Li S, Moreau LA, Neuberg D, Li C, Fox EA, Meyerson M, Diller L, Fortina P, Look AT, Maris JM: **Genome-wide analysis of neuroblastomas using high-density single nucleotide polymorphism arrays.** *PLoS One* 2007, **2**(2):e255.
45. Northcott PA, Nakahara Y, Wu X, Feuk L, Ellison DW, Croul S, Mack S, Kongkham PN, Peacock J, Dubuc A, Ra YS, Zilberberg K, McLeod J, Scherer SW, Sunil Rao J, Eberhart CG, Grajkowska W, Gillespie Y, Lach B, Grundy R, Pollack IF, Hamilton RL, Van Meter T, Carlotti CG, Boop F, Bigner D, Gilbertson RJ, Rutka JT, Taylor MD: **Multiple recurrent genetic events converge on control of histone lysine methylation in medulloblastoma.** *Nat Genet* 2009, **4:**465–472.
46. Kotliarov Y, Steed ME, Christopher N, Walling J, Su Q, Center A, Heiss J, Rosenblum M, Mikkelsen T, Zenklusen JC, Fine HA: **High-resolution global genomic survey of 178 gliomas reveals novel regions of copy number alteration and allelic imbalances.** *Cancer Res* 2006, **66**(19):9428–9436.

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Brief Correspondence

MicroRNA Expression Profile of Primary Prostate Cancer Stem Cells as a Source of Biomarkers and Therapeutic Targets

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Abstract

MicroRNA (miRNA) expression profiles were generated from prostate epithelial subpopulations enriched from patient-derived benign prostatic hyperplasia ($n = 5$), Gleason 7 treatment-naive prostate cancer (PCa) ($n = 5$), and castration-resistant PCa (CRPC) ($n = 3$). Microarray expression was validated in an independent patient cohort ($n = 10$). Principal component analysis showed that miRNA expression is clustered by epithelial cell phenotype, regardless of pathologic status. We also discovered concordance between the miRNA expression profiles of unfractionated epithelial cells from CRPCs, human embryonic stem cells (SCs), and prostate epithelial SCs (both benign and malignant). MiR-548c-3p was chosen as a candidate miRNA from this group to explore its usefulness as a CRPC biomarker and/or therapeutic target. Overexpression of miR-548c-3p was confirmed in SCs (fivefold, $p < 0.05$) and in unfractionated CRPCs (1.8-fold, $p < 0.05$). Enforced overexpression of miR-548c-3p in differentiated cells induced stemlike properties ($p < 0.01$) and radioresistance ($p < 0.01$). Reanalyses of published studies further revealed that miR-548c-3p is significantly overexpressed in CRPC ($p < 0.05$) and is associated with poor recurrence-free survival ($p < 0.05$), suggesting that miR-548c-3p is a functional biomarker for PCa aggressiveness. Our results validate the prognostic and therapeutic relevance of miRNAs for PCa management while demonstrating that resolving cell-type and differentiation-specific differences is essential to obtain clinically relevant miRNA expression profiles.

Patient summary: We report microRNA (miRNA) expression profiles of epithelial cell fractions from the human prostate, including stem cells. miR-548c-3p was revealed as a functional biomarker for prostate cancer progression. The evaluation of miR-548c-3p in a larger patient cohort should yield information on its clinical usefulness.

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The identification of improved biomarkers and treatment strategies for castration-resistant prostate cancer (CRPC) remains a priority in prostate cancer (PCa) research. Since their discovery, microRNAs (miRNAs) have shown promise in both fields [1]. Indeed, miRNA-focused research has yielded >2000 patents and several clinical trials for cancer management [2]; however, clinical translation of miRNA as a PCa biomarker and/or as a novel therapeutic target

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remains more limited. This situation is perhaps because of the considerable heterogeneity and discrepancies in PCa miRNA expression profiles [1,3]. Most miRNA expression patterns are cell type–specific, but they also change with cellular differentiation status, even in cancer [4]. We set out to investigate whether the failure to resolve cell type–specific and differentiation-specific differences has contributed to the significant variations in published PCa miRNA profiles.

We have previously shown that a $CD133^{+}\alpha_2\beta_1{}^{hi}$ subpopulation enriched from benign and cancerous prostate tissue expresses high levels of CD44 and exhibits stem cell (SC) properties [5,6]. Genome-wide miRNA expression analysis was performed on patient-derived stemlike cells (SC-$CD133^{+}\alpha_2\beta_1{}^{hi}$), transit-amplifying cells (TA-$CD133^{-}\alpha_2\beta_1{}^{hi}$), and committed basal (CB) cells (CB-$CD133^{-}\alpha_2\beta_1{}^{lo}$) enriched from briefly cultured primary prostate epithelial cells (Fig. 1a, Supplement, Supplementary Table 1) [5,6]. The validity of miRNA expression data was confirmed by examining the expression patterns of 11 randomly selected miRNAs using quantitative reverse transcription polymerase chain reaction analysis (Supplementary Fig. 1). Subsequent principal component analysis clearly demonstrated that each subpopulation, regardless of its pathologic status, had a distinct miRNA expression profile (Fig. 1a). The magnitude and the extent of differential miRNA expression in SCs compared with CB cells were also significantly higher than in benign prostatic hyperplasia (BPH) versus PCa or in BPH versus CRPC, indicating that the differentiation stage of a prostate epithelial cell is the primary determinant of its miRNA expression profile.

Further examination of the miRNA expression profiles led to the following interpretations. First, a prostate epithelial SC signature is conserved in BPH, PCa, and CRPC (Supplementary Table 2), suggesting that miRNAs may primarily regulate core SC properties (self-renewal, prolonged proliferation, and differentiation capability), which are common for the SC phenotype regardless of its pathologic status. Second, conserved prostate SC miRNA signatures share their miRNA expression pattern with human embryonic SCs (hESCs) [7], for example, higher expression of miR-302/372 families and suppression of the let-7 family (Table 1). Third, there is an overlap of approximately 60% between the miRNA expression profiles of SCs and those of previously published unfractionated CRPCs [8] (Fig. 1b). Several of these shared miRNAs potentially regulate key SC and cancer-associated proteins; for example, miRNAs potentially regulating c-MYC, KLF4, NANOG, and EZH2 are all suppressed in SCs and CRPCs. Fourth, it is possible to distinguish between PCa–cancer stemlike cell (CSC), CRPC-CSC, and normal SC signatures, as well as signatures from their respective differentiated progeny (Table 1). Fifth, composite PCa and CRPC miRNA signatures identified in this paper contain several previously known onco-miRs and tumour suppressor miRNAs (eg, miR-629 and miR-203) (Supplementary Table 3).

Our miRNA expression analysis of patient-derived prostate epithelial subpopulations has therefore identified several novel PCa-CSC–specific and CRPC-CSC–specific miRNA candidates. The analyses also identified previously well-established miRNAs associated with PCa (eg, consistent suppression of miR-299–5p, which is downregulated in metastatic cell lines compared with normal prostate epithelial cells) [9], CRPC (eg, miR-521, whose inhibition in LNCaP cells enabled acquisition of a radioresistant phenotype) [10], and CSCs (eg, miR-708, whose suppression allows upregulation of CD44 and Akt in prostrate xenograft–derived cells) [11]. These correlations also imply that the hESC maintenance program is partly conserved in adult human prostate epithelial SCs at the miRNA level, which is in turn hijacked by the malignant cells in CRPCs.

To illustrate the relevance of our data set, we decided to investigate the role of miR-548c-3p during prostate epithelial differentiation and carcinogenesis (based on criteria described in Supplementary Fig. 2). This miRNA is overexpressed approximately fivefold in prostate epithelial SCs compared with CBCs (Supplementary Fig. 3), and its overexpression has been associated with poor survival of PCa patients [12] ($p = 0.0389$, log-rank test) (Fig. 1c). Overexpression of miR-548c-3p in CB cells (Supplementary Fig. 3) resulted in dedifferentiation to a more stemlike phenotype as (1) the colony-forming efficiency increased by approximately 75%, which is a commonly used indicator for SC self-renewal (Fig. 1d); (2) expression of the prostate epithelial stem/progenitor cell proteins CD49b (integrin β_2) and CD49f (integrin β_6) increased by 50–80% (Fig. 1e); (3) there was an increase in mRNA expression of multiple SC-specific genes with a concomitant reduction in CB cell–specific genes (Supplementary Fig. 3); and (4) CB cells became radioresistant, as an increase in live cell count of approximately 25% was noted 48 h after exposure to 5-Gy radiation (Fig. 1f).

Analyses of potential miR-548c-3p targets (Supplementary Fig. 4), together with our functional data, implicate miR-548c-3p in SC maintenance and cell cycle regulation. An independent study has shown that over-expression of miR-548c-3p decreased doxorubicin-induced DNA damage in cervical cancer cell line (HeLa cells through inhibition of topoisomerase (DNA) II alpha 170kDa (*TOP2A*) [13]. A reduction in DNA damage, an increase in cell proliferation, and the acquisition of stemlike properties have all been reported in CRPCs. We indeed found miR-548c-3p to be significantly upregulated in uncultured CRPC-derived epithelial cells compared with BPH-derived epithelial cells (Fig. 1g), which eliminated the possibility of cell culture artifact. Others have further demonstrated that serum obtained from CRPC patients contained 2.8-fold higher miR-548c-3p levels compared with serum derived from low-risk PCa patients [14]. These results attest to the importance of miR-548c-3p as a strong diagnostic and prognostic candidate to improve CRPC patient management. Clinical validation in a larger patient cohort is now necessary to establish therapeutic relevance.

The molecular programs that drive epithelial SC lineage commitment toward a differentiated phenotype (in an adult human prostate) remain unexplained. This analysis

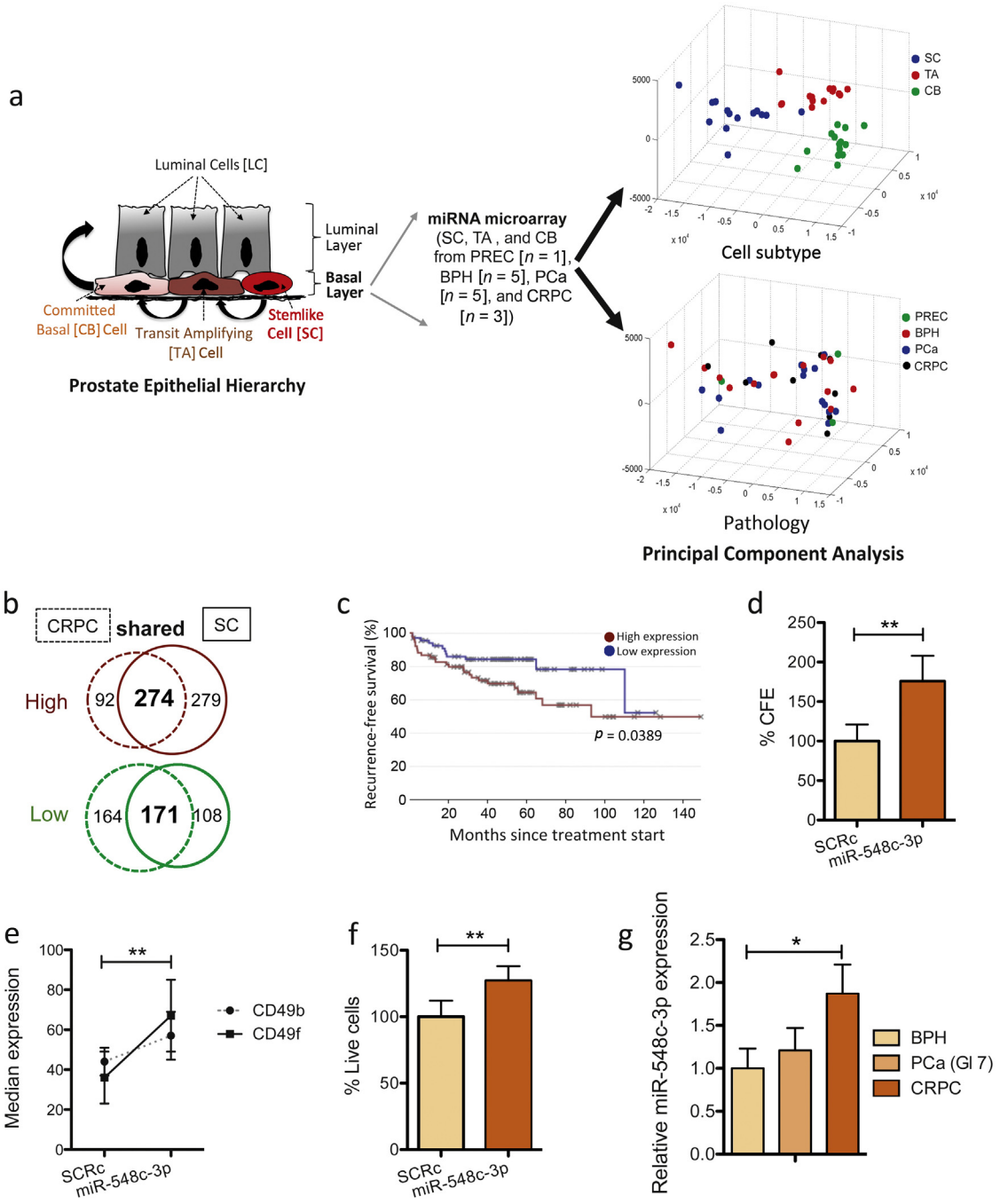

Fig. 1 – Cell subtype, rather than pathologic status, is a primary determinant of microRNA (miRNA) expression. (a) A schematic of human prostate epithelial hierarchy (left) showing a stemlike cell with a basal phenotype subsequently differentiating into luminal cells by way of committed basal (CB) cells. The subpopulations were enriched from normal human prostate epithelial cells, benign prostatic hyperplasia (BPH), and cancers (high Gleason grade, treatment-naive prostate cancer [PCa], and castration-resistant PCa [CRPC]). Principal component analysis was performed on miRNA microarray profiles of cultured stem cells (SCs) and CB cells at passage 2 (right). (b) Comparison of miRNA expression profiles for unfractionated CRPC tissue (vs BPH) [8] and prostate SC (vs CB). (c) Kaplan-Meier curve for PCa patient survival with differential miR-548c-3p expression using Taylor et al. [12]. (d) Colony-forming efficiency of miR-548c-3p transfected CB cells ($n = 3$ for BPH, $n = 3$ for PCa; each sample in triplicate). (e) Fluorescence-activated cell sorting analysis for CD49b (integrin β_2) and CD49f (integrin β_6) expression performed on CB cells transfected with either control or miR-548c-3p for 3 d ($n = 3$ for BPH, $n = 3$ for PCa; each sample in triplicate). (f) Live cell count of miR-548c-3p transfected CB cells 48 h after exposure to 5-Gy radiation ($n = 3$ for BPH, $n = 3$ for PCa; each sample in triplicate). (g) Quantitative reverse transcription polymerase chain reaction analysis for miR-548c-3p expression in epithelial cells enriched from freshly disaggregated uncultured BPH tissue ($n = 3$), PCa Gleason grade 7 tissue ($n = 5$), and CRPC tissue ($n = 3$). Each sample was assessed in triplicate. Data are expressed as mean plus or minus standard deviation. * $p < 0.05$ (student t test); ** $p < 0.01$ (student t test); *** $p < 0.001$ (student t test).

BPH = benign prostatic hyperplasia; CFE = colony-forming efficiency; CRPC = castration-resistant prostate cancer; miRNA = microRNA; PCa = prostate cancer; PREC = prostate epithelial cell.

Table 1 – MicroRNA signatures of conserved stem cells, prostate cancer stemlike cells, and castration-resistant prostate cancer stemlike cells

SC signature	Specific PCa CSC signature	Specific CRPC CSC signature
Upregulated	Upregulated	Upregulated
miR-548c-3p	miR-323-3p	miR-143
miR-484	miR-411*	miR-362-5p
miR-302 family	miR-33a*	miR-214*
miR-371 family	miR-532-3p	let-7i*
Downregulated	miR-181a-2*	miR-542-5p
miR-99a/100	miR-1271	miR-1913
miR-143	miR-487b	miR-136
miR-145	Downregulated	miR-545
miR-10 family	miR-302c	miR-516a-5p
miR-8 family	miR-1181	Downregulated
miR-17-92 family	miR-519c-3p	miR-125b-2*
let-7 family	miR-574-5p	miR-708

CRPC = castration-resistant prostate cancer; CSC = cancer stemlike cell; PCa = prostate cancer; SC = stem cell.
* Indicates the non-predominant product of a specific miRNA locus.

provides the first comprehensive input toward enabling an understanding of key miRNA expression changes during prostate epithelial differentiation. The overlap between the miRNA expression patterns of hESCs, prostate epithelial SCs, and unfractionated CRPCs clearly illustrates that embryonic signalling machinery is activated in the terminal stages of PCa.

In conclusion, our investigation identifies the failure to resolve cell subtype–specific miRNA expression differences as one of the reasons behind previously observed heterogeneous miRNA expression profiles of unfractionated prostate tumours. The data also provide novel and clinically relevant miRNA-based therapeutic candidates, including miR-548c-3p, for the management of CRPCs and CSCs. Further integration of this miRNA data set with mRNA data obtained from similarly fractioned subpopulations from PCa and CRPC should now enable the resolution of multidimensional transcriptional interrelationships in human prostate epithelium.

Author contributions: Norman J. Maitland had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Rane, Pellacani, Visakorpi, Maitland.
Acquisition of data: Rane, Scaravilli, Ylipää.
Analysis and interpretation of data: Rane, Ylipää, Pellacani, Nykter, Visakorpi, Maitland.
Drafting of the manuscript: Rane, Maitland.
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Statistical analysis: Rane, Ylipää.
Obtaining funding: Visakorpi, Maitland.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2014.09.005.

References

[1] Catto JW, Alcaraz A, Bjartell AS, et al. MicroRNA in prostate, bladder, and kidney cancer: a systematic review. Eur Urol 2011;59:671–81.
[2] van Rooij E, Purcell AL, Levin AA. Developing microRNA therapeutics. Circ Res 2012;110:496–507.
[3] Coppola V, De Maria R, Bonci D. MicroRNAs and prostate cancer. Endocr Relat Cancer 2010;17:F1–7.
[4] Lujambio A, Lowe SW. The microcosmos of cancer. Nature 2012;482:347–55.
[5] Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65:10946–51.
[6] Richardson GD, Robson CN, Lang SH, Neal DE, Maitland NJ, Collins AT. CD133, a novel marker for human prostatic epithelial stem cells. J Cell Sci 2004;117:3539–45.
[7] Leonardo TR, Schultheisz HL, Loring JF, Laurent LC. The functions of microRNAs in pluripotency and reprogramming. Nat Cell Biol 2012;14:1114–21.
[8] Jalava SE, Urbanucci A, Latonen L, et al. Androgen-regulated miR-32 targets BTG2 and is overexpressed in castration-resistant prostate cancer. Oncogene 2012;31:4460–71.
[9] Formosa A, Markert EK, Lena AM, et al. MicroRNAs, miR-154, miR-299-5p, miR-376a, miR-376c, miR-377, miR-381, miR-487b, miR-485-3p, miR-495 and miR-654-3p, mapped to the 14q32.31 locus, regulate proliferation, apoptosis, migration and invasion in metastatic prostate cancer cells. Oncogene 2014;33:5173–82.
[10] Josson S, Sung SY, Lao K, Chung LW, Johnstone PA. Radiation modulation of microRNA in prostate cancer cell lines. Prostate 2008;68:1599–606.
[11] Saini S, Majid S, Shahryari V, et al. miRNA-708 control of CD44(+) prostate cancer-initiating cells. Cancer Res 2012;72:3618–30.
[12] Taylor B, Schultz N, Hieronymus H, et al. Integrative genomic profiling of human prostate cancer. Cancer Cell 2010;18:11–33.
[13] Srikantan S, Abdelmohsen K, Lee EK, et al. Translational control of TOP2A influences doxorubicin efficacy. Mol Cell Biol 2011;31:3790–801.
[14] Nguyen HC, Xie W, Yang M, et al. Expression differences of circulating microRNAs in metastatic castration resistant prostate cancer and low-risk, localized prostate cancer. Prostate 2013;73:346–54.

MiR-1247-5p is Overexpressed in Castration Resistant Prostate Cancer and Targets MYCBP2

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BACKGROUND. Recently, there has been increasing attention on the role of microRNAs (miRNAs) in cancer development. Several expression profiling studies have provided evidence of aberrant expression of miRNAs in prostate cancer and have highlighted the potential use of specific miRNA expression signatures as prognostic or predictive markers. Here we report an expression analysis of miR-1247–5p, miR-1249, miR-1269a, miR-1271–5p, miR-1290, miR-1291, and miR-1299.

METHODS. qRT-PCR was performed to validate the differential expression of miRNAs in clinical samples, and the effect of miR-1247–5p was studied in prostate cancer cell lines transiently transfected with a miR-1247–5p mimic. The expression of miR-1247–5p's putative target *MYCBP2* was evaluated by qRT-PCR and Western blotting, and the interaction of the miRNA with the target gene was assessed using a luciferase assay.

RESULTS. We found a significant up-regulation of miR-1247–5p in castration-resistant prostate cancer (CRPC) samples compared to non-malignant prostate. The expression of miR-1247–5p was subsequently studied in prostate cancer (PC) cell lines where an up-regulation of miR-1247–5p was observed in the androgen-independent PC-3 model. Target prediction analysis for miR-1247–5p performed online revealed that MYCBP2 (myc-binding protein 2) was a high-scoring potential target. Functional studies in vitro performed using PC-3 and LNCaP models confirmed the down-regulation of MYCBP2 at the mRNA and protein levels, and a luciferase assay showed interaction between the miRNA and target gene.

CONCLUSION. miR-1247–5p is overexpressed in CRPC and targets MYCBP2. *Prostate*

KEY WORDS: **microRNA; castration resistant prostate cancer; *MYCBP2***

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INTRODUCTION

Prostate cancer (PC) is the most frequently diagnosed cancer among males in developed countries [1]. Despite the fact that surgery and/or radiation therapy are effective treatments for early stage disease, 30–40% of cases will progress to advanced disease. For advanced disease, androgen-deprivation is initially highly efficient, but patients will eventually develop castration-resistant prostate cancer (CRPC), which remains incurable [2–4]. Therefore, a deeper understanding of the mechanisms responsible for disease progression is needed to develop more effective therapeutic strategies. MicroRNAs (miRNAs) are short, single-stranded RNA molecules that are not translated into proteins [5] but function in the regulation of gene expression by repressing target mRNAs. Together with Ago proteins, mature miRNAs form a complex called RISC (RNA-induced silencing complex) and bind complementary sequences usually located in the 3′-UTR region of target mRNAs, causing their degradation or translational inhibition [6–8]. One miRNA can potentially have an effect on the expression of a large number of target genes [9]. It is currently estimated that 30% of the human coding genes are regulated by miRNAs [10].

Several expression profiling studies have provided evidence on the differential expression of miRNAs in prostate cancer [11–16] and have investigated the role of individual miRNAs in the molecular mechanisms of disease progression [17]. Aberrant expression of miRNAs in prostate cancer is driven by different mechanisms, including chromosomal alterations, epigenetic changes, androgen receptor (AR) signaling, and transcription regulation. [18–22]. Dysregulation of miRNA expression leads to alterations in key cellular processes responsible for apoptosis, cell cycle regulation, cell proliferation and migration, with an overall effect of enhancing cell survival and tumor progression and invasion [17]. Thus, miRNAs effectively function as oncogenes or tumor suppressors depending on the overall effect on cell growth [23]. Moreover, because of the differential expression between normal and malignant tissues and the relatively high stability in severe conditions, miRNAs represent attractive candidates for the discovery of new diagnostic and prognostic markers [24–26]. Despite the considerable evidence of differential expression in cancer, the limiting step for a thorough understanding of the molecular function of miRNAs is the identification of downstream target genes [27].

We have previously used the Agilent microarray platform for the expression profiling of PC and CRPC [14,19]. However, those arrays contained probes for only 723 miRNAs and lacked probes for more recently discovered miRNAs. Thus, we utilized the data from a deep-sequencing of eleven pools, each containing four samples of normal or malignant prostates (Martens-Uzunova ES, Jenster G et al., submitted). Based on those data, we selected miR-1247–5p, miR-1249, miR-1269a, miR-1271–5p, miR-1290, miR-1291, and miR-1299, which showed differential expression between malignant and non-malignant prostates, for expression profiling with qRT-PCR. We found that miR-1247–5p was overexpressed in CRPC and validated *MYCBP2* as one target gene for the miRNA in prostate cancer cell lines.

MATERIALS AND METHODS

Cell Culture and miRNA Transfection

The prostate cancer cell lines PC3, DU145, LNCaP, 22Rv1, and VCaP were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured according to the recommended conditions. HT-1080 cells were a kind gift from Olli Lohi, Tampere Center for Child Health Research.

The cells were transfected with 20 nM or 100 nM of human miRVana^{TM} microRNA mimic for miR-1247–5p or negative control (Thermo Fisher Scientific/Ambion, Waltham, MA). The INTERFERin^{TM} transfection reagent (Polyplus-transfection, Illkirch, France) was used according to themanufacturer's instructions.

Clinical Material

Two sets of clinical samples were used for miRNA expression analysis and were both obtained from Tampere University Hospital (TAUH). The first set included 54 freshly frozen samples of 5 benign prostate hyperplasia (BPH) and 28 untreated primary prostate tumors obtained from radical prostatectomy specimens as well as 7 BPH and 14 CRPC tumors obtained from transurethral resection of the prostate (TURP). The second set included 81 hormonally untreated, freshly frozen PC prostatectomy samples. The samples were confirmed to contain a minimum of 70% cancerous or hyperplastic cells by hematoxylin-eosin staining. The mean age at diagnosis for the second set of samples was 62.1 years (range: 47.4–71.8) and the mean PSA at diagnosis was 11.8 (range: 3.15–51.5). The use of clinical material was approved by the ethical committee of the Tampere University Hospital. Written informed consent was obtained from the subjects donating the samples.

TRI-reagent (Molecular Research Center Inc., Cincinnati, OH) was used to collect total RNA from

the freshly frozen clinical samples and cell lines, according to the manufacturer's instructions.

qRT-PCR

aqMan® microRNA assays (Thermo Fisher Scientific, Waltham, MA) were used to study the expression of selected miRNAs, according to the manufacturer's protocol. The analysis was performed on CFX96 qPCR equipment (Bio-Rad Laboratories, Hercules, CA), and the raw expression data were normalized against *RNU6B*.

Expression analysis of *MYCBP2* and *SOX9* was performed using Maxima™ SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA) on the same equipment. Specific primers for *MYCBP2* and *SOX9* were designed based on the internet database Primer Bank: *MYCBP2*_for 5′-GGGGACGGATTCTACCCAG-3′ and *MYCBP2*_rev 5′-ATTGAGCGCAGCGGTATAAAT-3′; *SOX9*_for 5′-AGCGAACGCACATCAAGAC-3′ and *SOX9*_rev 5′-CTGTAGGCGATCTGTTGGGG-3′. The raw expression data were normalized against *TBP* (*TBP* for 5′-GAATATAATCCCAAGCGGTTTG-3′ and *TBP* rev 5′-ACTTCACATCACAGCTCCCC-3′).

Western Blot

Total proteins were extracted from cell lines using RIPA lysis buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 4% polyacrylamide gel. The proteins were subsequently wet-transferred to Whatman™ nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). The membranes were incubated for 2 hr with a rabbit polyclonal antibody against MYCBP2 (ab86078, Abcam, Cambridge, UK), a rabbit polyclonal against SOX9 (ab26414, Abcam, Cambridge, UK), a mouse monoclonal against vinculin as a loading control for MYCBP2 (ab18058, Abcam, Cambridge, UK) and a mouse monoclonal against pan actin as a loading control for SOX9 (NeoMarkers, Freont, CA). After washing, the membranes were incubated with secondary antibodies (anti-rabbit IgG-horseradish peroxidase-conjugated and anti-mouse IgG-horseradish peroxidase-conjugated (Dako, Glostrup, Denmark)), and the protein bands were visualized using the Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA). The density of the protein bands was quantified using ImageJ, image processing and analysis software (http://imagej.nih.gov/ij/). The values for the MYCBP2 bands were normalized against vinculin, and the values for the SOX9 bands were normalized against pan actin. Each experiment was performed in duplicate.

Luciferase Assay

A luciferase assay was performed using SwitchGear Genomics GoClone reporter constructs co-transfected with a LightSwitch miRNA mimic and non-targeting control (SwitchGear Genomics, Menlo Park, CA) according to the manufacturer's instructions. In brief, HT-1080 human fibrosarcoma cells were seeded overnight to yield 90% confluence in a 96-well plate. The cells were subsequently co-transfected with 30 ng/μl of individual GoClone reporter vectors (3′-UTR sequence for MYCBP2; 3′-UTR for ACTB (beta-actin); random 3′-UTR; empty vector control) and 100 nM of the miR-1247–5p mimic or non-targeting control, using the DharmaFECT Duo transfection reagent (Thermo Fisher Scientific, Waltham, MA). Each transfection was repeated for a total of 8 replicates per sample. The next day, 100 μl of the LightSwitch Assay Solution was added to each well of co-transfected cells and the luciferase signal was measured on a Wallac EnVision™ 2104 multilabel plate reader luminometer (Perkin Elmer, Waltham, MA), according to protocol settings. The difference in luciferase signal intensity for miR-1247–5p transfected cells was calculated for each construct versus the non-targeting control. Data from housekeeping, random and empty constructs were used to control for non-UTR-specific treatment effects.

Statistics

Significant differences of the qRT-PCR results were evaluated by Mann–Whitney *U*-test using GraphPad Prism statistics software (GraphPad Software Inc., La Jolla, CA). Student's *t*-test was used to evaluate the statistical significance of the luciferase assay. Spearman's rank correlation was used to compare reference genes for qRT-PCR normalization.

RESULTS

Normalization of miRNA Expression

To reliably normalize qRT-PCR expression values for miRNAs in clinical material, four commonly used reference genes (*RNU6B*,*RNU44*, *RNU24*, and *RNU48*) were assessed. We compared qRT-PCR expression data of five miRNAs, miR-17–5p, 32–5p, 96–5p, 141–5p and 182–5p normalized with these different RNUs, with our previously generated microarray [19] and unpublished small RNA deep-sequencing of individual sample data (Figs. S1 and S2). The normalization of qRT-PCR with RNU6B revealed data that was the most similar to that of microarray hybridization and deep-sequencing. Subsequently, we analyzed the individual expression of *RNU44*, *RNU24*, and *RNU48* in the same

set of samples, using *RNU6B* as a reference gene. Consistent with a previously published study [28], we found significant up-regulation of *RNU44, 24 and 48* in cancer compared with the normal samples, confirming *RNU6B* as the most stably expressed reference gene in our sample cohort (Fig. S3).

miRNA Expression Analysis

The expression of seven miRNAs, miR-1247–5p, miR-1249, miR-1269a, miR-1271–5p, miR-1290, miR-1291, and miR-1299, was first analyzed using qRT-PCR in the first sample set (Fig. 1A and Fig. S4). Most notably, miR-1247–5p was significantly up-regulated in CRPC samples compared to BPH. In the second sample set, consisting of 81 PCs obtained by radical prostatectomy, miR-1247–5p did not show an association with Gleason score, pathological stage of the disease (Fig. 1B), or prognosis (data not shown). Next, the expression of miR-1247–5p was measured in PC cell lines PC-3, DU145, LNCaP, 22Rv1, and VCaP and was found to be highly expressed in the androgen-independent PC-3 cells (Fig. 2A). miR-1290 showed a slight reduction in PC compared to BPH, but there was no difference in CRPC compared to BPH. The other miRNAs that were measured showed no significant differential expression between any of the groups (Fig. S4).

MYCBP2 is a Target of miR-1247–5p in Prostate Cancer Cell Lines

To identify putative target genes for miR-1247–5p, we queried the online target prediction programs TargetScan 6.2 (www.targetscan.org) and miRanda (www.micorrna.org). Both online tools identified *MYCBP2* (myc-binding protein 2) as a highest-scoring potential target gene, based on the sequence complementarity of miR-1247–5p with the 3′-UTR of the gene. Thus, we measured the expression of *MYCBP2* in the clinical samples and found it to be down-regulated in CRPC samples compared with BPH (Fig. 2B). In the prostate cancer cell lines, there was an inverse correlation between miR-1247–5p and *MYCBP2* (Fig. 2A). Next, we studied the effect of miR-1247–5p on *MYCBP2* mRNA levels in prostate cancer by transiently transfecting PC-3 and LNCaP cells (intrinsically expressing high and low levels of miR-1247–5p, respectively) with 100 nM miR-1247–5p mimic or negative control. First, overexpression of the miRNA was confirmed by qRT-PCR (Fig. 3A and B). We found a significant reduction in MYCBP2 mRNA levels in miR-1247–5p-transfected cells (Fig. 3A and B). MYCBP2 expression was studied at a protein level in PC-3 cells transiently transfected with 100 nM miR-1247–5p mimic or negative control, confirming the down-regulation in cells overexpressing miR-1247–5p (Fig. 3C).

A recent study has reported that miR-1247–5p downregulates the expression of the transcription factor SOX-9 in isolated human chondrocytes by non-canonical binding to the coding region of the gene and is downregulated by SOX-9 in a negative feedback loop [29]. Dysregulation of the *SOX9*-dependent pathway has been recently shown to induce senescence bypass and tumor invasion in prostate cancer [30]. In addition, it was recently reported that the expression of SOX-9 is induced in DU145 cells

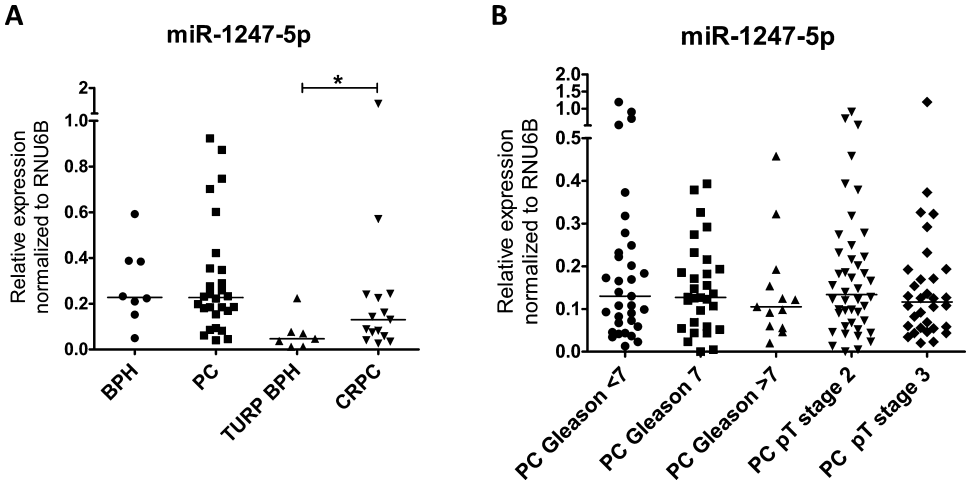

Fig. 1. Expression of miR-1247–5p (qRT-PCR) in clinical samples. (**A**) Set 1 consisting of 54 freshly frozen samples of 5 benign prostate hyperplasia (BPH) and 28 untreated primary prostate tumors (PC) obtained by radical prostatectomy and 7 BPH and 14 castration-resistant prostate cancer (CRPC) obtained by transurethral resection of the prostate. (**B**) Set 2 consisting of 81 hormonally untreated, freshly frozen PC prostatectomy samples. Graphs represent relative expression values, normalized against reference gene. (*$P < 0.05$).

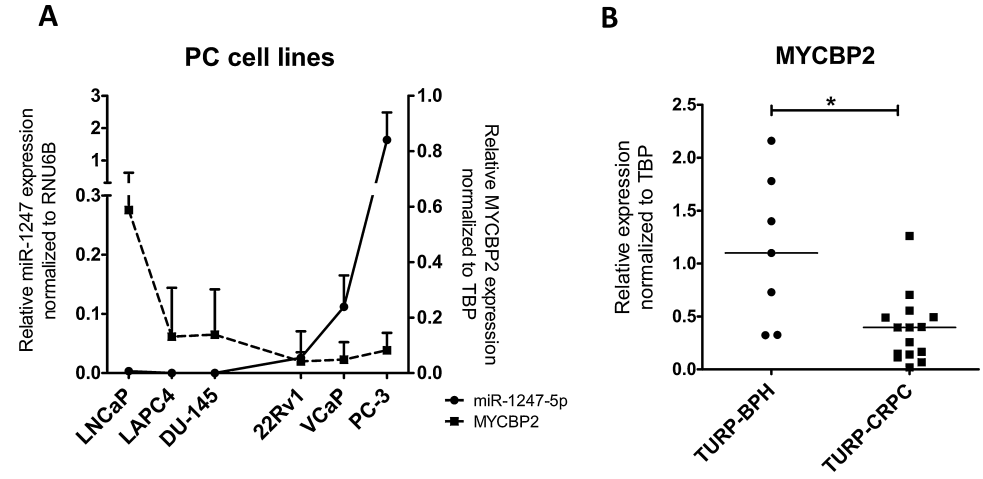

Fig. 2. (**A**) Expression of miR-1247–5p and *MYCBP2* in prostate cancer cell lines (relative expression values normalized against reference genes). (**B**) Expression of *MYCBP2* in clinical samples of TURP-BPH versus TURP-CRPC. Graph represents relative expression values normalized against TBP (*$P < 0.05$).

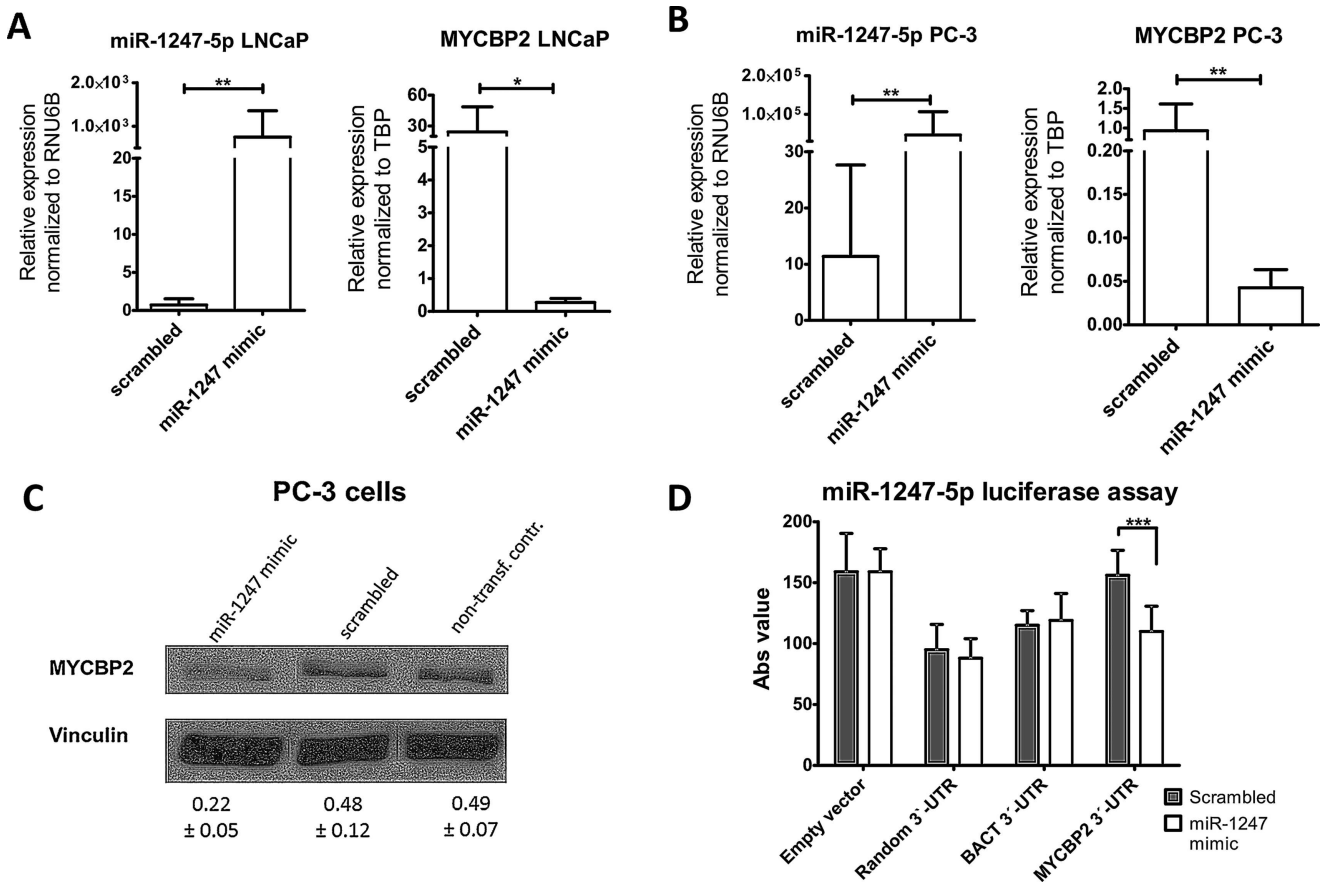

Fig. 3. qRT-PCR expression data for miR-1247–5p and *MYCBP2* in LNCaP (**A**) and PC-3 (**B**) cells, both transiently transfected with 100 nM miR-1247–5p mimic vs. negative control. Graphs represent relative expression values, normalized against reference gene. (*$P < 0.05$, **$P < 0.01$). (**C**) Western blot for MYCBP2 in PC-3 cells transiently transfected with 20 nM miR-1247–5p mimic versus negative control. Values shown represent mean of two replicate experiments for MYCBP2 protein intensity, normalized against loading control (Vinculin) ± S.D. (**D**) Absorbance values of Luciferase signal in HT-1080 cells cotransfected with 100 nM miR-1247–5p mimic versus negative control and 30 ng/μl MYCBP2 3′-UTR vector. Empty vector, ACTB 3′-UTR vector and random-sequence vector were used in the same conditions to control for non-UTR-specific cotransfection effects. Graph represents mean of 8 replicates ± S.D. (***$P < 0.001$).

stimulated with Hepatocyte Growth Factor (HGF) and contributes to phenotypic stem-like cell induction [31]. Thus, we decided to assess *SOX9* expression in miR-1247–5p transiently transfected PC-3 and LNCaP cells. We found a non-significant reduction in the level of *SOX9* mRNA, but no reduction was found at the protein level upon miR-1247–5p overexpression (Fig. S5).

To investigate the interaction between miR-1247–5p and putative target MYCBP2, a luciferase reporter assay was performed in HT-1080 cells co-transfected with a construct vector expressing the 3′-UTR of MYCBP2 downstream of the luciferase gene and a miR-1247–5p mimic or negative control. Vectors expressing the 3′-UTR of the housekeeping gene *ACTB* (beta-actin) or random sequences were used to control for non-specific interactions of the miR-1247–5p mimic. A significant reduction in the luciferase signal was observed in cells co-transfected with the MYCBP2 3′-UTR vector and the miR-1247–5p mimic compared to controls (Fig. 3D).

DISCUSSION

Accumulating evidence links altered miRNA expression patterns to prostate cancer tumorigenesis and tumor progression. Here, we studied the expression of 7 miRNAs in prostate cancer with qRT-PCR. The miRNAs were selected based on our deep-sequencing of pools of clinical samples of normal and malignant prostates (Martens-Uzunova ES, Jenster G, et al., submitted). Although most of the miRNAs showed an overall low expression level in qRT-PCR experiments, we discovered that miR-1247–5p was the most significantly differentially expressed and up-regulated miRNA in CRPC. We have recently performed small RNA deep sequencing of individual clinical samples belonging to the first sample set used here in the qRT-PCR (manuscript in preparation). That set of data also shows that miR-1247–5p is up-regulated in CRPC.

In a recent study, the expression profile of miR-1247–5p was investigated in pancreatic cancer, revealing downregulation in cancer compared to normal tissues. Neuropilin 1 (*NRP1*) and neuropilin 2 (*NRP2*) were also shown to be targets of miR-1247–5p by Western blotting and luciferase reporter assays [32]. Another study validated *SOX9* as a target of miR-1247–5p in human chondrocytes [29]. However, our data suggest that SOX9 is not the primary target of miR-1247–5p in prostate cancer, although a slight reduction in *SOX9* mRNA was seen in miR-1247–5p transfected cells. Instead, we showed interaction between miR-1247–5p and the 3′-UTR of *MYCBP2* and confirmed downregulation at both the mRNA and protein levels in prostate cancer cell lines transiently overexpressing miR-1247–5p. Therefore, the data suggest that miR-1247–5p might target different genes depending on the cell context.

We studied the effects of transient downregulation of miR-1247–5p in the same cell lines, using miR-1247–5p inhibitors, but we did not find significant expression changes in miR-1247–5p upon inhibition (data not shown), making the results of transient downregulation inconclusive.

MYCBP2 encodes a very large 510 kDa E3-ubiquitin ligase, also known as protein associated with myc (PAM). It was originally identified as a protein that interacts directly with the transcriptional activating domain of the transcription factor Myc [33]. However, there is no strong evidence that MYCBP2 is functionally associated with MYC. MYCBP2 is highly expressed in peripheral and central neurons [34], where it has been shown to be responsible for regulating neuronal outgrowth and synaptogenesis by regulating the cAMP [35], Smad4 [36], mTOR [37], and p38 MAPK-signaling pathways [38]. However, the role of MYCBP2 in cancer is currently unknown. Interestingly, recent data revealed the existence of a novel biological phenomenon in tumors called cancer-related axonogenesis and neurogenesis [39]. Nerve density is increased in cancer and in preneoplastic areas of the prostate compared to non-malignant areas confirming that cancer cells induce neurite outgrowth.

In conclusion, we have shown that miR-1247–5p is overexpressed in CRPC and targets MYCBP2. Further studies on the functional role of miR-1247–5p/MYCBP2 in the emergence of castration-resistant prostate cancer are now warranted.

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REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69–902.

2. Scher HI, Sawyers CL. Biology of progressive, castration-resistant prostate cancer: Directed therapies targeting the androgen-receptor signaling axis. J Clin Oncol 2005;23:8253–8261.
3. Seruga B, Ocana A, Tannock IF. Drug resistance in metastatic castration-resistant prostate cancer. Nat Rev Clin Oncol 2011;8-(1):12–23.
4. Hotte SJ, Saad F. Current management of castrate-resistant prostate cancer. Curr Oncol 2010;17(Suppl. 2):S72–S79.
5. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 2004;116:281–297.
6. Eulalio A, Huntzinger E, Izaurralde E. Getting to the root of miRNA-mediated gene silencing. Cell 2008;132:9–14.
7. Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature 2008;455:64–71.
8. Pillai RS, Artus CG, Filipowicz W. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. RNA 2004;10:1518–1525.
9. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs down-regulate large numbers of target mRNAs. Nature 2005;433:769–773.
10. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15–20.
11. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA 2006;103:2257–2261.
12. Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL, Visakorpi T. MicroRNA expression profiling in prostate cancer. Cancer Res 2007;67:6130–6135.
13. Ambs S, Prueitt RL, Yi M, Hudson RS, Howe TM, Petrocca F, Wallace TA, Liu CG, Volinia S, Calin GA, Yfantis HG, Stephens RM, Croce CM. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. Cancer Res 2008;68:6162–6170.
14. Martens-Uzunova ES, Jalava SE, Dits NF, van Leenders GJ, Møller S, Trapman J, Bangma CH, Litman T, Visakorpi T, Jenster G. Diagnostic and prognostic signatures from the small non-coding RNA transcriptome in prostate cancer. Oncogene 2012;31(8):978–991.
15. Szczyrba J, Löprich E, Wach S, Jung V, Unteregger G, Barth S, Grobholz R, Wieland W, Stöhr R, Hartmann A, Wullich B, Grässer F. The microRNA profile of prostate carcinoma obtained by deep sequencing. Mol Cancer Res 2010; 8:529–538.
16. Schaefer A, Jung M, Mollenkopf HJ, Wagner I, Stephan C, Jentzmik F, Miller K, Lein M, Kristiansen G, Jung K. Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. Int J Cancer 2010;126:1166–1176.
17. Fang YX, Gao WQ. Roles of microRNAs during prostatic tumorigenesis and tumor progression. Oncogene 2014;33-(2):135–147.
18. Ribas J, Ni X, Haffner M, Wentzel EA, Salmasi AH, Chowdhury WH, Kudrolli TA, Yegnasubramanian S, Luo J, Rodriguez R, Mendell JT, Lupold SE. MiR-21: An androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. Cancer Res 2009;69:7165–7169.
19. Jalava SE, Urbanucci A, Latonen L, Waltering KK, Sahu B, Jänne OA, Seppälä J, Lähdesmäki H, Tammela TL, Visakorpi T. Androgen-regulated miR-32 targets BTG2 and is overexpressed in castration-resistant prostate cancer. Oncogene 2012;31:4460–4471.
20. Shi XB, Xue L, Ma AH, Tepper CG, Kung HJ, White RW. MiR-125b promotes growth of prostate cancer xenograft tumor through targeting pro-apoptotic genes. Prostate 2011;71:538–549.
21. Porkka KP, Ogg EL, Saramäki OR, Vessella RL, Pukkila H, Lähdesmäki H, van Weerden WM, Wolf M, Kallioniemi OP, Jenster G, Visakorpi T. The miR-15a-miR-16-1 locus is homozygously deleted in a subset of prostate cancers. Genes Chromosomes Cancer 2011;50(7):499–509.
22. Rauhala HE, Jalava SE, Isotalo J, Bracken H, Lehmusvaara S, Tammela TL, Oja H, Visakorpi T. MiR-193b is an epigenetically regulated putative tumor suppressor in prostate cancer. Int J Cancer 2010;127(6):1363–1372.
23. Zhang B, Pan X, Cobb GP, Anderson TA. MicroRNAs as oncogene and tumor suppressors. Dev Biol 2007;302:1–12.
24. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA 2008;105:10513–10518.
25. Yun SJ, Jeong P, Kim WT, Kim TH, Lee YS, Song PH, Choi YH, Kim IY, Moon SK, Kim WJ. Cell-free microRNAs in urine as diagnostic and prognostic biomarkers of bladder cancer. Int J Oncol 2012;41:1871–1878.
26. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Zhang Y, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Wang J, Zen K, Zhang J, Zhang CY. Characterization of microRNAs in serum: A novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 2008;18:997–1006.
27. O'Kelly F, Marignol L, Meunier A, Lynch TH, Perry AS, Hollywood D. MicroRNAs as putative mediators of treatment response in prostate cancer. Nat Rev Urol 2012;9(7):397–407.
28. Gee HE, Buffa FM, Camps C, Ramachandran A, Leek R, Taylor M, Patil M, Sheldon H, Betts G, Homer J, West C, Ragoussis J, Harris AL. The small-nucleolar RNAs commonly used for microRNA normalisation correlate with tumour pathology and prognosis. British Journal of Cancer 2011;104(7):1168–1177.
29. Martinez-Sanchez A, Murphy CL. MiR-1247–5p functions by targeting cartilage transcription factor SOX9. J Biol Chem 2013;288(43):30802–30814.
30. Wang G, Lunardi A, Zhang J, Chen Z, Ala U, Webster KA, Tay Y, Gonzalez-Billalabeitia E, Egia A, Shaffer DR, Carver B, Liu XS, Taulli R, Kuo WP, Nardella C, Signoretti S, Cordon-Cardo C, Gerald WL, Pandolfi PP. Zbtb7a suppresses prostate cancer through repression of a Sox9-dependent pathway for cellular senescence bypass and tumor invasion. Nat Genet 2013;45-(7):739–746.
31. van Leenders GJ, Sookhlall R, Teubel WJ, de Ridder CM, Reneman S, Sacchetti A, Vissers KJ, van Weerden W, Jenster G.

Activation of c-MET induces a stem-like phenotype in human prostate cancer. Plos One 2011;6(11):e26753.

32. Shi S, Lu Y, Qin Y, Li W, Cheng H, Xu Y, Xu J, Long J, Liu L, Liu C, Yu X. MiR-1247-5p is correlated with prognosis of pancreatic cancer and inhibits cell proliferation by targeting neuropilins. Curr Mol Med 2014;14(3):316–327.
33. Guo Q, Xie J, Dang CV, Liu ET, Bishop JM. Identification of a large Myc-binding protein that contains RCC1-like repeats. Proc Natl Acad Sci USA 1998;95(16):9172–9177.
34. Yang H, Scholich K, Poser S, Storm DR, Patel TB, Goldowitz D. Developmental expression of PAM (protein associated with MYC) in the rodent brain. Brain Res Dev Brain Res 2002;136:35–42.
35. Scholich K, Pierre SC, Patel TB. Protein associated with Myc (PAM) is a potent inhibitor of adenylyl cyclases. J Biol Chem 2001;276:47583–47589.
36. McCabe BD, Hom S, Aberle H, Fetter RD, Marques G, Haerry TE, Wan H, O'Connor MB, Goodman CS, Haghighi AP. Highwire regulates presynaptic BMP signaling essential for synaptic growth. Neuron 2004;41:891–905.
37. Han SWR, Santos TM, Polizzano C, Sabatini BL, Ramesh V. Pam (Protein associated with Myc) functions as an E3 Ubiquitin ligase and regulates TSC/mTOR signaling. Cell Signal 2008;20:1084–1091.
38. Nakata KAB, Grill B, Goncharov A, Huang X, Chisholm AD, Jin Y. Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. Cell 2005;120:407–420.
39. Ayala GE, Dai H, Powell M, Li R, Ding Y, Wheeler TM, Shine D, Kadmon D, Thompson T, Miles BJ, Ittmann MM, Rowley D. Cancer-related axonogenesis and neurogenesis in prostate cancer. Clin Cancer Res 2008;14(23):7593–7603.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

TITLE: A comprehensive repertoire of tRNA-derived fragments in prostate cancer

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ABSTRACT

Prostate cancer (PCa) is the most common cancer among men in developed countries. Although its genetic background is thoroughly investigated, rather little is known about the role of small non-coding RNAs (sncRNA) in this disease. tRNA-derived fragments (tRFs) represent a new class of sncRNAs, which are present in a broad range of species and have been reported to play a role in several cellular processes. Here, we analyzed the expression of tRFs in fresh frozen patient samples derived from normal adjacent prostate and different stages of PCa by RNA-sequencing. We identified 598 unique tRFs, many of which are deregulated in cancer samples when compared to normal adjacent tissue. Most of the identified tRFs are derived from the 5' and 3' end of mature cytosolic tRNAs, but we also found tRFs produced from other parts of tRNAs, including pre-tRNA trailers and leaders, as well as tRFs from mitochondrial tRNAs. The 5'-derived tRFs comprise the most abundant class of tRFs in general and represent the major class among upregulated tRFs. 3'-derived tRFs types are dominant among downregulated tRFs in PCa. We validated the expression of three tRFs using qPCR. The ratio of tRFs derived from $tRNA^{LysCTT}$ and $tRNA^{PheGAA}$ emerged as a good indicator of progression-free survival and a candidate prognostic marker. This study provides a systematic catalogue of tRFs and their dysregulation in PCa and can serve as the basis for further research on the biomarker potential and functional role of tRFs in this disease.

INTRODUCTION

Prostate cancer (PCa) is the second most common cancer in men worldwide [1]. The treatment of PCa is hampered by the lack of reliable markers for disease outcome prediction leading to incorrect patient stratification, overtreatment and consequent side effects from prostatectomy and radiation therapy [2]. A better understanding of the molecular mechanisms behind the onset and progression of PCa is needed in order to discover better markers and develop new therapeutic strategies. The role of small non-coding RNAs (sncRNAs) other than microRNAs (miRNAs) in PCa is poorly understood. The rapid progress and popularity of high throughput sequencing led to the discovery of a novel class of sncRNAs derived from tRNAs and named tRNA-derived fragments (tRFs) [3-5]. tRFs are present across all domains of life [6-8]. While initially considered random degradation products of tRNA turnover, their abundance and ubiquitous expression suggest that tRFs are actual biological entities [6, 7].

tRFs are generated by endonucleases such as ribonuclease T2 (Rny1p) in yeast and angiogenin or dicer 1 in human. Based on size, they can be divided into two groups. The first group consists of tRFs with a size of 30 to 35 nt, which are generally referred to as tRNA halves or stress-induced tRFs. tRNA halves are produced by endonucleolytic cleavage at the anticodon loop of the full-length tRNA. The second group consists of tRFs with a size of about 20 nt and can be further divided into 5'- and 3'-derived tRFs, originating from the 5′- and 3′-parts of mature tRNAs, respectively [4, 9, 10]. The small RNAs derived from the 5'-leader and 3'-trailer sequences of the precursor tRNAs (pre-tRNAs) are also classified as tRFs [5, 11, 12].

Expression of tRFs is detected in different cancer cell lines, including the PCa cell lines LNCaP and C4-2 [4, 5, 13-15]. In a previous study, we reported the discovery and differential expression of tRFs in clinical samples of PCa [16]. This suggests that tRFs might play an important role in the pathogenesis of cancer. The mechanism behind the function of tRFs appears to be diverse. Several reports demonstrate that tRF levels are elevated by cellular stress conditions and particularly under oxidative stress such as hypoxia [10, 13, 15, 17]. tRFs are also involved in post-transcriptional regulation of gene expression via direct inhibition of protein synthesis by displacing the eIF4G translation initiation factor from mRNA [18-20]. Moreover, a 3′-derived tRF identified in B-cell lymphoma cells possesses the functional characteristics of a guide RNA that suppresses proliferation and modulates response to DNA damage in a miRNA-fashion [21]. It has also been shown that tRFs can compete for the binding sites of the RNA-binding protein YBX1, which is involved in the stabilization of oncogenic transcripts suppressing cell growth and invasion [15]. In this way, tRFs antagonize the activity of YBX1 and act as tumor suppressors. Taken together, these findings strongly suggest a functional role of tRFs in tumorigenesis.

Very recently, it was proposed that although tRFs are defined biological entities, their composition and abundance in the transcriptome is dependent on gender, tissue, disease and even disease subtype [22].This suggests that tRFs can be explored as novel sensitive biomarkers of disease. Yet, studies providing systematic insight into the composition and expression of the tRF transcriptome throughout various disease stages are still missing. Here, we analyze tRF expression in an extended cohort of clinical samples representing progressing stages of PCa. We construct a database of tRFs expressed across PCa samples and identify the

most differentially expressed tRFs. Finally, we perform a qPCR quantification in two cohorts of clinical samples to validate the differential expression of selected tRFs.

RESULTS

Inventory of tRFs expressed in PCa

In order to obtain a global overview of the tRF repertoire in PCa, we analyzed tRFs across normal adjacent prostate (NAP), benign-prostate hyperplasia (BPH), PCa from radical prostatectomies, trans-urethral resected tissue from castration resistant PCa (TURP_PCa), and lymph node metastasis (LN_PCa) using next-generation RNA sequencing (Table 1). All 21 cytosolic tRNA isotypes (including selenocystein tRNAs) were found to produce tRFs in variable amounts (Figure 1A). $tRNA^{Ala}$ and $tRNA^{Lys}$ showed the highest numbers of mapped tRFs, while the least tRFs were produced from $tRNA^{Ile}$ and $tRNA^{Asp}$. The raw sum of tRFs weakly correlated with the number of tRNA genes per isotype or anticodon, as well as with the percentage of codon usage (Supplementary Figure 1; codon usage from http://gtrnadb.ucsc.edu/Hsapi19/Hsapi19-summary-codon.html). tRFs derived from 15 out of 20 mitochondrial tRNAs (mtRNAs) were also detected (Figure 1A). We could not detect tRFs corresponding to the mitochondrial tRNA isotypes $mtRNA^{Gln}$, $mtRNA^{Glu}$, $mtRNA^{Lys}$, $mtRNA^{Trp}$ and $mtRNA^{Val}$. With the exception of $mtRNA^{Phe}$, most mtRNA isotypes had a lower number of mapped tRFs, compared to cytosolic tRNAs. The read count of $mtRNA^{Phe}$ in the NAP group was 83 fold higher than the average of all other mtRNA read counts.

In order to quantify the expression of tRFs we assembled a PCa tRF-database. The read-coverage of mature cytoplasmic tRNAs across all groups was analyzed using the fragment detection algorithm FlaiMapper [25]. Initially, 1175 tRFs were identified and mapped to 386 unique cytosolic tRNAs [23]. However, since tRNA sequences are highly conserved within tRNA isotypes, some tRFs were mapped to more than one unique tRNA (Supplementary Figure 2) and the total read-count in the initial mapping was equally divided across them. Upon further examination, we noticed that this causes underrepresentation of sequence counts for tRFs that had identical sequence but could be mapped to multiple tRNA isotypes. Therefore, tRFs with identical sequences were merged into single entries, even if they could be derived from different tRNAs, and their corresponding reads were summed. After this correction, a total of 598 unique tRFs were identified (Supplementary Table 1). Multiple fragmentation patterns, in combination with low read-count, caused low reliability in the automated prediction of tRFs derived from mtRNAs (Supplementary Figure 3). Therefore, these tRFs were omitted from further analysis.

Based on their size, tRNA-derived fragments can be generally separated into two major categories: tRNA halves, with size of 30-35 nt and small tRNA fragments (tRFs), with a size of approximately 20 nt. In our dataset, small tRFs were predominant and their sizes ranged from 15 to 23 nt (Figure 1B). The most abundant tRFs, however, were between 18 to 21 nt, while 40% of tRFs were 19 nt long (Figure 1B). A group of longer tRFs, with sizes between 25 and 29 nt, was also identified.

In addition to tRFs derived from mature tRNAs, we were also able to detect fragments corresponding to the 5'-pre-tRNA leader (5'U-tRFs) and 3'-pre-tRNA trailer (3'U-tRFs) sequences of various tRNAs (Supplementary Table 2). The length of 5'U-tRFs and 3'U-tRFs varied between 15 and 25 nt. Most 5'U-tRFs were 17 nt long and most 3'U-tRFs were 18 nt long (Supplementary Figure 4A). Interestingly, more than 54% of 3'U-tRFs and 30% of 5'U-tRFs were derived from sequences right next to or 1 nt off the mature tRNA sequence (Supplementary Figure 4B-C), suggesting that they are produced during the normal processing of pre-tRNA. Both 5'U-tRFs and 3'U-tRFs showed overall low expression values (data not shown), with the exception of tRF-1001/cand45. This fragment was previously detected in PCa cell lines, as well as in human colon carcinoma and human embryonic kidney cells [5, 11]. In our libraries tRF-1001/cand45 showed read counts from 40 000 in the NAP and PCa (average) groups to 110 000 in the LN_PCa group.

tRFs derived from the 5'-end are dominant in PCa

The majority of tRFs identified in our samples originate from the 5′- and the 3′-end of tRNAs (Figure 1C). This is in concordance with previous studies collectively reporting on the existence of short tRFs derived from the 5′- and the 3′-end of mature tRNAs [5, 26, 27]. To analyze the relative abundance of each tRF class in our dataset we examined the start and end positions of all unique tRFs on their precursor tRNAs. All the

fragments with a 3'-end nucleotide at position ≤40 on the mature tRNA sequence were considered as 5′-derived, whereas all fragments with the first 5'-nucleotide at position ≥30 on the mature tRNA sequence were considered as 3′-derived. Based on fragment uniqueness, we found comparable rates of tRF types, *i.e.* 51.7% corresponded to 5′-derived tRFs and 44.2% to 3′-derived tRFs. Nevertheless, when relative fragment abundance was taken into account a strong bias towards the 5′-derived (84.7%) *vs.* the 3'-derived tRFs was observed.

To get a more precise overview of the localization of tRFs, we also analyzed their start- and end- position frequencies. Interestingly, more than 26% of all unique tRFs, which in the terms of abundance account for over 80% of all tRFs, were found to start at position 1 on the mature tRNA sequence (Figure 1D-E). Most of these tRFs have the end at position 19 on the mature tRNA. Based on the peaks generated by the start positions of all unique fragments (Figure 1D), we observed that the tRF pool constitutes of several distinct classes (note the peak appearing before 20 nt, another at around 40 nt and another before 60 nt of the mature tRNA). While categorizing tRFs into 5′- or 3′-derived tRFs is very common, we found that at least 5 different classes are present across our samples. Therefore, we classified tRFs into (i) 5e-tRFs with a start position in the first nucleotide of the 5'-end of the tRNA ("e" stands for "end"); (ii) D-tRFs with a start position between nucleotides 12-23 and overlapping the D-loop of the precursor-tRNA; (iii) A-tRFs starting between nucleotides 31-39 and overlapping with the anticodon loop; (iv) V-tRFs with a start between nucleotides 45-49 and overlapping the variable loop; and finally, (v) 3e-tRFs starting between nucleotides 50-60 and overlapping the T loop (Figure 1F). While 5e-tRFs represent the most abundant class of tRFs (approximately 75%), other classes of tRFs appear to have very similar expression (<10% abundance) compared to each other (Figure 1G). Interestingly, similar tRF types have been detected in the lower eukaryote *Tetrahymena thermophile,* suggesting the existence of an evolutionary conserved tRNA processing mechanism [28]. Moreover, the position of these peaks was found to overlap with all tRNA loops, indicating that endonucleolytic cleavage occurs in the single-stranded loop regions of tRNAs.

Several tRFs are deregulated in PCa

To investigate whether tRF production is dysregulated in PCa we compared the expression levels of tRFs in normal tissue and in samples from different clinical stages representing progressing disease (Table 1). While the expression levels of other types of sncRNAs correlated well between the two libraries representing non-malignant tissue, *i.e.* NAP and BPH (Pearson r=0.89, P-value <0.0001; median fold-change -0.002), tRFs showed lower correlation and very high one-directional deviation towards increased expression in the BPH library (Pearson r=0.81; P-value <0.0001; median fold-change -0.758; Supplementary Figure 5). These results indicate that tRFs, as opposed to other sncRNAs, might be differentially expressed in benign prostate hyperplasia. This difference can be explained by the different anatomical origin of the BPH and NAP/PCa samples. While, BPH occurs exclusively in the transition zone of the prostate, prostate tumors are predominantly localized in the peripheral zone. Both zones are characterized by distinct expression profiles indicating differential regulation of a large number of genes [29]. For this reason BPH was excluded as a control sample from further analyses.

We found several tRFs to be significantly differentially expressed in PCa when compared to NAP (Kal's Z-test with Bonferroni correction, p-value < 0.05) (Figure 2 and Supplementary table 3-4). The number of differentially expressed tRFs varied slightly between the stages of PCa, with a minimum of 27 differentially expressed tRFs in PCa6_recur group and a maximum of 61 differentially expressed tRFs in the LN_PCa group (Figure 2, Supplementary Table 4). We identified 12 tRFs to be commonly differentially expressed between recurrent PCa groups with Gleason grade 6, 7, or 8 (Supplementary Table 5). Of these, 5 were upregulated, 6 downregulated and 1 was downregulated in PCa6 group but upregulated in PCa7 and PCa8 groups (Supplementary Table 5). This result indicates that a small subset of differentially expressed tRFs can be found across increasing grades of PCa.

In summary, we found 110 differentially expressed tRFs across our dataset, out of which 72 were upregulated, 24 downregulated and 13 that were upregulated in one but downregulated in other group.

tRFs deregulated in PCa belong to distinct classes

It has been proposed that 5′- but not 3′-derived tRFs, play a role in stress granule assembly or inhibition of protein synthesis *in vitro* [19, 30]. On the other hand, some 3′-derived tRFs are able to repress their mRNA targets in a miRNA-like fashion and may exert tumor suppressive functions [21, 31]. Interestingly, our results

indicate that the deregulation of 5'-derived tRFs differs from that of 3'-derived tRFs (Figure 2). In order to study which tRF types are present among the downregulated and upregulated tRFs in PCa we compared the percentage of different tRF types among our groups of upregulated and downregulated tRFs (Figure 3A-D). We noticed major differences in the abundance of tRF types in both lists. Most of the upregulated tRFs were 5e-tRFs (50%) and most downregulated were 3e-tRFs (50%). We selected tRFs originating from 6 different tRNAs for further analysis and qPCR validation. All of them were commonly differentially regulated in recurrent PCa groups with Gleason grade 6, 7, or 8 (Supplementary Table 5). Out of these, 4 tRFs, three 5e-tRFs and one D-tRF, were upregulated in PCa (Figure 3E-H), and 2, both belonging to 3e-tRF class, were downregulated (Figure 3I-J).

Specific tRF signatures can serve as prognostic marker of recurrent prostate cancer

The expression levels of tRFs selected for validation by qPCR were studied in a cohort of clinical samples obtained from Erasmus MC, Rotterdam (cohort 1) and a cohort of samples from Tampere University Hospital, Tampere (cohort 2). The NAP samples were identical for both cohorts and were processed independently in cohort 1 and cohort 2 to account for technical differences in sample treatment. Using custom designed primers, we could detect three tRFs (Figure 4A-C). tRF-544 (derived from $tRNA^{PheGAA}$) was significantly downregulated in the recurrent PCa compared to NAP or cured PCa in cohort 1 (Figure 4A). In cohort 2, tRF-544 was downregulated in PCa with Gleason score higher than 7 or in PCa with pathological stage 3 suggesting association with aggressive or late stage disease. The differential expression of this tRF was also confirmed in a second deep-sequencing analysis of a sub-set of PCa samples from Tampere University Hospital (unpublished data). tRF-315 (derived from $tRNA^{LysCTT}$) was significantly upregulated in all PCa groups of cohort 2 (Figure 4B). We could not detect statistically significant difference in the expression of tRF-315 in the smaller cohort 1. Nevertheless, there was a clear trend of tRF-315 upregulation in the PCa samples. tRF-562 (derived from $tRNA^{GlyTCC}$) was significantly downregulated in PCa recurrent *vs*. NAP group in the cohort 1 and in the PCa pT3 *vs*. NAP group in the cohort 2 (Figure 4C).
Interestingly, tRF-544 was consistently downregulated in samples from patients that developed recurrent disease compared to samples from patients that were cured by radical prostatectomy in both cohorts. Furthermore, tRF-544 expression was lower in high- (Gleason score $\geq$7) compared to low-grade (Gleason score <7) tumors (Figure 4A). *Vice versa,* tRF-315 demonstrated a clear trend of upregulation in recurrent disease and its expression was higher in high-grade tumors (Figure 4B). Therefore, we reasoned that the expression of these two tRFs might be prognostic for aggressive tumor growth and disease recurrence after radical prostatectomy. We took advantage of the opposing expression patterns of these two tRFs and calculated the expression ratio tRF-315/tRF-544 for both cohorts (Figure 4D). The tRF-315/tRF-544 ratio showed significant differences, clearly distinguishing high from low grade PCa and cured from recurrent disease. Moreover, high expression ratio was significantly associated with poorer progression-free survival and shorter period to disease relapse (Figure 4E), suggesting that the tRF-315/tRF-544 ratio might represent a helpful clinical biomarker of disease progression.

DISCUSSION

The technical progress in sequencing technologies and the rapid increase in the number of studies on sncRNA led to the discoveries of novel small RNA classes including tRFs. Since their initial identification, tRFs have been described in a plethora of species and knowledge about their function in the cell is starting to accumulate. Although several studies describe expression of tRFs in human cell lines, their actual repertoire in human tissues remains largely unknown [5, 15, 16, 22].
Here, we studied the composition and expression of tRFs in clinical PCa samples representing progressing disease stages. We found that all cytosolic tRNAs produced tRFs in the size range of 18-21 nt, representing the small class of tRFs. The longer tRNA halves were not as common, which is a consequence of the size selection (~15-35 nt) applied for the isolation of sncRNAs fraction in our study. We found a significant but weak correlation between the expression of tRFs per tRNA and the codon usage of tRNAs, suggesting that although tRF expression is dependent on the expression levels of their precursors, most likely additional mechanisms control tRF levels in the cell.
The accurate quantification of fragments derived from small RNAs in RNA sequencing data requires a precise annotation of the exact position of the fragment on its precursor transcript. To predict the locations of tRFs and quantify their expression we used the program FlaiMapper [25]. We identified 598 unique tRFs derived

from mature tRNAs. Based on the part of mature tRNA from which fragments originate, we could distinguish 5 different tRF classes. Out of these, the 5e-tRFs class was the most abundant of all and contained the highest number of unique tRFs. This finding is in agreement with other reports showing higher abundance of 5'-end derived tRFs [4, 5, 22, 32-34]. Given the role of 5'-derived tRFs in the inhibition of proteosynthesis and their role in the assembly of stress granules, a type of stress-induced cytoplasmatic foci with high concentration of untranslated mRNPs [10, 19, 30], it would be interesting to test their potential to inhibit translation and induce the assembly of stress granules *in vitro* in PCa cell lines using the set of upregulated 5'-derived tRFs identified in our study. The importance of tRFs in stress granule assembly becomes even more intriguing thanks to the latest indications that stress granules might play an important role in cancer via the negative regulation of mTORC1-hyperactivation-induced apoptosis [35]. This suggests that upregulation of tRFs might be indirectly linked with the suppression of apoptosis in cancer cells.

Our discovery cohort included patient-derived PCa samples with different clinico-pathological characteristics. The major difference in tRF expression (at least 110 unique differentially expressed tRFs) was observed between NAP and PCa tissue indicating that global upregulation of tRF production is associated with malignant transformation. Interestingly, 5e-tRFs were the predominant class upregulated in PCa. Recently, 5'-tRFs were found to induce translational inhibition in siRNA-independent way [36]. It was shown that the repressing activity of 5'-derived tRFs was dependent on the presence of a conserved "GG" dinucleotide at their 3'-end, which is a common feature of ~75% of the upregulated 5e-tRFs described in this study.

Comparing our data set with an external tRF data set of PCa cell lines generated by Lee *et al.* [5] demonstrated that all tRFs originating from 3'-pre-tRNA trailers and 32 out of 36 5'-tRFs described by Lee *et al*. were detected in our study. This suggests that tRFs in prostate (cancer) tissue and cell lines are common and discrete biological entities produced by defined molecular mechanisms. For 3'-derived tRFs we found a small overlap of only 6 out of 77 tRFs. A possible reason for that could be that 3'-derived tRFs represent a class of tRFs with a less stable expression. On the other hand, our results demonstrate that most of the downregulated tRFs are 3e-tRFs, which might be a general feature of PCa and PCa cell lines. If that is the case, the limited overlap of 3'-derived tRFs between both data sets might be caused by the less reliable detection of low expressed transcripts. Downregulation of 3'-derived tRFs might be an important event at the onset of cancer [21]. For example, the expression of the 3´-derived tRF CU1276 in B-cell lymphoma cells suppresses proliferation and modulates the response to DNA damage [21]. Future investigations should address the extent of gene regulation in PCa affected by the downregulation of 3'-derived tRFs.

Due to high conservation of tRNAs we were unable to identify specific sequences that would serve as a recognition site of tRNA nucleases that discriminate and preferably cleave particular tRNAs. Recently, it was proposed that certain tRNAs switch from canonical to alternative folding and the ability to do so might cause the specific upregulation of their tRFs. For example, besides the canonical cloverleaf structure, $tRNA^{Ile}$ has the potential to form a long hairpin [37]. $tRNA^{Asp}$ also adopts an alternative folding in order to bind to the Alu element insertion in the 3' UTR of the mRNA of its own aminoacyl-tRNA synthethase [38]. Since nucleotide modifications are known to affect hybridization, it is tempting to speculate to what extend they affect the alternative folding of tRNAs [39].

Finally, Q-PCR analysis of tRFs differentially expressed in different grade PCa demonstrated that the expression ratio tRF-544, derived from $tRNA^{PheGAA}$ and tRF-315 derived from $tRNA^{LysCTT}$ effectively discriminates high from low grade prostate tumors and cured from recurrent disease. This establishes tRFs as novel candidate biomarkers for the early detection of recurrent aggressive PCa.

In conclusion, our study provides a comprehensive catalogue of tRFs expressed in various stages of PCa and provides leads for the further investigation of biological role and marker potential of these novel RNA entities in prostate cancer.

MATERIALS AND METHODS

Sample cohorts and processing

The discovery set used in this study consists of 10 sequencing libraries generated as previously described [23]. Briefly, each library was constructed from an RNA pool prepared from four individual patient samples with similar pathological or genetic characteristics [24]. Different groups represent: normal adjacent prostate tissue (NAP), prostate tumors with Gleason score 6, 7, or 8 (PCa6, PCa7, PCa8), metastatic lymph nodes (LN_PCa), all obtained by radical prostatectomy; benign prostate hyperplasia tissue (BPH) obtained by cystoprostatectomy; and castration resistant prostate tumors obtained by trans-urethral resection of the prostate

(TURP_PCa) [23]. NAP and BPH samples were used as controls. The clinical parameters of each group are summarized in the Table 1. PCa groups with Gleason score 6 were divided into cured and recurrent disease groups or into groups with or without TMPRSS2-ERG fusion or ETV abnormalities. Sample material was obtained from the tissue banks of the Erasmus University Medical Center, Rotterdam, The Netherlands (Erasmus MC, Rotterdam, The Netherlands) and Tampere University Hospital (TAUH, Tampere, Finland). Collection and use of patient material was performed according to the national legislations concerning ethical requirements and approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act (MEC-2004- 261), and the Ethical Committee of the Tampere University Hospital. Samples were snap frozen and stored in liquid nitrogen. Gleason score and the percentage of cancer cells were evaluated from the histological sections by two pathologists. Only samples with more than 70% of tumor cells were used for sequencing library preparation. All samples that were used for the normal prostate pool contained 0% of tumor cells. Total RNA was extracted using RNABee reagent (Campro Scientific, GmbH, Berlin, Germany) according to the manufacturer´s protocol.

qPCR validation was performed in two separate cohorts. (clinical parameters available in Supplementary Tables 6-7). The first cohort (cohort 1) consists of 65 samples obtained from Erasmus MC. The samples were collected, handled and evaluated as mentioned in the previous paragraph. The second cohort (cohort 2) consists of 104 hormonally untreated primary prostate tumors from radical prostatectomy specimens obtained from Tampere University Hospital. The samples were confirmed to contain a minimum of 70% cancerous or hyperplastic cells by hematoxylin/eosin staining. Histological evaluation and Gleason grading for the second set were performed by a pathologist based on hematoxylin/eosin stained slides. Follow-up data was available for 74 of these samples. The use of clinical material was approved by the ethical committee of the Tampere University Hospital. Written informed consent was obtained from the subjects donating the samples. TRI-reagent (Molecular Research Center Inc., Cincinnati, OH, USA) was used to collect total RNA from the freshly frozen clinical samples, according to the manufacturer's instructions.

RNA sequencing and expression analysis

RNA pools were outsourced for library construction and sequencing to BGI (Beijing Genomics Institute, Beijing, China). Shortly, total RNA samples were size-separated on denaturing polyacrylamide gel. RNA in the size range of 15-35 nt was recovered from the gel and used for the preparation of sequencing libraries. The libraries were sequenced by Illumina deep sequencing. The tRNA database used to map the reads was constructed from the Genomic tRNA Database (http://gtrnadb.ucsc.edu/) as previously described [23, 25]. Shortly, tRNA genes with identical sequences were merged into single entries. Intronic sequences in tRNAs were removed, to allow mapping of tRFs derived from mature, spliced tRNAs. Genomic tRNAs in the database were modified by extending the 3'-ends with a single "CCA" sequence. Sequencing reads were mapped to tRNA database using CLC-Bio Genomics Workbench (Aarhus, Denmark). Subsequently, tRFs were predicted using the FlaiMapper program and a tRF database was constructed [25]. The final read counts used for expression analysis were generated by mapping the sequencing reads to the tRF database. tRFs derived from 5'-pre-tRNA leaders (5'U-tRFs) and 3'-pre-tRNA trailers (3'U-tRFs) were identified by mapping the sequencing reads to a tRNA reference database in which the genomic sequence of each tRNA gene was extended by 50 bp on both sides. The length, position and type of tRF were calculated from the sum of the read counts of the following groups: NAP, PCa6_cur, PCa6_nofusion, PCa6_TERG, PCa6_recur, PCa7_recur, PCa8_recur, TURP_PCa, and LN_PCa. To identify differentially expressed tRFs, read counts were normalized as "parts per million" and Kal's Z-test on proportions followed by Bonferroni correction was subsequently performed. The generated adjusted p-values lower than 0.05 were considered significant.

Quantitative real-time PCR and statistics

Total RNA extracted from clinical samples was reverse transcribed using miRCURY Universal cDNA Synthesis kit (Exiqon, Vedbaek, Denmark). The provided UniSp6 spike-in RNA was added to the reverse transcription reaction to control for the efficiency of the reaction. The amplification was performed using miRCURY LNA™ SYBR® Green Master Mix (Exiqon, Vedbaek, Denmark) and specific custom LNA™ primers (Exiqon, Vedbaek, Denmark) were used for each tRF. The names of tRFs with their sequences are shown in Table 2. Quantitative real-time PCR (qPCR) was performed on an Applied Biosystems ABI 7900

thermocycler (Applied Biosystems, Waltham, Massachusetts, USA) for the cohort 1 and on Bio-Rad CFX96 Real Time System (Bio-Rad Laboratories, Hercules, California, USA) for the cohort 2. Data were analyzed using the ΔΔCT method and the expression of each tRF was normalized against the small nucleolar RNA SNORD38B (Reference gene primer set 2039, Exiqon, Vedbaek, Denmark). Statistical significance of qPCR expression data was assessed using Mann-Whitney U test. The log-rank test was used to compare progression-free survival distributions of the tumor samples. P-values lower than 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 6.0g for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com").

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015; 136:E359-386.
2. Nam RK, Cheung P, Herschorn S, Saskin R, Su J, Klotz LH, Chang M, Kulkarni GS, Lee Y, Kodama RT and Narod SA. Incidence of complications other than urinary incontinence or erectile dysfunction after radical prostatectomy or radiotherapy for prostate cancer: a population-based cohort study. Lancet Oncol. 2014; 15:223-231.
3. Kawaji H, Nakamura M, Takahashi Y, Sandelin A, Katayama S, Fukuda S, Daub CO, Kai C, Kawai J, Yasuda J, Carninci P and Hayashizaki Y. Hidden layers of human small RNAs. BMC Genomics. 2008; 9:157.
4. Cole C, Sobala A, Lu C, Thatcher SR, Bowman A, Brown JW, Green PJ, Barton GJ and Hutvagner G. Filtering of deep sequencing data reveals the existence of abundant Dicer-dependent small RNAs derived from tRNAs. RNA. 2009; 15:2147-2160.
5. Lee YS, Shibata Y, Malhotra A and Dutta A. A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). Genes Dev. 2009; 23:2639-2649.
6. Thompson DM and Parker R. Stressing out over tRNA cleavage. Cell. 2009; 138:215-219.
7. Martens-Uzunova ES, Olvedy M and Jenster G. Beyond microRNA--novel RNAs derived from small non-coding RNA and their implication in cancer. Cancer Lett. 2013; 340:201-211.
8. Kumar P, Anaya J, Mudunuri SB and Dutta A. Meta-analysis of tRNA derived RNA fragments reveals that they are evolutionarily conserved and associate with AGO proteins to recognize specific RNA targets. BMC Biol. 2014; 12:78.
9. Thompson DM and Parker R. The RNase Rny1p cleaves tRNAs and promotes cell death during oxidative stress in Saccharomyces cerevisiae. J Cell Biol. 2009; 185:43-50.
10. Yamasaki S, Ivanov P, Hu GF and Anderson P. Angiogenin cleaves tRNA and promotes stress-induced translational repression. J Cell Biol. 2009; 185:35-42.
11. Haussecker D, Huang Y, Lau A, Parameswaran P, Fire AZ and Kay MA. Human tRNA-derived small RNAs in the global regulation of RNA silencing. RNA. 2010; 16:673-695.
12. Kumar P, Mudunuri SB, Anaya J and Dutta A. tRFdb: a database for transfer RNA fragments. Nucleic Acids Res. 2015; 43:D141-145.

13. Thompson DM, Lu C, Green PJ and Parker R. tRNA cleavage is a conserved response to oxidative stress in eukaryotes. RNA. 2008; 14:2095-2103.
14. Liao JY, Ma LM, Guo YH, Zhang YC, Zhou H, Shao P, Chen YQ and Qu LH. Deep sequencing of human nuclear and cytoplasmic small RNAs reveals an unexpectedly complex subcellular distribution of miRNAs and tRNA 3' trailers. PLoS One. 2010; 5:e10563.
15. Goodarzi H, Liu X, Nguyen HC, Zhang S, Fish L and Tavazoie SF. Endogenous tRNA-Derived Fragments Suppress Breast Cancer Progression via YBX1 Displacement. Cell. 2015; 161:790-802.
16. Martens-Uzunova ES, Jalava SE, Dits NF, van Leenders GJ, Moller S, Trapman J, Bangma CH, Litman T, Visakorpi T and Jenster G. Diagnostic and prognostic signatures from the small non-coding RNA transcriptome in prostate cancer. Oncogene. 2012; 31:978-991.
17. Nawrot B, Sochacka E and Duchler M. tRNA structural and functional changes induced by oxidative stress. Cell Mol Life Sci. 2011; 68:4023-4032.
18. Gebetsberger J, Zywicki M, Kunzi A and Polacek N. tRNA-derived fragments target the ribosome and function as regulatory non-coding RNA in Haloferax volcanii. Archaea. 2012; 2012:260909.
19. Ivanov P, Emara MM, Villen J, Gygi SP and Anderson P. Angiogenin-induced tRNA fragments inhibit translation initiation. Mol Cell. 2011; 43:613-623.
20. Zhang S, Sun L and Kragler F. The phloem-delivered RNA pool contains small noncoding RNAs and interferes with translation. Plant Physiol. 2009; 150:378-387.
21. Maute RL, Schneider C, Sumazin P, Holmes A, Califano A, Basso K and Dalla-Favera R. tRNA-derived microRNA modulates proliferation and the DNA damage response and is down-regulated in B cell lymphoma. Proc Natl Acad Sci U S A. 2013; 110:1404-1409.
22. Telonis A, Loher, P., Honda, S., Jing, Y., Palazzo, J., Kirino, Y., & Rigoutsos, I. Dissecting tRNA-derived fragment complexities using personalized transcriptomes reveals novel fragment classes and unexpected dependencies. Oncotarget. 2015.
23. Martens-Uzunova ES, Hoogstrate Y, Kalsbeek A, Pigmans B, Vredenbregt-van den Berg M, Dits N, Nielsen SJ, Baker A, Visakorpi T, Bangma C and Jenster G. C/D-box snoRNA-derived RNA production is associated with malignant transformation and metastatic progression in prostate cancer. Oncotarget. 2015.
24. Hendriksen PJ, Dits NF, Kokame K, Veldhoven A, van Weerden WM, Bangma CH, Trapman J and Jenster G. Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res. 2006; 66:5012-5020.
25. Hoogstrate Y, Jenster G and Martens-Uzunova ES. FlaiMapper: computational annotation of small ncRNA derived fragments using RNA-seq high throughput data. Bioinformatics. 2014.
26. Couvillion MT, Sachidanandam R and Collins K. A growth-essential Tetrahymena Piwi protein carries tRNA fragment cargo. Genes Dev. 2010; 24:2742-2747.
27. Wei C, Salichos L, Wittgrove CM, Rokas A and Patton JG. Transcriptome-wide analysis of small RNA expression in early zebrafish development. RNA. 2012; 18:915-929.
28. Couvillion MT, Bounova G, Purdom E, Speed TP and Collins K. A Tetrahymena Piwi bound to mature tRNA 3' fragments activates the exonuclease Xrn2 for RNA processing in the nucleus. Mol Cell. 2012; 48:509-520.
29. van der Heul-Nieuwenhuijsen L, Hendriksen PJ, van der Kwast TH and Jenster G. Gene expression profiling of the human prostate zones. BJU Int. 2006; 98:886-897.
30. Emara MM, Ivanov P, Hickman T, Dawra N, Tisdale S, Kedersha N, Hu GF and Anderson P. Angiogenin-induced tRNA-derived stress-induced RNAs promote stress-induced stress granule assembly. J Biol Chem. 2010; 285:10959-10968.
31. Yeung ML, Bennasser Y, Watashi K, Le SY, Houzet L and Jeang KT. Pyrosequencing of small non-coding RNAs in HIV-1 infected cells: evidence for the processing of a viral-cellular double-stranded RNA hybrid. Nucleic Acids Res. 2009; 37:6575-6586.
32. Cai P, Piao X, Hao L, Liu S, Hou N, Wang H and Chen Q. A deep analysis of the small non-coding RNA population in Schistosoma japonicum eggs. PLoS One. 2013; 8:e64003.
33. Garcia-Silva MR, Frugier M, Tosar JP, Correa-Dominguez A, Ronalte-Alves L, Parodi-Talice A, Rovira C, Robello C, Goldenberg S and Cayota A. A population of tRNA-derived small RNAs is actively produced in Trypanosoma cruzi and recruited to specific cytoplasmic granules. Mol Biochem Parasitol. 2010; 171:64-73.

34. Li Z, Ender C, Meister G, Moore PS, Chang Y and John B. Extensive terminal and asymmetric processing of small RNAs from rRNAs, snoRNAs, snRNAs, and tRNAs. Nucleic Acids Res. 2012; 40:6787-6799.
35. Thedieck K, Holzwarth B, Prentzell MT, Boehlke C, Klasener K, Ruf S, Sonntag AG, Maerz L, Grellscheid SN, Kremmer E, Nitschke R, Kuehn EW, Jonker JW, et al. Inhibition of mTORC1 by astrin and stress granules prevents apoptosis in cancer cells. Cell. 2013; 154:859-874.
36. Sobala A and Hutvagner G. Small RNAs derived from the 5' end of tRNA can inhibit protein translation in human cells. RNA Biol. 2013; 10.
37. Babiarz JE, Ruby JG, Wang Y, Bartel DP and Blelloch R. Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. Genes Dev. 2008; 22:2773-2785.
38. Rudinger-Thirion J, Lescure A, Paulus C and Frugier M. Misfolded human tRNA isodecoder binds and neutralizes a 3' UTR-embedded Alu element. Proc Natl Acad Sci U S A. 2011; 108:E794-802.
39. Helm M. Post-transcriptional nucleotide modification and alternative folding of RNA. Nucleic Acids Res. 2006; 34:721-733.

TABLES

Table 1: Clinical parameters of the samples used for the RNA-sequencing

	Number of patient samples per group	*TMPRSS2_ERG fusion*	*ETV1 abnormalities*	*% cancer*	*Gleason score*	*Status after radical prostatectomy**
NAP	4	0 (0 %)	0	0	N/A	N/A
BPH	4	N/A	N/A	0	N/A	N/A
PCa6_cur	4	4 (100 %)	0	70-90	3+3	cured
PCa6_nofusion	4	0 (0 %)	0	70-90	3+3	recurrent
PCa6_TERG	4	4 (100 %)	0	80-90	3+3	recurrent
PCa6_recur	4	4 (100 %)	0	80-90	3+3	recurrent
PCa7_recur	4	2 (50 %)	1 (fusion)	80-100	4+3	recurrent
PCa8_recur	3	1 (25 %)	1 (overexp)	90-100	4+4(5)	recurrent
TURP_PCa (castration resistant)	4	1 (25 %)	1 (fusion)	90-100	(3+4) to (5+4)	recurrent
LN_PCa	4	3 (75 %)	1 (fusion)	100	4+4(5)†	N/A

* patients were considered cured if there was no biochemical relapse or detection of metastasis after radical prostatectomy
† Gleason score of the primary tumor

Group abbreviations: NAP - normal adjacent prostate; BPH - benign prostatic hyperplasia; PCa - organ-confined prostate cancer; cur/recur - cured/recurrent after radical prostatectomy; PCa6_nofusion-PCa Gleason score 3+3 with no TMPRSS2-ERG fusion or ETV abnormalities; PCa6_TERG- PCa33 with TMPRSS2-ERG fusion; TURP_PCa- trans-urethral resection of the prostate, castration resistant; LN_PCa- PCa metastasis from lymph nodes; FFPE- formalin-fixed paraffin-embedded

Table 2: tRFs selected for validation by qPCR

tRF ID	**tRNA isotype**	**Anticodon**	**Sequence 5'- 3'**
tRF-544	Phe	GAA	TCCCTGGTTCGATCCCGGGTTTCGGC
tRF-159	Arg	CCT	ATGGATAAGGCATTGGCCT
tRF-368	Arg	TCT	GGCTCCGTGGCGCAATGGA
tRF-562	Gly	TCC	TCGATTCCCGGCCAACGC
tRF-542	Glu	CTC	TCCCTGGTGGTCTAGTGGTTAG
tRF-315	Lys	CTT	CCCGGCTAGCTCAGTCGGTAGAGCATGG

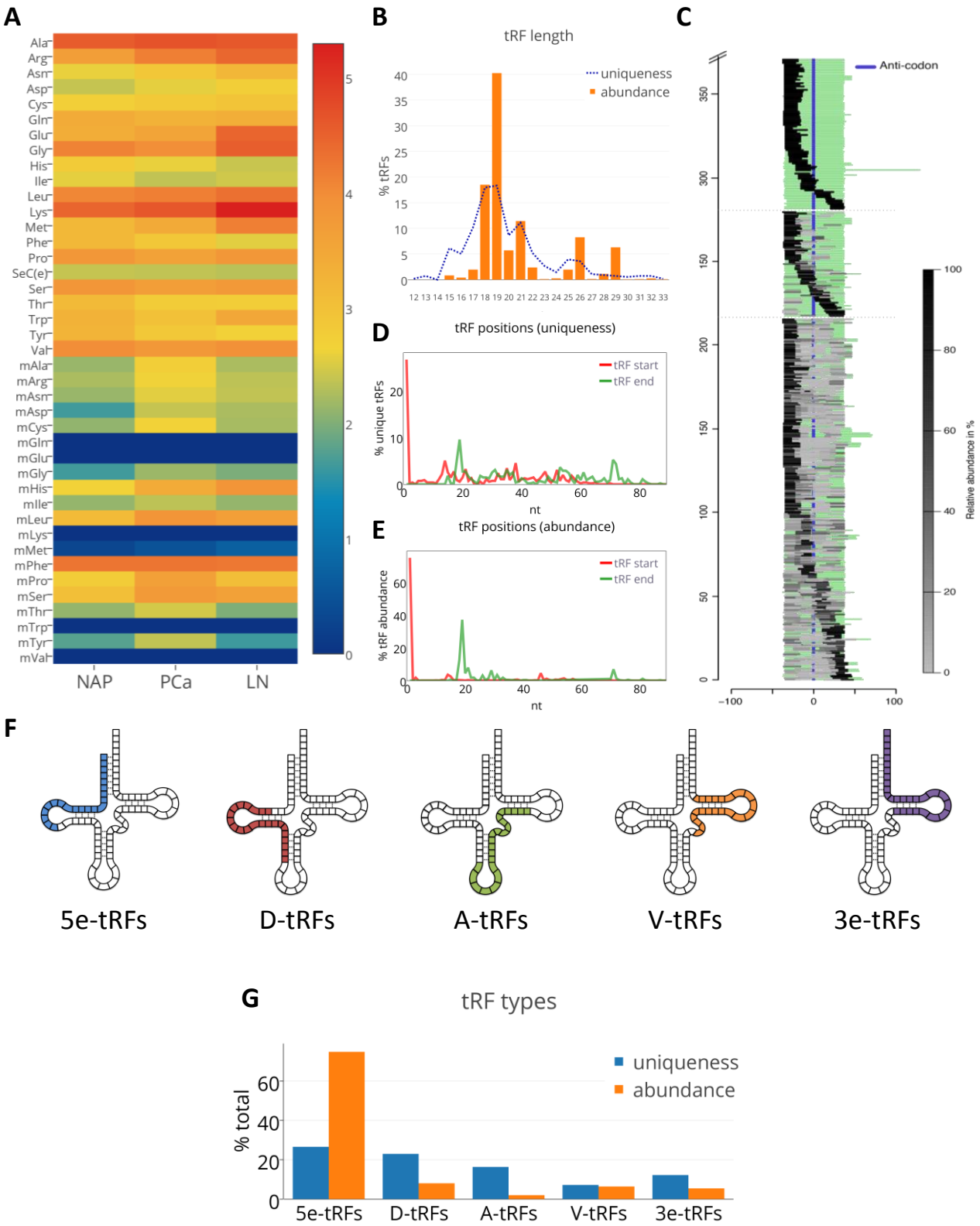
FIGURE LEGENDS

Figure 1: tRF types in prostate cancer. (A) Heatmap showing the total read counts mapped to individual tRNA isotypes in three study groups: NAP-normal adjacent prostate; PCa- prostate cancer group (consisting of 6 different sample pools, the average value is shown); LN-PCa lymph node metastasis. The color and its corresponding value in $\log_{10}$ scale are depicted on the right. (B) tRF length as based on the read abundance and uniqueness. (C) Graph depicting the locations of mapped tRFs on the sequences of mature tRNAs. Full-length tRNA sequences are aligned to the middle using the anticodon position. tRFs mapped to these tRNAs are depicted as grey bars which relative abundance per particular tRNA is reflected by the color intensity (light grey – low abundance, black – high abundance). tRNAs with only one mapped tRF are clustered at the top, tRNAs with two mapped tRFs in the middle and tRNAs with multiple mapped tRFs are at the bottom. (D-E) Start (red line) and end (green line) positions of tRFs on the mature tRNA sequence. Relative abundance of each tRF type based on the uniqueness (D) or abundance (E) is shown. Approximate locations of 5 tRF classes are indicated above tRF start peaks (D). (F) An illustration of various tRF classes and their approximate location on the secondary structure of tRNA. (G) Ratio of each tRF class in our dataset as based on the uniqueness (% of unique independent reads) or abundance (% of total number of reads).

Figure 2: Differentially expressed tRFs in prostate cancer. Normalized read count values of each tRF in the normal healthy prostate versus various stages of prostate cancer are plotted. The baseline value for tRFs that are not expressed is 1. Full lines represent 4-fold change borderlines. Colored points represent significantly changed tRFs (Kal's Z-test on proportions, Bonferroni corrected p-values, $p<0.05$) and were further discriminated into 5'-derived (magenta) and 3'-derived (green) tRFs. tRFs with the 3'-nucleotide at a position ≤40 on the precursor tRNA sequence were considered as 5´-derived and tRFs with the start nucleotide at a position ≥30 on the precursor tRNA sequence were considered as 3´-derived. tRFs that could not fall into any of these two categories are shown in blue. Positions of tRF-310, tRF-315, and tRF-389 are indicated as an example of three differentially expressed tRFs. The graph at the bottom right corner summarizes the total number of differentially expressed tRFs per group. tRFs with ≥4-fold differential expression are indicated with dark red (upregulated) or dark green (downregulated) color.

Figure 3: Frequency of tRF types among differentially expressed tRFs. (A-D) Start (red line) and end (green line) positions of tRFs on the mature tRNA sequence and the quantification of each tRF type for 72 upregulated (A-B) and 24 downregulated (C-D) tRFs. (E-J) Graphs showing the exact positions of 6 selected tRFs on their tRNAs precursors. Their mean read counts in PCa (dark color) or NAP (light color) are indicated. Upregulated and donwregulated tRFs are depicted in blue and red, respectively. Expression of other tRFs from the same tRNA are indicated in grey.

Figure 4: qPCR validation of tRF-544, tRF-315, and tRF-562. (A-C) RNA expression of tRF-544 (A), tRF-315 (B), and tRF-562 (C) in cohorts of clinical samples obtained from Erasmus MC (cohort 1) and Tampere University Hospital (cohort 2). The red line indicates mean. (D) Ratio of tRF-315 (derived from $tRNA^{LysCTT}$) to tRF-544 (derived from $tRNA^{PheGAA}$). (E) Progression-free survival curves of the tRF-315/tRF544 ratios. Legend: NAP-normal adjacent prostate, PCa-prostate cancer, PCa cur/recur- PCa cured/reccurent, PCa Gl <7/>7- PCa with Gleason score <7/>7, PCa pT2/pT3-PCa pathological stage 2/3, *P-value ≤0.05, ** P-value ≤0.01, ***P-value ≤0.001.


A
Ala
Arg
Asn
Asp
Cys
Gln
Glu
Gly
His
Ile
Leu
Lys
Met
Phe
Pro
SeC(e)
Ser
Thr
Trp
Tyr
Val
mAla
mArg
mAsn
mAsp
mCys
mGln
mGlu
mGly
mHis
mIle
mLeu
mLys
mMet
mPhe
mPro
mSer
mThr
mTrp
mTyr
mVal
NAP
PCa
LN
5
4
3
2
1
0
B
tRF length
uniqueness
abundance
% tRFs
12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
C
Anti-codon
Relative abundance in %
D
tRF positions (uniqueness)
tRF start
tRF end
% unique tRFs
nt
E
tRF positions (abundance)
tRF start
tRF end
% tRF abundance
nt
F
5e-tRFs
D-tRFs
A-tRFs
V-tRFs
3e-tRFs
G
tRF types
uniqueness
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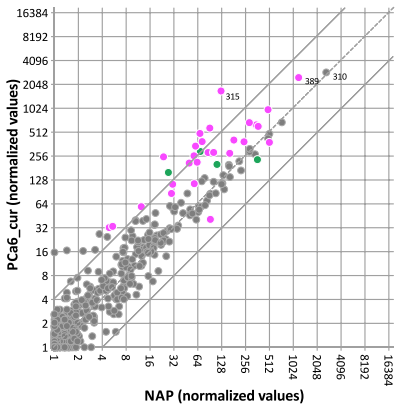
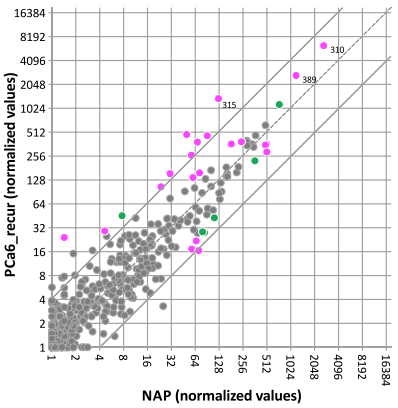
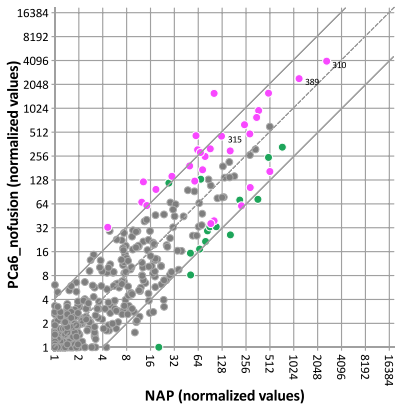
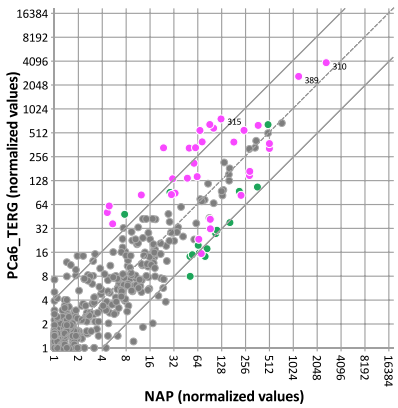
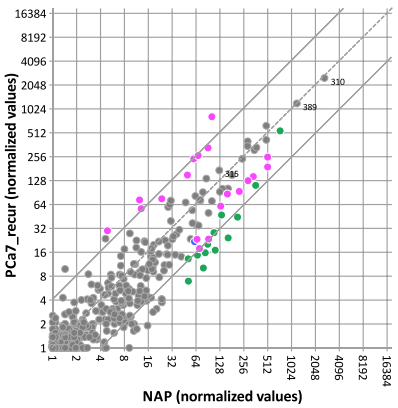
D-tRFs
A-tRFs
V-tRFs
3e-tRFs

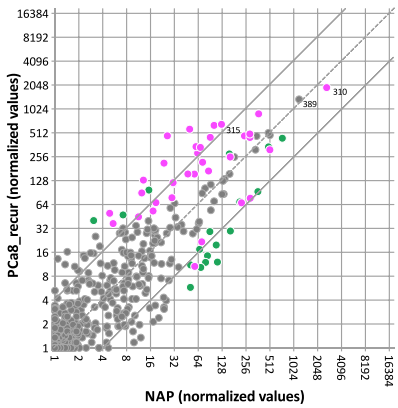

PCa6_cur (normalized values)
NAP (normalized values)
315
389
310

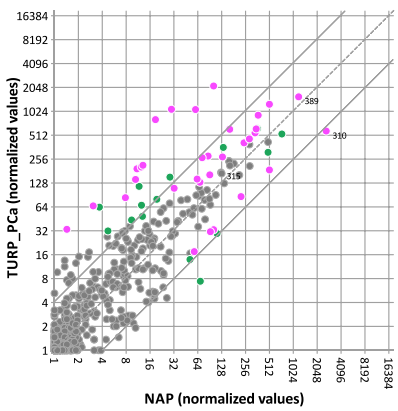

PCa6_recur (normalized values)
NAP (normalized values)
315
389
310

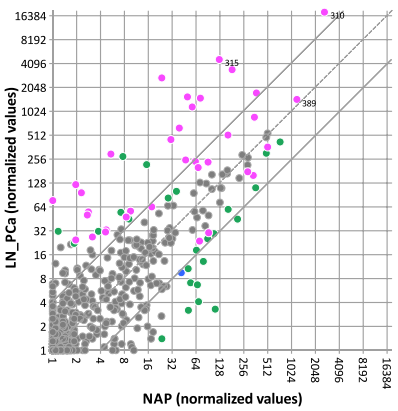

PCa6_nofusion (normalized values)
NAP (normalized values)
315
389
310

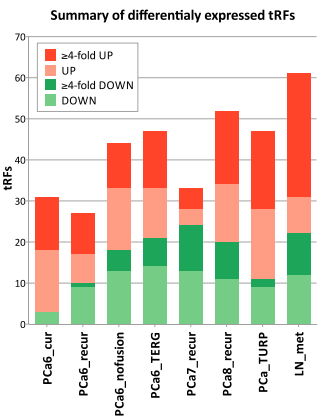

PCa6_TERG (normalized values)
NAP (normalized values)
315
389
310

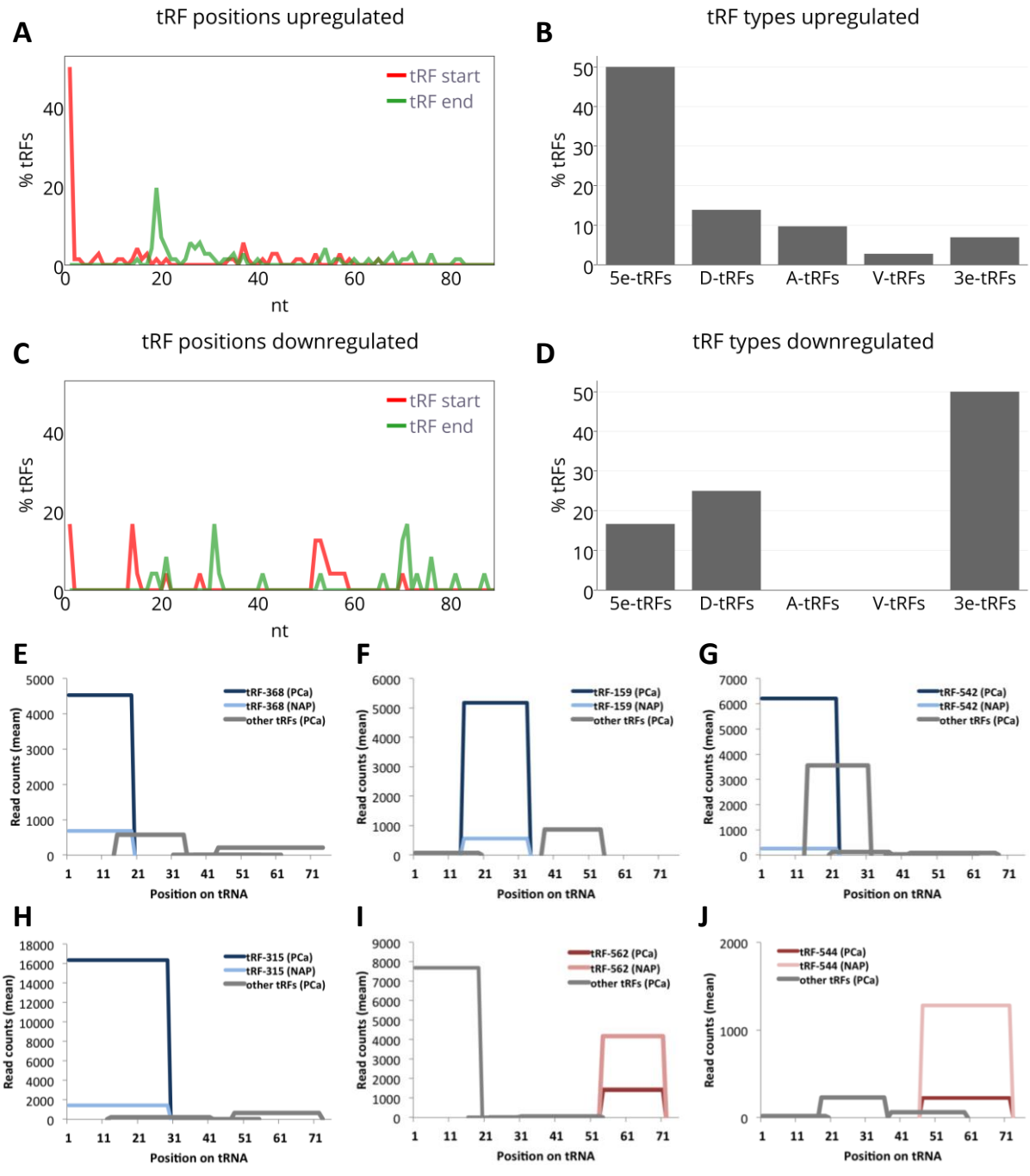

PCa7_recur (normalized values)
NAP (normalized values)
315
389
310

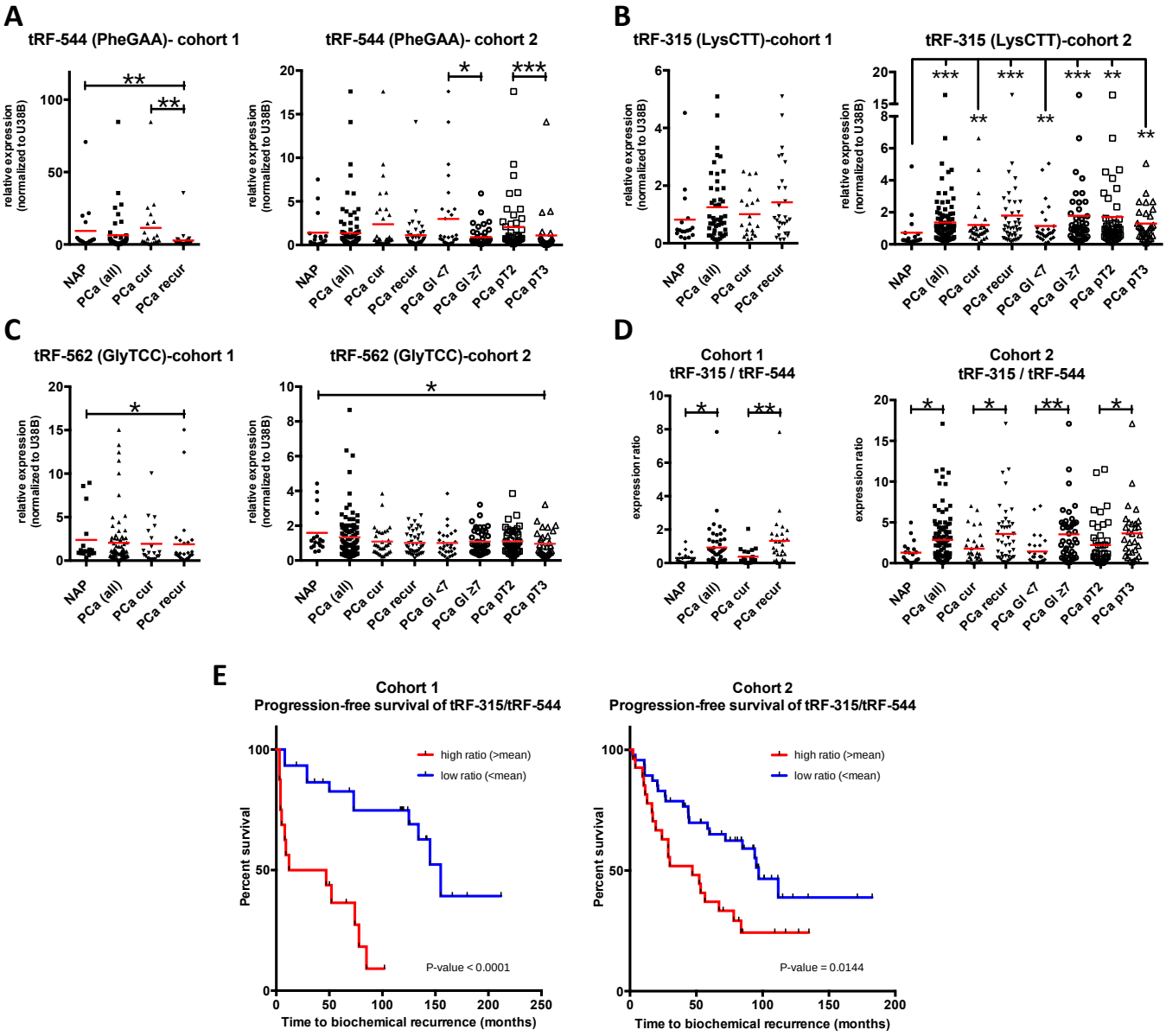

PCa8_recur (normalized values)
NAP (normalized values)
315
389
310


TURP_PCa (normalized values)
NAP (normalized values)
315
389
310


LN_PCa (normalized values)
NAP (normalized values)
315
389
310

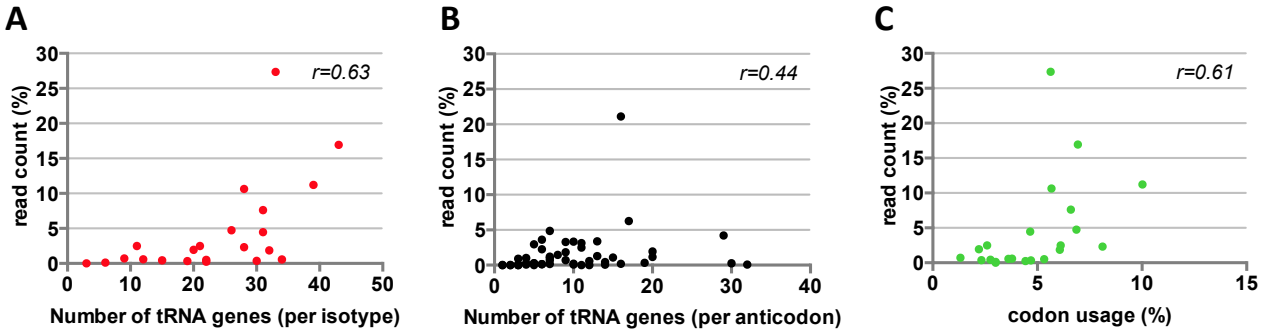

Summary of differentialy expressed tRFs
≥4-fold UP
UP
≥4-fold DOWN
DOWN
tRFs
PCa6_cur
PCa6_recur
PCa6_nofusion
PCa6_TERG
PCa7_recur
PCa8_recur
PCa_TURP
LN_met


A
tRF positions upregulated
tRF start
tRF end
% tRFs
nt
B
tRF types upregulated
% tRFs
5e-tRFs
D-tRFs
A-tRFs
V-tRFs
3e-tRFs
C
tRF positions downregulated
tRF start
tRF end
% tRFs
nt
D
tRF types downregulated
% tRFs
5e-tRFs
D-tRFs
A-tRFs
V-tRFs
3e-tRFs
E
tRF-368 (PCa)
tRF-368 (NAP)
other tRFs (PCa)
Read counts (meam)
Position on tRNA
F
tRF-159 (PCa)
tRF-159 (NAP)
other tRFs (PCa)
Read counts (mean)
Position on tRNA
G
tRF-542 (PCa)
tRF-542 (NAP)
other tRFs (PCa)
Read counts (mean)
Position on tRNA
H
tRF-315 (PCa)
tRF-315 (NAP)
other tRFs (PCa)
Read counts (mean)
Position on tRNA
I
tRF-562 (PCa)
tRF-562 (NAP)
other tRFs (PCa)
Read counts (mean)
Position on tRNA
J
tRF-544 (PCa)
tRF-544 (NAP)
other tRFs (PCa)
Read counts (mean)
Position on tRNA

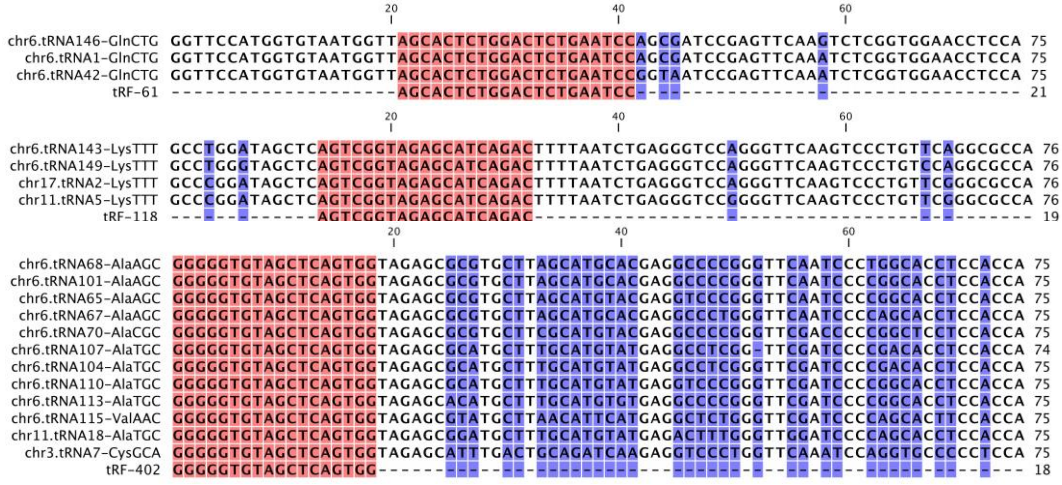

A
tRF-544 (PheGAA)- cohort 1
tRF-544 (PheGAA)- cohort 2
relative expression (normalized to U38B)
NAP
PCa (all)
PCa cur
PCa recur
PCa GI <7
PCa GI ≥7
PCa pT2
PCa pT3
B
tRF-315 (LysCTT)-cohort 1
tRF-315 (LysCTT)-cohort 2
C
tRF-562 (GlyTCC)-cohort 1
tRF-562 (GlyTCC)-cohort 2
D
Cohort 1
tRF-315 / tRF-544
Cohort 2
tRF-315 / tRF-544
expression ratio
E
Cohort 1
Progression-free survival of tRF-315/tRF-544
Cohort 2
Progression-free survival of tRF-315/tRF-544
high ratio (>mean)
low ratio (<mean)
Percent survival
P-value < 0.0001
P-value = 0.0144
Time to biochemical recurrence (months)

Supplementary Figures

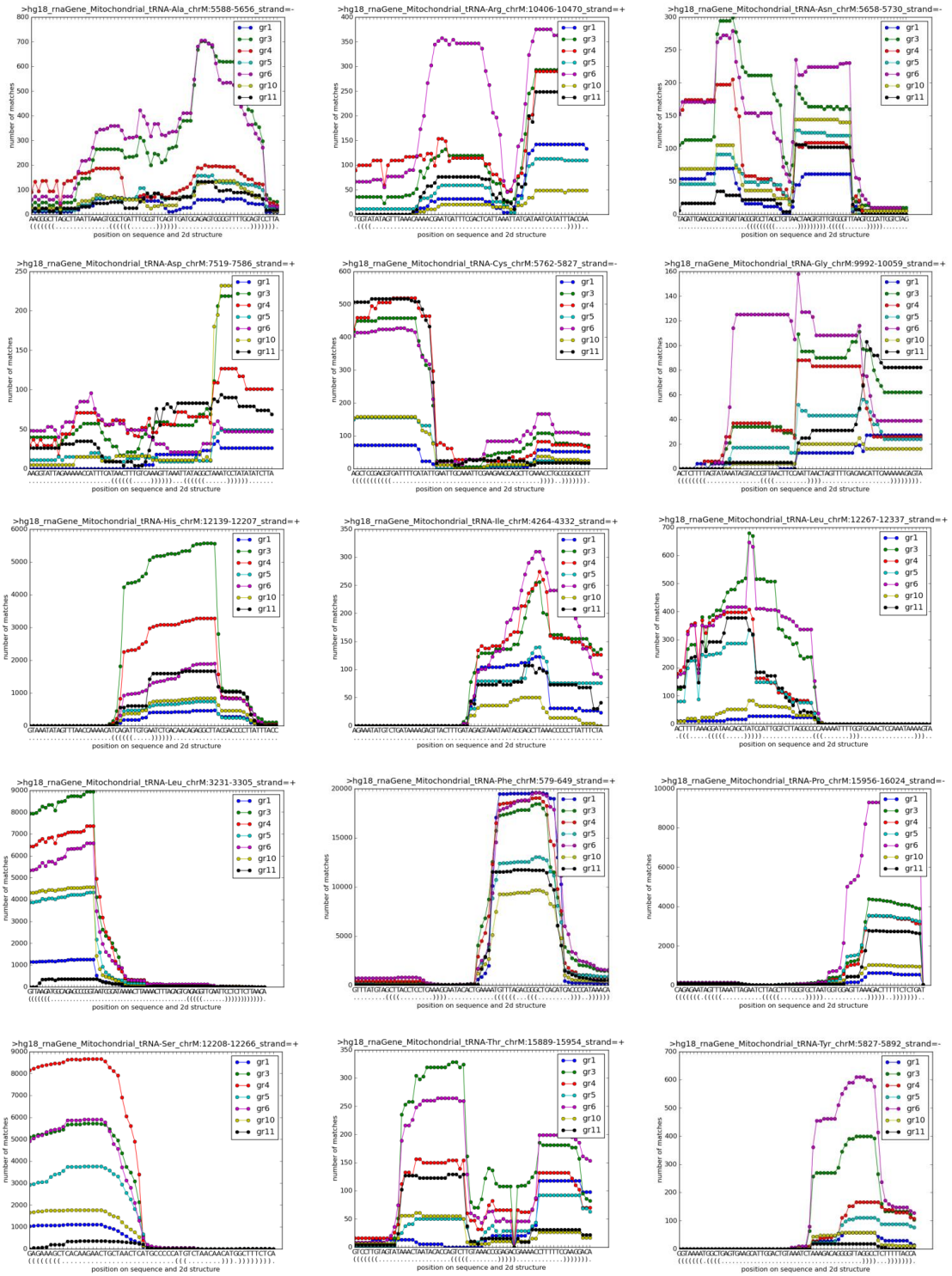
Supplementary Figure 1: Correlation of tRF levels with their estimated precursor levels. Scatter plots depicting the relationship between the expression of tRFs and their precursors. Correlation between the percentage of tRF read counts and the number of tRNA genes per isotype (A), anticodon (B) or codon usage (C). Spearman correlation coefficient (r) is indicated in the graph. All correlations were significant (P-value <0.05). Due to the high sequence similarities between tRNAs, several reads mapped to multiple tRNA loci and thus were omitted from the analysis (percentage of tRFs mapped to multiple loci in figure: A=2 %, B=21 %, C=2 %).



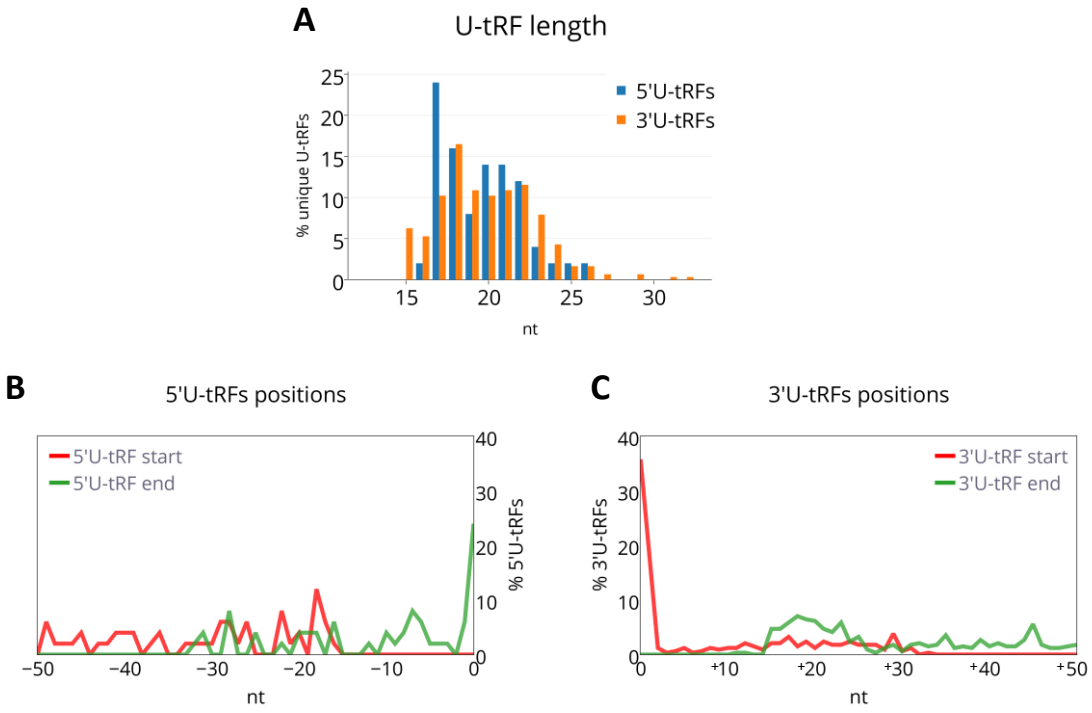
Supplementary Figure 2: Sequence alignments of three tRF examples (tRF-61, tRF-118, and tRF-402) that mapped to different tRNA loci. The tRF location is highlighted in red. Nucleotide positions that are dissimilar between tRNA sequences are highlighted in blue.



Supplementary Figure 3: Fragments derived from mitochondrial tRNAs. Graphs showing the coverage of tRF nucleotides projected on mature mtRNA for each study group, reveal the complicated fragmentation pattern of mitochondrial tRNAs. The x-axis shows the sequence of the mtRNA (in the 5' to 3' direction) to which tRFs were mapped. The experimental groups are shown with different colors. Legend: gr1-NAP; gr3-PCa6_cur; gr4-PCa6_recur; gr5-PCa7_recur; gr6-PCa8_recur; gr10-PCa6_nofusion; gr11-PCa6_TERG



Supplementary Figure 4: U-tRF types in prostate cancer. The size distribution and location of 5'-pre-tRNA leaders (5'U-tRFs) and 3'-pre-tRNA trailers (3'U-tRFs) was analyzed. (A) U-tRF length as based on the uniqueness. (B-C) Start (red line) and end (green line) positions of 5'U-tRFs (B) and 3'U-tRFs (C) on the 5'-leaders and 3'-trailers of pre-tRNAs. The values on X-axis represent number of nucleotides from the start or end of mature tRNA sequence.



Supplementary Figure 5: tRF expression is affected in BPH. Scatter plots of log2-transformed normalized values of tRFs (A) and other sncRNAs (B) including miRNAs, snoRNA and sdRNA, in NAP *vs* BPH group. The red line indicates no change in the expression between NAP and BPH.