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Defining new biotypes in Prostate Cancer for diagnosis, prognosis and therapeutic intervention

DOCTORAL THESIS

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"Dejamos de temer aquello que hemos aprendido a entender"

Marie Curie

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CERTIFICAN QUE:

La presente tesis doctoral "Defining new biotypes in Prostate Cancer for diagnosis, prognosis and therapeutic intervention" ha sido realizada por Dña. Irene Casanova Salas en el Laboratorio de Biología Molecular de la Fundación Instituto Valenciano de Oncología bajo nuestra dirección, y reúne todos los requisitos para su depósito y lectura.

Y para que así conste, firman la presente en Valencia, Abril 2015

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Abbreviations

%	Percentage
2D	Bidimensional
ADT	Androgen deprivation therapy
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3
AMO	Anti-miRNA oligonucleotide
AR	Androgen receptor
ARE	Androgen-regulated elements
ATP	Adenosine triphosphate
AUC	Area under the curve
BPFS	Biochemical progression free survival
BPH	Benign prostatic hyperplasia
BTB	Brick-a-brack/Tamtrack/Broad complex
ChIP	Chromatin immunoprecipitation
CI	Confidence interval
CRPC	Castration-resistant prostate cancer
cT	Clinical stage
Cul3	Cullin 3
CYP17	17 α-hydroxylase/C17,20-lyase
DES	Diethyltilbestrol
DHT	Dihydrotestosterone
DIGE	Difference gel electrophoresis
DNA	Deoxiribonucleic acid
dNTP	Deoxinucleotide triphosphate
DRE	Digital rectal examination
dsRNA	Double-stranded RNA
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immuno-Sorbent Assay
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
ERSPC	European randomized study of screening for prostate cancer
ETS	E26 transformation-specific
EZH2	Enhancer of zeste homolog 2
FC	Fold-change
FFPE	Formalin-fixed paraffin embedded
FDA	Food and drug administration
FISH	Fluorecent in-situ hybridization
FUBx	Follow-up biopsy
GEO	Gene expression omnibus
HAbs	Human monoclonal antibodies
Her-2	Human epidermal growth factor receptor 2
HR	Hazard ration
HSP	Heat-shock protein
IBx	Initial biopsy
ICGC	International cancer genome consortium
i.e	Id est
IGF	Insulin growth factor
IGF-IR	Insulin growth factor receptor 1
IGFBP	Insulin growth factor binding protein

IGFBPrP	IGFBP-related protein
IHC	Immunohistochemistry
IL-6	Interleukin 6
IR	Insulin receptor
kD	Kilodalton
LC	Liquid chromatography
LHRH	Luteinizing hormone-releasing hormone
m/z	Mass-to-charge
MALDI-TOF	Matrix-assisted laser deorption-ionization tie-of-flight
MAPK	Mitogen-activated protein kinase
ml	Mililiter
miR	MicroRNA
miRNA	MicroRNA
mRNA	Messenger RNA
MS	Mass spectrometry
ng	Nanogram
NGS	Next-generation sequencing
nm	Nanometre
NPV	Negative predictive value
Nt	Nucleotide
PCa	Prostate cancer
PCA3	Prostate cancer antigen 3
PCR	Polymerase chain reaction
PFS	Progression free survival
PI3K	Phosphoinositide-3-kinase
PIN	Prostate intraepithelial neoplasia
PPi	Pyrophosphate
PIP2	Phosphoinositide 4,5-biphosphate
PIP3	Phosphoinositide 3,4,5-triphosphate
PLCO	Prostate, Lung, Colorectal, and Ovarian Cancer Screening
Pol II	Polymerase II
PPV	Positive predictive value
pT	Pathological stage
PTEN	Phosphatase and tensin homologue tumor supressor
PSA	Prostate specific antigen
qPCR	Quantitative PCR
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROC	Receiver operating characteristic
RT	Retrotranscription
SD	Standard deviation
SPOP	Speckle-type Poz protein
SRC-3	Steroid receptor coactivator 3
sRNA	Small RNA
T2E	TMPRSS2-ERG
TCGA	The cancer genome atlas
TKI	Tyrosine kinase inhibitor
TNM	Tumor-node-metastasis
TP53	Tumor protein 53
UTR	Untranslated region

Resumen

El cáncer de próstata (CaP) es el segundo tumor más frecuente en hombres y la sexta causa de muerte por cáncer. Así pues, esta enfermedad constituye un problema socio-sanitario prioritario para el sistema de Salud Pública. Actualmente, las herramientas para orientar el diagnóstico en CaP (PSA y DRE) no son cáncer específicas y presentan distintas limitaciones tales como el alto número de falsos positivos (aproximadamente un 70% en un rango de PSA de 4-10 ng/ml) que dan lugar a complicaciones asociadas con el proceso de biopsia. Además, un gran número de los CaP diagnosticados son tumores de bajo grado implicando un sobre-diagnóstico y sobre-tratamiento de esta enfermedad. Sin embargo, otros CaP tendrán un comportamiento pronóstico más agresivo que dará lugar a la progresión de la enfermedad y en último término a la muerte del paciente. Estas diferencias en el comportamiento clínico del CaP se explican por una alta heterogeneidad molecular presente en este tumor. En este contexto de heterogeneidad molecular nuestro objetivo se centra en la búsqueda de nuevos biomarcadores identificables mediante procedimientos no invasivos y capaces de clasificar a los pacientes con CaP de acuerdo a biotipos moleculares asociados con diferentes parámetros clínico-patológicos y distinto riesgo de progresión. En este trabajo exploramos el papel que tienen los miRNAs como nueva fuente de biomarcadores en CaP y encontramos que el miR-182 y el miR-187 juegan un papel clave en la patogénesis y el desarrollo del CaP en ambos contextos, el diagnóstico (miR-187) y el pronóstico (miR-182). Además, identificamos *ALDH1A3*, un gen regulado por andrógenos, como diana del miR-187 y como potencial biomarcador en CaP. En nuestra búsqueda de nuevos biomarcadores estudiamos también el papel que tiene el gen *SPOP* en CaP confirmando su pérdida de expresión y mutaciones en CaP y siendo el primer grupo en describir la asociación de estas alteraciones moleculares con el pronóstico en CaP. Además en nuestro trabajo también intentamos ofrecer nuevas alternativas terapéuticas para el tratamiento del CaP avanzado de acuerdo con el biotipo molecular. Así, nuestro hallazgo de la asociación directa entre IGF-IR y *TMPRSS2-ERG* y la mayor sensibilidad de este grupo a los inhibidores de IGF-IR nos

llevaron a proponer este subgrupo de pacientes como población diana -biotipo- para la inhibición de IGF-IR.

Abstract

Prostate cancer (PCa) is the second most frequent tumor in men and the sixth cause of cancer death. Hence, this disease constitutes a primary socio-sanitary and Public Health problem. Currently, the tools to orientate the PCa diagnosis (PSA and DRE) are not cancer specific and present several limitations such as the high rate of false positives (approximately 70% in the PSA range 4-10 ng/ml) leading to biopsy-associated complications. Furthermore, a high percentage of diagnosed PCa are low-grade tumors meaning a high overdiagnosis and overtreatment. On the other hand, other PCa will have a more aggressive prognostic behavior that could lead to disease progression and patient death. This different clinical behavior is translated into a high molecular heterogeneity. In this context of molecular heterogeneity we aimed to find new biomarkers identifiable by non-invasive procedures able to classify PCa patients according to molecular biotypes associated with different clinico-pathological parameters and risk of progression. In this work we explored the role of miRNAs as a source of new biomarkers in PCa and we found that miR-182 and miR-187 play a key role in the pathogenesis and development of PCa in both the diagnostic (miR-187) and prognostic settings (miR-182). Furthermore, we identified *ALDH1A3*, an androgen-regulated gene, as a target of miR-187 that also plays a role as biomarker for PCa. In our search for new biomarkers we also assessed the role of *SPOP* gene in PCa confirming its loss of expression and mutations in PCa but also being the first group to describe the association of these molecular alterations with PCa prognosis. Moreover in our work we also tried to offer new therapeutic alternatives for advanced PCa treatment according to the molecular biotype. Our finding of a direct association between IGF-IR and *TMPRSS2-ERG* and the higher sensitivity of this group to IGF-IR inhibitor agents lead us to propose this subgroup of patients as a target candidate population -biotype- for IGF-IR inhibition.

General Introduction

1. Epidemiology of Prostate Cancer (PCa)

Prostate cancer (PCa) is the first most common cancer in men with around 899000 diagnosed men each year [1]. Approximately one in three men over the age of 50 years shows histological evidence of this tumor, however only 10% will be diagnosed with clinically significant PCa [2-4].

The strongest risk factors for PCa are older age, a positive family history, and black race. The frequencies of PCa increase dramatically with age, beginning with low frequencies in middle-aged men and progressing to 90% by age of 90 years [5]. The median age at diagnosis is 67 years, and the median age at death is 81 years. Furthermore the risk of PCa is two times higher among patients who have a first-degree relative with a PCa diagnosis than among patients who do not have a first-degree relative with this diagnosis, indicating a heritage factor in this disease [5, 6].

An increase in PCa incidence has been reported during the last 25 years mainly due to the increase of the median population age, early diagnosis programs, prostate specific antigen (PSA) and image techniques (**Figure 1**). However in spite of this high incidence there is still controversy regarding the detection programs, optimal evaluation, classification and treatment for each stage of the disease [7, 8].

1.1. The diagnostic dilemma of PCa

Since 1980s, when PSA testing was developed, the current standard for the diagnosis of PCa has consisted of a serum test for PSA and digital rectal examination (DRE) [9] which will direct to the performance of a transrectal biopsy. DRE remains the primary test for the initial clinical assessment of the prostate. DRE was the first screening test to be evaluated and is still routinely used along with PSA testing. It has the benefit of detecting non-PSA-secreting tumours. In the many studies performed since the first investigation of its accuracy in 1956, the positive predictive value of DRE has been around 50%. However, the DRE is a test with only fair reproducibility, even in the hands of experienced examiners. It misses a

substantial number of cancers and, compared with PSA, it detects cancers at a more advanced pathological stage

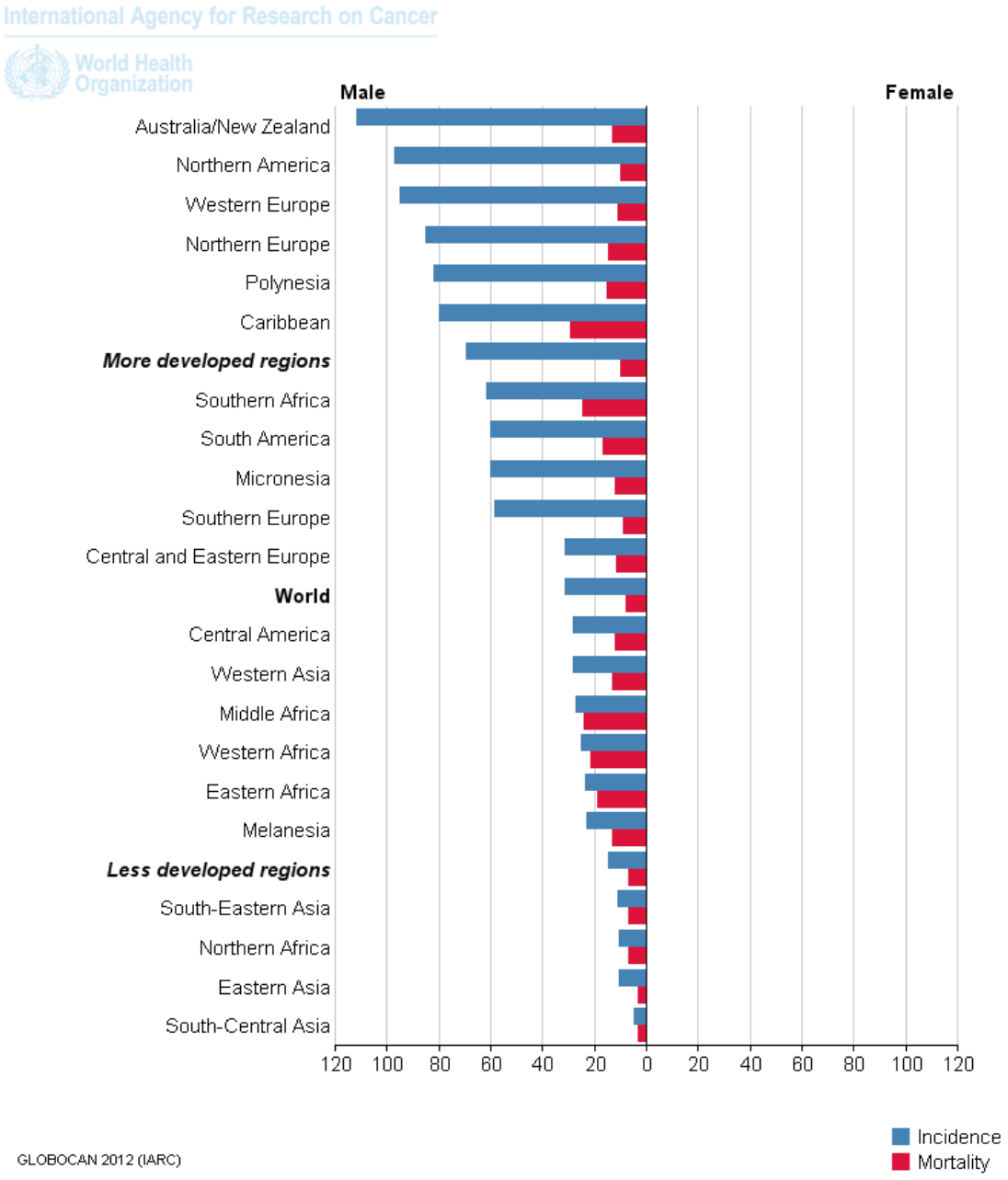

Figure 1. Prostate cancer estimated incidence and mortality worldwide in 2012. PCa incidence varies more than 25-fold worldwide; the rates are highest in Australia/New Zealand and Northern America and in Western and Northern Europe, because the practice of PSA testing and subsequent biopsy has become widespread in those regions. However there are not significant differences in mortality pointing out the incapability of the current screening tools to distinguish between indolent and more aggressive tumors. *Adapted from GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worlwide in 2012 (http://globocaniarcfr/Defaultaspx)*

PSA is a 34-kilodalton (kD), single-chain glycoprotein of 237 amino acids that was first described in 1979 as a serine protease, member of the kallikrein gene family [10]. It is produced by the prostatic epithelium and periurethral glands and is present in large amounts in prostatic secretions.

PSA serum levels have improved the detection and management of this disease but despite screening with PSA is widespread [11] it is clear that PSA has significant limitations as an early detection biomarker for PCa [12] as it is not cancer-specific . The first normal reference range for serum PSA was set rather arbitrarily in a very limited number of patients and probably not attending actual rules for biomarkers settling and management as less than 4 ng/mL, with a diagnostic grey area between 4 and 10 ng/mL [13]. However serum levels are also commonly elevated in benign conditions. Other urological conditions such as bening prostatic hyperplasia (BPH), prostatitis, urine infection or vesical lithiasis will also lead to a raise of PSA values (>4 ng/ml). On the other hand 15% of histologically confirmed PCa will harbor PSA levels below 4 ng/ml and a normal DRE. Hence, only 26% of the biopsies performed with a PSA between 4 -10 ng/ml are positive for PCa leading to a 74% of unnecesary biopsies performed [14].

Although the use of PSA test for PCa screening since 1990s has led to increased early diagnosis, there are several studies in conflict about the risks and benefits of routine PCa screening [15].Currently there are two main random screening studies based on PSA testing which aimed to reduced mortality however their results are contradictory. The European Randomized Study of Screening for PCa (ERSPC **ISRCTN49127736**) [8, 16, 17], with 13 years of follow-up and 162388 PSA screened men showed that absolute reduction in PCa moratlity was 0.13% although screening of 781 men is needed to be able to prevent one death from PCa. On the other hand the American study (PLCO, **NCT00002540**) [18], with 7-10 years of follow-up and 76693 screened men resulted in a 22% increase in cancer detection although it did not show any reduction in overall or PCa mortality. Moreover this study has been very criticized due to unacceptable rates of contamination in the control group, which makes its results not valuable. These observations confirm

that reduction of cancer associated deaths is associated with a higher risk of overdiagnosis, leading to the performance of large number of unnecessary biopsies, not free of morbidity and constituting important social and health costs [19]. Hence, methods to integrate other new markers to improve the cost-effectiveness of prostate biopsies and detect clinically significant PCa are needed. In this context the emergence of a commercial kit (Progensa *PCA3* test) for clinical application to detect the expression of *PCA3* gene in urine have provided promising results with a biopsy sparing rate between 40 and 67% and an area under the curve (ROC) of 0.7 [20].

1.2. Role of biomarkers in cancer

The 'Holy-grail' of a cancer-screening program is to have a biomarker that detects tumors at an early stage and in a sufficiently noninvasive and inexpensive way to allow widespread applicability. According to the National Cancer Institute, a biomarker *is a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease such as cancer* (*www.cancer.gov*). Cancer cells display a broad spectrum of genetic alterations that include gene rearrangements, point mutations and gene amplifications leading to disturbances in molecular pathways regulating cell growth, survival, and metastasis. When such changes manifest in a majority of patients with a specific type of tumor, these can be used as biomarkers. Cancer biomarkers can be DNA, mRNA, miRNA, proteins, metabolites or processes. These markers can be used for screening purposes, differential cancer diagnosis, estimating risk of disease, distinguishing benign from malignant findings or one type of malignancy from another, predict disease progression and monitoring the status of the disease, either to detect recurrence or determine response or progression to therapy. Hence, an ideal biomarker should be measured easily, reliably and cost-effectively with high specificity- understood as the proportion of control individuals (without tumor) who test negative for the biomarker- and sensitivity –considered as the proportion of individuals with confirmed disease who test positive for the biomarker- [21]. A common graphical representation to evaluate the sensitivity and specificity of a

novel biomarker is based on ROC curve evaluation. This curve is used to evaluate the efficacy of a tumor marker at various cut-off points leading to a quantitative value known as the maximum area under the curve (AUC)[22] .

The first step in the search for new biomarkers begins with the development of pre-clinical studies comparing tumor tissue with non-tumor tissue. Currently, this phase of biomarker identification is frequently performed using a "discovery" approach, using techniques such as high-throughput sequencing, gene expression arrays and mass spectroscopy (MS) to quickly identify individual or groups of biomarkers that differ between cohorts [23]. Once a technically valid assay has been developed, the biomarker must be studied to determine if it has clinical or "biologic" validity. The potential biomarker should be assessed initially in relatively small cohorts of patients (training set) and afterwards those biomarkers that are informative in phase I are tested in larger independent, well-characterized cohorts of patients, including retrospective analysis of the material. Candidate biomarkers with supportive data from phases I and II are assessed prospectively in randomized control trials that usually implies several institutions [24] (**Figure 2**).

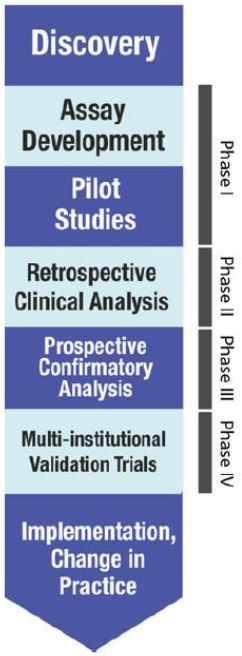

Figure 2. Pipeline of biomarker discovery and development. The National Cancer Institute strategy for biomarker development starts with a discovery phase to identify promising candidates for further validation. In the following phase an assay is developed to detect the new promising candidate which is initially tested in a training set (Phase I) and once utility is demonstrated is further tested in a larger independent cohort of patients (Phase II). Candidate biomarkers will be assessed prospectively during Phase III and their clinical utility will be finally tested in a multi-institutional prospective randomized controlled trial (Phase IV). *Adapted from [23].*

In the future, the emergence of genetic discoveries, molecular biotyping and development in areas of research such as microarrays, proteomics and immunology may allow improved identification of men at risk of PCa and personalized screening protocols.

2. Pathology of PCa

Over 95% of PCas are adenocarcinomas that arise from prostatic epithelial cells. Other rare histologies have been described, including mucinous or signet-ring cell carcinomas, adenoid cystic carcinomas, carcinoid tumors, large prostatic duct carcinomas (including the endometrioid type adenocarcinomas) and small-cell undifferentiated cancers (**Figure 3**) [25].

The prostate is composed of branching glands, with ducts that are lined with secretory epithelial cells and basal cells. Secretory epithelial cells represent the major cell type in the gland, are androgen-dependent for growth, and produce PSA and prostatic acid phosphatase. The main function of the prostate is performed by the gland cells which produce the fluid portion of semen and the control of urine flow by muscle fibers [26].

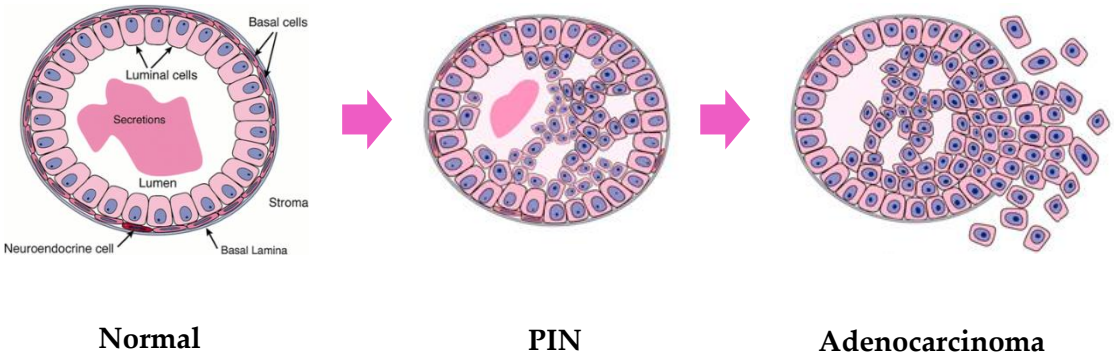

Figure 3. Schematic view of the cell types within a human prostatic duct during disease progression. Within the prostatic epithelium, there are three distinct cell types that can be distinguished by their morphological characteristics and functional significance. The predominant cell type is the secretory luminal cell, which produces prostatic secretory proteins, followed by basal cells and neuroendocrine cells, which provide paracrine signals that support the growth of luminal cells. Prostate cancer progresses from an enlargement (BPH) to precursor lesions (prostate intraepithelial neoplasia [PIN]) on to invasive carcinomas and ultimately to metastases. *Modified from [27].*

Tumors predominantly arise from epithelial cells in the peripheral zone of the gland. Tumors that progress, if untreated, will extend into the prostatic capsule and seminal vesicles, and will ultimately metastasize to regional and distant sites such as lymph nodes and bone [28]. Metastatic hormone-refractory disease is the most important cause of morbidity, treatment failure, and subsequent mortality from PCa. The two main issues for clinicians and pathologists involved in PCa are early detection of the cancer and identification of the prognostic and predictive factors that predict outcome in individual patients.

Since its inception in 1958, the **TNM system** has provided a standardized, anatomical basis for staging cancer disease. This staging system is based on the anatomical extent of the disease, which is assessed using a combination of tumor size or depth (T), lymph node spread (N), and presence or absence of metastasis (M). It provides a basis for prediction of survival, choice of initial treatment and stratification of patients [29]. In PCa this staging classification is subdivided into clinical (cT) and pathological stage (pT). cT staging is based on the clinical evaluation of the tumor extension performed by the facultative through the physical examination (DRE), laboratory analysis, prostate biopsy and imaging techniques, where multiparametric magnetic resonance imaging is gaining an important role as the best local/regional characterization of cT [30]. While, on the other hand, pT evaluation is based on the anatomic and pathological examination of the tissue after the surgery. The numeric indexes for both, clinical and pathological, evaluations indicate the extension of the tumor [31] (**Table 1**).

Table 1. TNM staging system [31].

Tumor size (T)	
T1c	Tumor identified on biopsy
T2a	Tumor palpable in half of one lobe
T2b	Tumor palpable in more than half of one lobe
T2c	Tumor palpable in both lobes
T3a	Tumor extends prostatic capsule
T4	Tumor reaches bladder and/or rectum
Lymph node spread (N)	
N0	Without extension to lymph nodes
N1	With extension to lymph nodes
Metastasis (M)	
M0	There is no distant metastasis
M1	There is distant metastasis

Another histological evaluation commonly used to assess PCa risk of progression is Gleason score. This parameter is based on the evaluation of the histology and the loss of normal gland tissue architecture. Five distinct glandular patterns are graded progressively from most to least differentiated. The grades of the two predominant patterns present in a surgical specimen are added to yield the final **Gleason score** (**Figure 4**). Patients with well-differentiated lesions (Gleason scores 2-4) usually have early-stage disease and a good prognosis. Gleason scores 8-10, however, are associated with a poor prognosis. Hence, Gleason score is the main prognostic variable for most of the stages of the disease therefore every Department of Pathology should follow the ISUP recommendations established in a consensus meeting in 2005 [32].

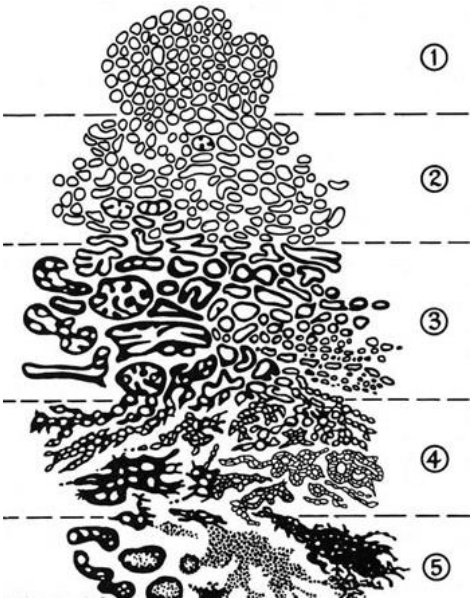

Figure 4. Gleason score grading. Gleason grading system assigns a score to different histological patterns within the prostate gland. The final score is calculated by the addition of the scores of the two main predominant histological patterns. Small, uniform glands with minimal nuclear changes are graded with Gleason score 1. Medium-sized acini, still separated by stroma but closely arranged constitute Gleason score 2. Score 3 corresponds to those histologies with a marked variation in glandular size and organization with stroma infiltration. Gleason score 4 is defined by atypical cells with extensive infiltration into surrounding tissues. Finally the score of 5 is assigned to markedly undifferentiated cancer cells. *Adapted from [33].*

In 1998, D'Amico and colleagues first proposed a three-group risk stratification system to predict post-treatment biochemical failure after radical prostatectomy and external-beam radiotherapy [34]. This classification is one of the most widely used and is a good starting point for risk assessment. This system divided non-metastatic patients into low-, intermediate-, and high-risk based on initial PSA, cT and biopsy Gleason score. Low-risk PCa was defined as T1/T2a, and PSA ≤10 ng/ ml, and Gleason score ≤6. Intermediate-risk PCa was defined as T2b, and/or PSA 10-20 ng/mL and/or Gleason 7 disease. High-risk disease was classified as having any one of the following high-risk features: cT >T2c, PSA >20 ng/mL or Gleason 8-10 disease. These risk groups predict for biochemical relapse based on post-treatment increases in PSA levels, and clinical relapse defined as local (to prostatic fossa), regional (to lymph nodes) or distant progression (metastasis). The time to biochemical and clinical relapse are defined as biochemical progression free survival (BPFS) and clinical progression free survival (PFS) respectively.

The current clinical prognostic factors of T category, PSA and Gleason score explain only a moderate proportion of the observed heterogeneity in clinical outcome [35]. Clinical phenotypes of PCa vary from an indolent disease requiring no treatment to one in which tumors metastasize and escape local therapy even when with early detection [36]. Thus far, little is known about what makes some PCa biologically

aggressive and more likely to progress to metastatic and potentially lethal disease. Heterogeneity exists both within and in between patients, therefore is critical to define the different genetic profiles that exists in PCa disease.

3. Genetics of PCa

The development of solid tumors is generally thought to be a multistep process, whereby successive genetic events occur in a normal cell to render it increasingly malignant. Recently, high-throughput large-scale genome analysis have demonstrated that the mutational landscape of cancer is complex, indicating that cancer may evolve through driver mutations in as many as 138 cancer-associated genes [37]. The appearance of these new technologies has enabled characterization of individual human cancers in an unprecedented level of molecular detail, with potential to identify cancer phenotypes and understand the clinically variable behavior of this disease. The high molecular heterogeneity found in cancer explains the diversity in clinical outcome and therapeutic response leading to new challenges on clinical practice (diagnosis and prognosis) and on the development of new therapeutic strategies [23].

Advances in sequencing technology have initiated several coordinated national and international efforts including The Cancer Genome Atlas (TCGA 2008) and the International Cancer Genome Consortium (ICGC 2010), to generate comprehensive catalogues of genomic, transcriptomic, and epigenomic changes in multiple tumor types [23]. The emergence of these new tools leads to a change in the traditional classification of cancers based on organ morphology into a "biotype" classification according to the molecular profile (**Figure 5**). Biotyping tumors into a collection of homogeneous subtypes identifiable by molecular criteria, associated with distinct risk profiles and therapeutic management, will lead to a better understanding of tumor biology.

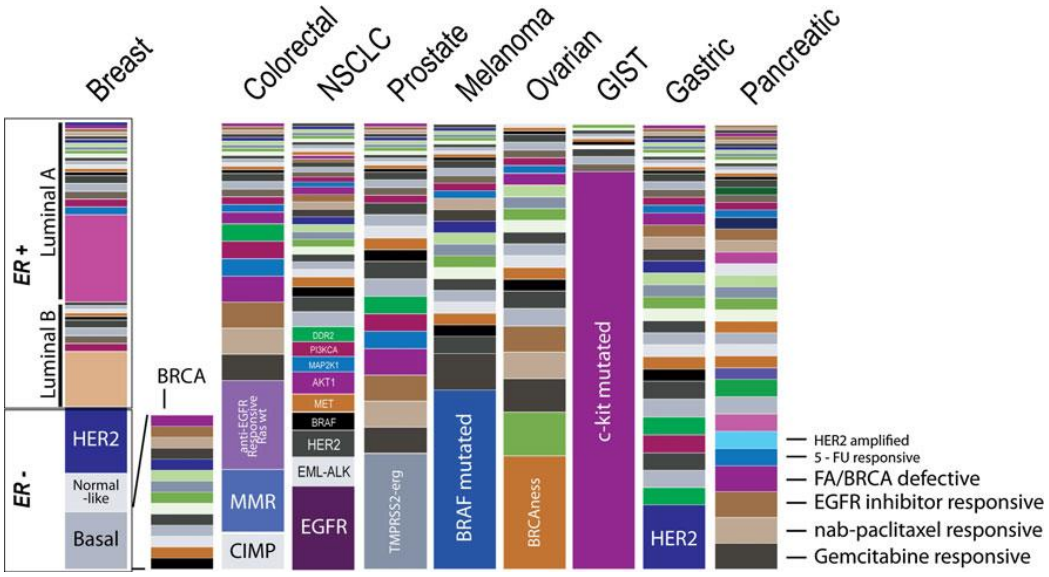

Figure 5. Biotype classification of human tumors according to molecular alterations. Emerging molecular taxonomy of tumors allows classifying the molecular heterogeneity within each tumor in a collection of homogenous subtypes with specific molecular alterations. Understanding this molecular heterogeneity will lead to the development of better diagnostic and prognostic tools as well as more accurate targeted therapeutic solutions. *Adapted from [23].*

Current understanding of PCa molecular heterogeneity is based on the emergence and application of these new technologies. Epidemiological studies indicate that germline variations caused by dominantly inherited susceptibility genes with high penetrance may cause 5% to 10% of all PCa cases [38]. However, the most common genetic variations in PCa comprise somatic alterations leading to structural genomic changes such as deletions, amplifications and translocations while punctual mutations are less common [12]. A large number of important somatic alterations have been identified as gains or losses of chromosomal regions, including gains at 8q and losses at 3p, 8p, 10q, 13q, and 17p. These alterations imply deletions involving the *NKX3.1* (8p21) and phosphatase and tensin homologue tumour suppressor genes (*PTEN*) (10q23), and amplifications of the androgen receptor (*AR*) (Xq12) and *MYC* (8q24) genes. More recent work reveals that majority of PCas harbor recurrent *ETS* gene fusions [36].

In addition to general genes and pathways that are commonly altered in cancer, **there are some genetic lesions that are highly specific of PCa**. Comprehensive understanding of these different molecular biotypes will orientate diagnosis, prognosis and therapeutics of PCa into an individualized handling of this disease. Hence,

amplification or mutation of androgen receptor (*AR*) (30% of castration-resistant PCa) and the fusion of *TMPRSS2* (an androgen-dependent serine protease) with the family of ETS transcription factors (*ERG* or *ETV4*) (50% of PCa) constitute frequent events in the development of PCa. It is also well known that the phosphoinositide-3-kinase (PI3K) pathway is among the most commonly altered signaling pathway in human PCa (25-75% of PCa). Epigenetic perturbations are also believed to represent important contributing factors in prostate carcinogenesis, and may provide useful biomarkers for disease progression [39]. In this context an emerging field of research in recent years has been the microRNAs (miRNAs), which regulate gene expression of mRNAs at the post-transcriptional level, and have been found to be deregulated in several tumors including PCa [40].

3.1. PCA3

In 1999, Bussemakers et al. identified *PCA3* (also known as *DD3*) as a potential biomarker for PCa [41]. *PCA3* (Prostate Cancer Antigen 3) is a long non-coding prostate-specific RNA highly expressed (about 34-fold increase) in 95% of prostate tumors. *PCA3* score (normalized with cellular PSA expression) correlates with the likelihood of a positive biopsy, hence constitutes the current most specific clinically available biomarker for PCa. *PCA3* determination in urine has been translated into a commercial assay, APTIMA (Hologic-Gene-Probe®, San Diego, CA, USA; PROGENSA in Europe), recently approved by FDA to assess the probability of a positive biopsy in the context of a second biopsy. *PCA3* has shown great promise in the general population as a diagnostic and prognostic marker for PCa since urine *PCA3* score has also demonstrated a significant association with extracapsular extension, tumor volume, and Gleason score [42, 43].

In our institution *PCA3* testing has been incorporated since 2009, in the context of an opportunistic screening program, into the diagnostic routine of decision making for the diagnosis of PCa for both initial biopsy (IBx) and follow-up biopsy (FUBx) [19, 20]. Since the initiation of the screening program 3865 men (169 men were excluded from the original 4034 men recruited) were tested with a median follow-up of 19 months, median age of 57 years old, with a mean PSA of 1.53 ng/ml

(SD = 1.49) and 19.8% with family history of PCa. Five hundred thirty men (13.7%) with normal DRE underwent per protocol *PCA3* testing; 447 (84.3%) were enrolled at first round, and then 73, 9 and 1 at the 2nd, 3rd and 4th rounds respectively (**Figure 6**).

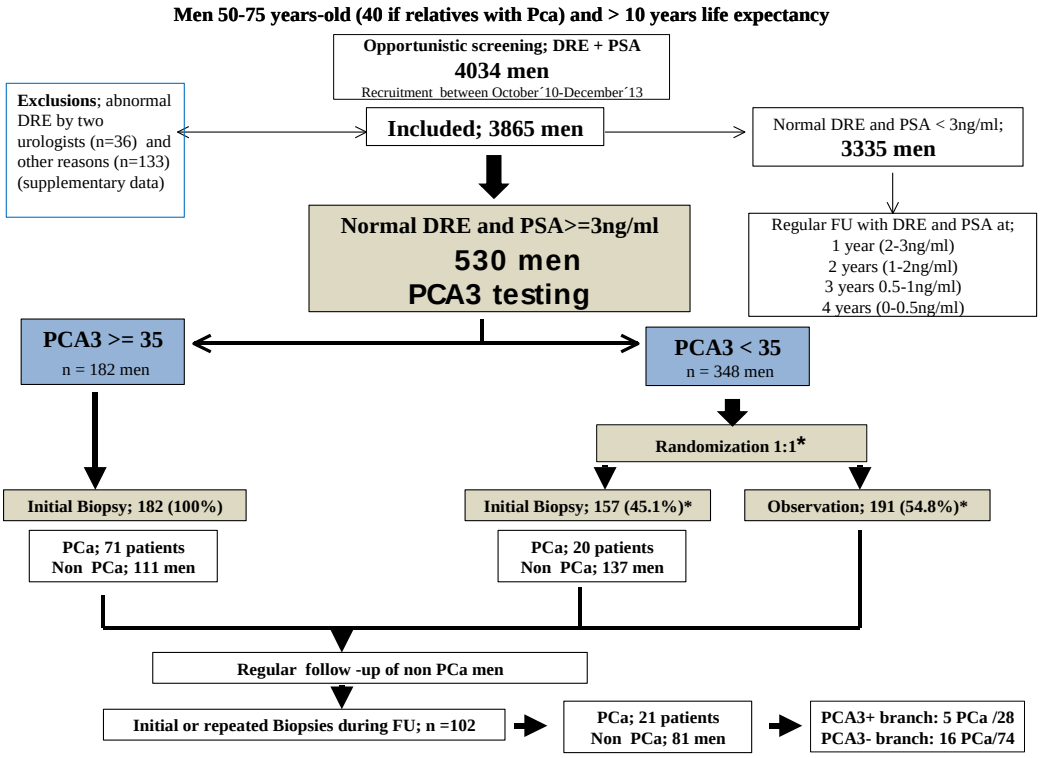

Figure 6. Algorithm of the dual protocol applied for the opportunistic screening program conducted in our institution (ref. number. 2010-20) and men allocated to each branch. *Adapted from [44].*

The overall sensitivity for the *PCA3* score (cut-off point of 35) assessment in our series was 61.6%, the specificity 73.2%, the positive predictive value (PPV) 39%, and the negative predictive value (NPV) 87.3%. Furthermore, in our experience the best results for *PCA3* are obtained at the IBx setting were parameters of sensitivity, specificity, PPV, and NPV are better for the subgroup of patients without previous biopsy [20]. Our results confirm that the routine use of the *PCA3* score both as a continuous and categorical variable, complementary to the PSA and rectal examination, can advise a patient with suspected PCa whether to undergo biopsy or not and make biopsies profitable.

3.2. *TMPRSS2-ERG* (*T2E*)

Chromosomal rearrangements leading to gene fusion have long been known to be involved in the pathogenesis of lymphoma, leukemia and sarcomas. In 2005 Tomlins et al described for the first time in PCa a series of genetic rearrangements between the promotor of a serine protease *TMPRSS2* (21q22) and some members of the ETS family of transcription factors, such as *ERG* (21q22), *ETV1* (7p21), *ETV3* and *ETV4* (17q21) [45]. *TMPRSS2* is an androgen regulated gene encoding a transmembrane serine protease that is highly expressed in normal and cancerous prostate cells (**Figure 7**). To date more than 20 *TMPRSS2-ETS* fusion transcripts have been described with *TMPRSS2* (exon 1) and *ERG* (exon 4) being the most common ones. Although functional overlap among different members of the ETS family exists, individual ETS factors also serve distinct roles. Thus, the expression pattern of ETS members through development varies, along with their repertoire of target genes, biological processes regulated and oncogenic potentials. The networks regulated by *ERG* are associated with cell cycle and DNA replication, whereas those controlled by *ETV1* are related to synthesis of lipids and other metabolic pathways. These different networks controlled by distinct ETS members will lead to different pathological consequences.

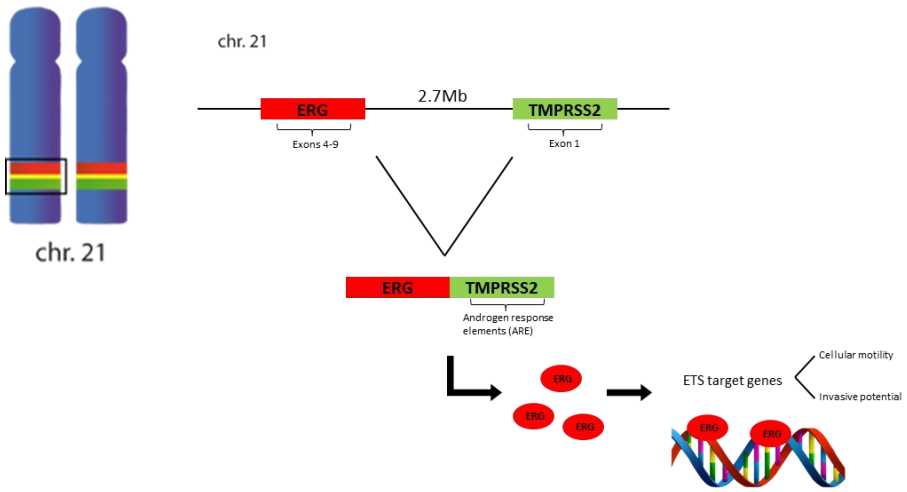

Figure 7. *TMPRSS2-ERG* translocation in PCa. Translocation within chromosome 21 between the androgen-regulated promotor of *TMPRSS2* and the transcription factor *ERG* is a common event in PCa. The product of this fusion gene (ERG) is able to control several processes such as genomic damage, epigenetic reprogramming, cell invasion, inflammation, and differentiation.

The prevalence of ETS rearrangements ranges from 27% to 79% in radical prostatectomy and biopsy samples and it is believed to constitute an early event in PCa development. Because of the high prevalence of ETS fusions together with the potential association with clinico-pathological parameters [46-48], division into ETS-positive and ETS-negative subclasses has been established to molecularly subdivide PCa specimens and the association with both clinico-pathological and prognostic factors have also been found to be different within each group [46]. In this context, the appearance of next-generation sequencing (NGS) technologies has supported the classification of ETS-positive and –negative tumors as distinct biological entities. Therefore, some molecular alterations such as *PTEN* or *TP53* deletions are enriched in ETS-positive tumors while mutations in *SPOP* gene, *CHD1* deletions or *SPINK1* overexpression are events that only occur in ETS-negative tumors.

Clinical studies of the prevalence and prognostic significance of ETS fusions in PCa have yielded discrepant results, possibly related to differences in the genetics of the evaluated populations and diversity in methods used. Several studies suggest that ETS fusions are associated with a worse prognosis [47-49], whereas others have failed to confirm the correlation with clinical outcome [50]. Despite these controversies it is clear that ETS proteins are involved in the regulation of essential processes such as cell growth, differentiation and apoptosis. Furthermore, *T2E* plays a critical role in cancer progression by disrupting the AR lineage-specific differentiation program of the prostate. Evidence exists that *ERG* interferes AR signaling by inhibiting *AR* expression via recruiting H3K27 methyltransferase, a Polycomb group protein named as enhancer of zeste homolog 2 (Drosophila) (EZH2). Therefore, repression of AR by *T2E* may provide a malignant selection pressure contributing to recurrent tumors with *AR* amplification.

3.3. Androgen receptor (AR)

The AR is a steroid hormone receptor that is cytoplasmic in its unbound state, forming a complex with heat-shock protein (HSP)-90 while upon androgen binding it undergoes a conformational change allowing nuclear translocation, DNA binding

and regulation of gene transcription through binding to androgen-response elements (AREs) within chromatin of AR regulated genes [51]. The most abundant androgen is testosterone, which is synthesized by the testis and converted into the more active metabolite dihydrotestosterone (DHT) in prostate tissue trough the activity of 5α-reductase enzyme [39].

AR plays a vital role in development of the normal prostate as well as PCa disease progression since gonadal-testosterone-AR axis constitutes a major stimulus for prostate growth [52]. The ablation of this axis leads to castration-resistant PCa (CRPC), an advanced cancer stage where tumors no longer respond to androgen-deprivation therapy and are associated with a poor prognosis (mean survival of 16-18 months) [53]. The progression of PCa into a castration-resistant state evolves from the development of mechanisms of resistance to therapies that accumulate different alterations in AR signaling. *AR* undergoes multiple alterations leading to an increased activity such as gene amplification (25% of CRPC), point mutations (10-30% CRPC) and splicing variants together with alterations in interacting proteins (co-activators) that can modulate AR activity [39, 53]. With *AR* amplification there is increased receptor available for ligand-binding and nuclear translocation, gain-of-function mutations of *AR* may confer increased protein stability while *AR* splice variants results in AR isoforms with constitutive nuclear localization despite the absence of ligand-binding. Another mechanism for increased AR signaling activity, although not as commonly observed, is the endogenous expression of androgen synthetic enzymes by tumor tissue, which can lead to de novo androgen synthesis or conversion of weaker adrenal androgens into testosterone and DHT. Furthermore, ligand-independent activation of AR through different growth factors, cytokines and receptors such as EGFR, IL-6 receptor, IGF-IR, Her-2 receptor or Src can also take place [53]. In this sense it has been described a potential interaction resulting in a negative feedback regulation between AR and PI3K/Akt signaling pathways. Carver et al. found that *PTEN* deleted mice showed lower levels of AR and they also described how the inhibition of PI3K/Akt with small tyrosine kinase inhibitors (TKIs) such as BEZ-235 up-regulates AR and leads to the activation of *AR* gene expression [54]. Hence, the

crosstalk between AR and PI3K/Akt pathways supports the rationale for combined inhibition of both signaling pathways.

3.4. PI3K pathway

The PI3K pathway is altered in 25-70% of PCa, with metastatic tumors having a higher incidence. This pathway is activated by alterations in different signal components and affects cell proliferation, survival and invasion.

PI3K catalyzes the conversion of membrane phosphoinositide 4,5-biphosphate (PIP2) into phosphoinositide 3,4,5-triphosphate (PIP3). The accumulation of PIP3 creates a docking site for Akt which induce a conformational change leading to Akt phosphorylation and activation. The activation of PI3K can be counterbalanced by the action of *PTEN* a lipid phosphatase and tumor suppressor that dephosphorylates PIP3 back to PIP2, controlling the activation of Akt [36].

In nearly 50% of PCas, the PI3K-Akt survival pathway has been shown to be constitutively up-regulated because of loss of function and/or mutations of *PTEN*. Heterozygous and homozygous deletions of *PTEN* occur in approximately 40% of primary PCa while inactivating mutations are more common in advanced disease and occur in 5-10% of PCa. Hence, *PTEN* loss is an early event in prostate carcinogenesis and it has also been described to be correlated with the progression to CRPC. Inactivation of *PTEN* has been shown to cooperate with loss of function of the *NKX3.1* homeobox gene, up-regulation of the *c-MYC* proto-oncogene, or the *T2E* fusion [39].

Like *PTEN*, there are other genes such as *PIK3CA*, *PHLPP1*, *MAGI2*, *CDKN1*, and *GSK3B* that also harbor mutations or deletions in PCa. The presence of several recurrent lesions in multiple nodes of the PI3K pathway reinforces the important role of this pathway in PCa [36].

3.5. IGF pathway

Different growth factors like insulin growth factor (IGF) and epidermal growth factor (EGF) represent important mitogens for PCa. IGF and EGF bind to a tyrosine kinase receptor at the cell surface and activate downstream signal cascades like

PI3K/Akt pathway to increase PCa cell growth and proliferation [55]. The IGF family consists of two ligands (IGF-I and IGF-II), IGF-I and IGF-II receptor (IGF-IR IGF-IIR), six high-affinity IGF-binding proteins (IGFBP1-6), and other low-affinity IGFBP-related proteins (IGFBPrP). The effects of IGF-I are mediated by the receptors, IGF-IR and IGF-IIR [56]. The main event following IGF-IR phosphorylation is the stimulation of PI3K-Akt signaling pathway, leading to cell survival. The second pathway consists of Ras, Raf and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) activation, leading to tumor growth and proliferation [57] (**Figure 8**).

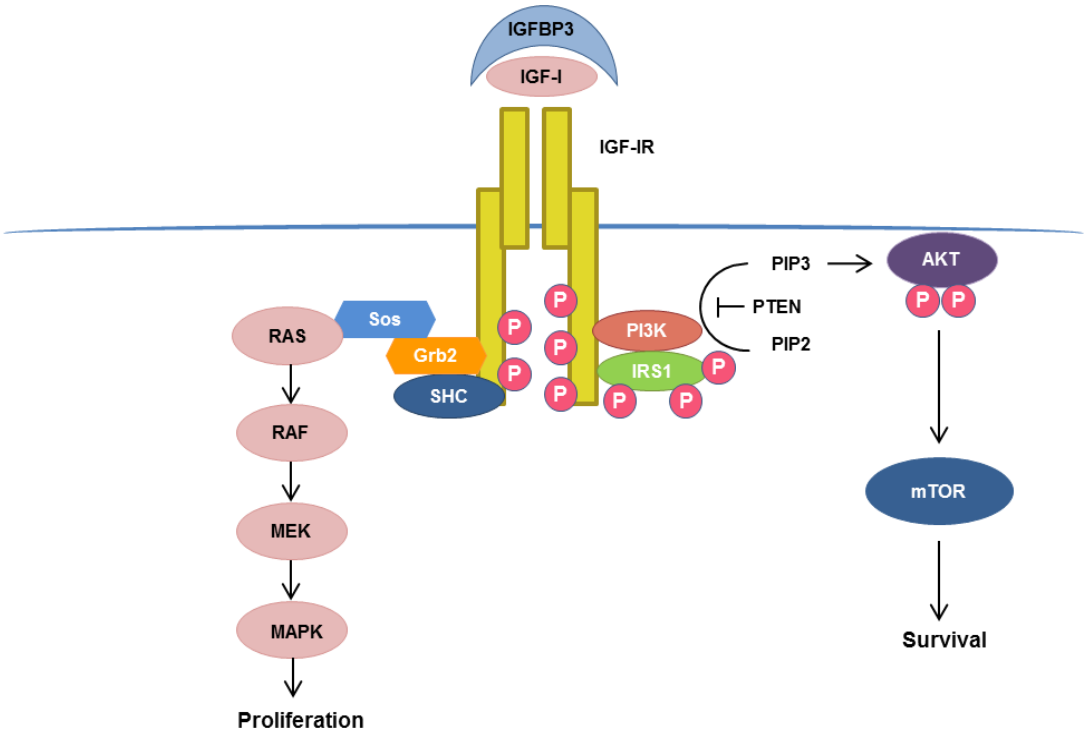

Figure 8. IGF system. The IGF system is composed by different ligands (IGF-I and IGF-II), receptors (IGF-IR and IGF-IIR) and binding proteins (IGFPB). Ligand binding to the IGF-IR induces its phosphorylation and activates two main signaling pathways (PI3K and MAPK) leading to cell proliferation and/or survival.

In prostate, IGF-IR plays a critical role in normal gland growth and development as well as in cancer initiation and progression [58]. Epidemiologic studies have associated levels of circulating IGF-I to risk of developing disease [59-61]. There is, however, no consensus regarding relative levels of IGF-IR expression

in benign and malignant prostate epithelium and the role of the IGF-IR in metastasis [62]. Analysis of the IGF axis in human PCa tissue specimens has lent further support to the hypothesis that activation of IGF-IR plays an important role in the pathogenesis of CRPC and IGF-IR was found in several works to be over-activated in PCa via increased expression of the receptor.

3.6. Speckle-type Poz Protein (SPOP)

Despite point mutations occur less commonly in PCa, the recently discovered gene *SPOP* has been found to be the most frequently non synonymous mutated gene in PCa [63] (**Figure 9**).

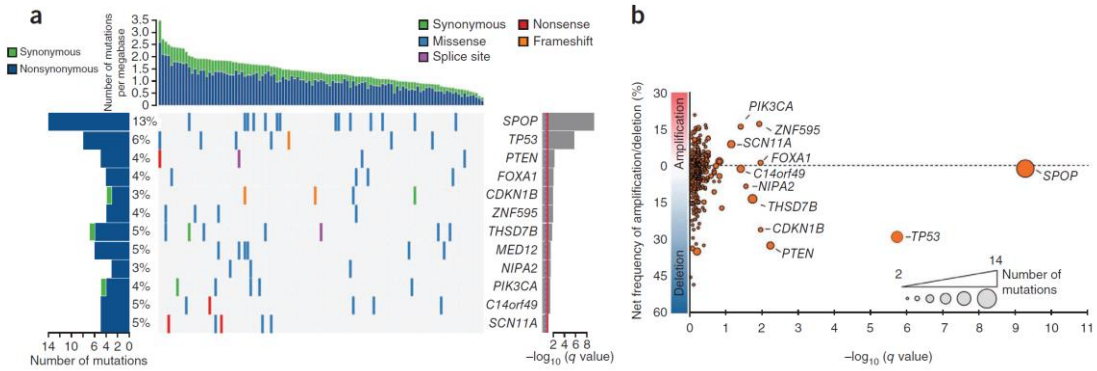

Figure 9. *SPOP* is the most frequently mutated gene in PCa. Exome sequencing analysis of more than 100 primary PCa tumors showed that *SPOP* gene was the most frequently mutated gene. *Adapted from [63].*

SPOP gene encodes for the substrate-recognition component of a Cullin3-based E3-ubiquitin ligase (Cul3) involved in the ubiquitination system. *SPOP* was first discovered in Hela cells in 1997, although it was also found to be expressed in other tissues such as brain, breast, lung, liver, pancreas, kidney and prostate [64].

SPOP gene is located in the 17q21 locus, which has been described to be a region with a high allelic imbalance in primary tumors [65]. Structurally, SPOP consists of an N-terminal MATH domain that recruits substrate proteins and a C-terminal BTB (Bric-a-brac/Tamtrack/Broad complex) domain that interacts with Cul3, which belongs to the ubiquitination scaffold [66] (**Figure 10**).

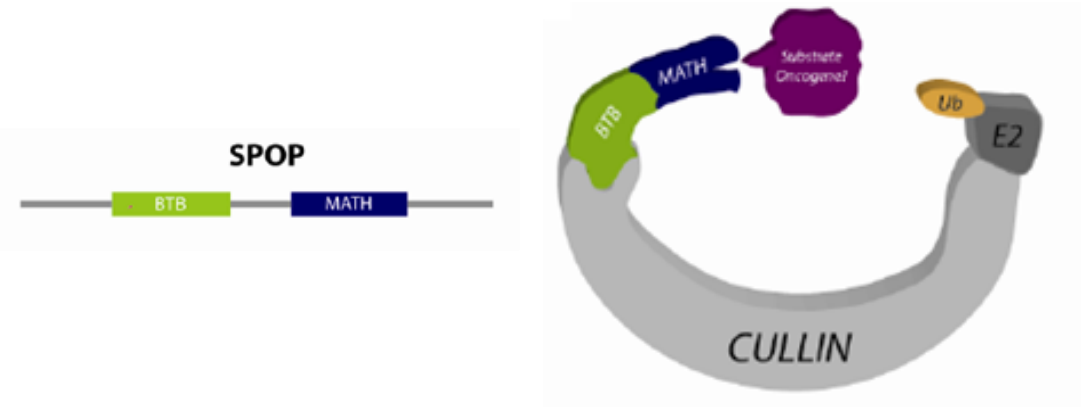

Figure 10. SPOP structure. SPOP protein is composed by two domains, an N-terminal MATH domain that recruits substrate proteins and a C-terminal BTB domain that interacts with Cul3. The BTB domain allows the binding to the cullin scaffold while the MATH domain specifically interacts with the substrate or protein to ubiquitinate.

SPOP is involved in several signaling pathways, including Hedgehog, c-Jun-N-terminal kinase and steroid signaling pathway [67].

Recurrent mutations in *SPOP* occur in 6–12% of PCa and are exclusively found in the substrate- binding cleft (MATH domain) of the protein, which is involved in the binding of those substrates to ubiquitinate. Hence, mutations in the MATH domain of *SPOP* gene will block the ubiquitination and degradation in the proteasome of the target proteins leading to its accumulation. In this sense, the oncogene *SRC-3* has been described to be a target of *SPOP* in PCa, reinforcing the potential role of this gene as a tumor suppressor in prostate tumors [68].

Furthermore, *SPOP* mutations have been found to be mutually exclusive with other common alterations in PCa such as *T2E* fusion, PI3K pathway or *TP53* alterations supporting the role of these alterations as a new molecular biotype in PCa [36, 69].

3.7. miRNAs

In cancer cells, genes and their functional products are either modified by mutations, or through epigenetic modifications that alter gene-expression patterns. In this context, an emerging field of research in recent years has been the miRNAs. miRNAs are small (17–27 nt) non-coding single-stranded RNA molecules that negatively regulate gene expression by binding to imperfect complementary sites

within the 3′ untranslated region (UTR) of their mRNA target at the post-transcriptional level [70].

miRNA biogenesis initiates with the transcription of a miRNA gene by RNA Polymerase II (Pol II) into a large molecule called pri-miRNA. A complex consisting of the proteins DGCR8 and Drosha processes the pri-miRNA to pre-miRNA, which is then exported by Exportin 5 to the cytoplasm and cleaved by the protein Dicer into a small dsRNA duplex (miRNA:miRNA*). This duplex is composed of a mature miRNA and a complementary strand (miRNA*). The mature miRNA is subsequently incorporated in an RNA-induced silencing complex (RISC) where it is free to interact with various mRNA targets [70] (**Figure 11**).

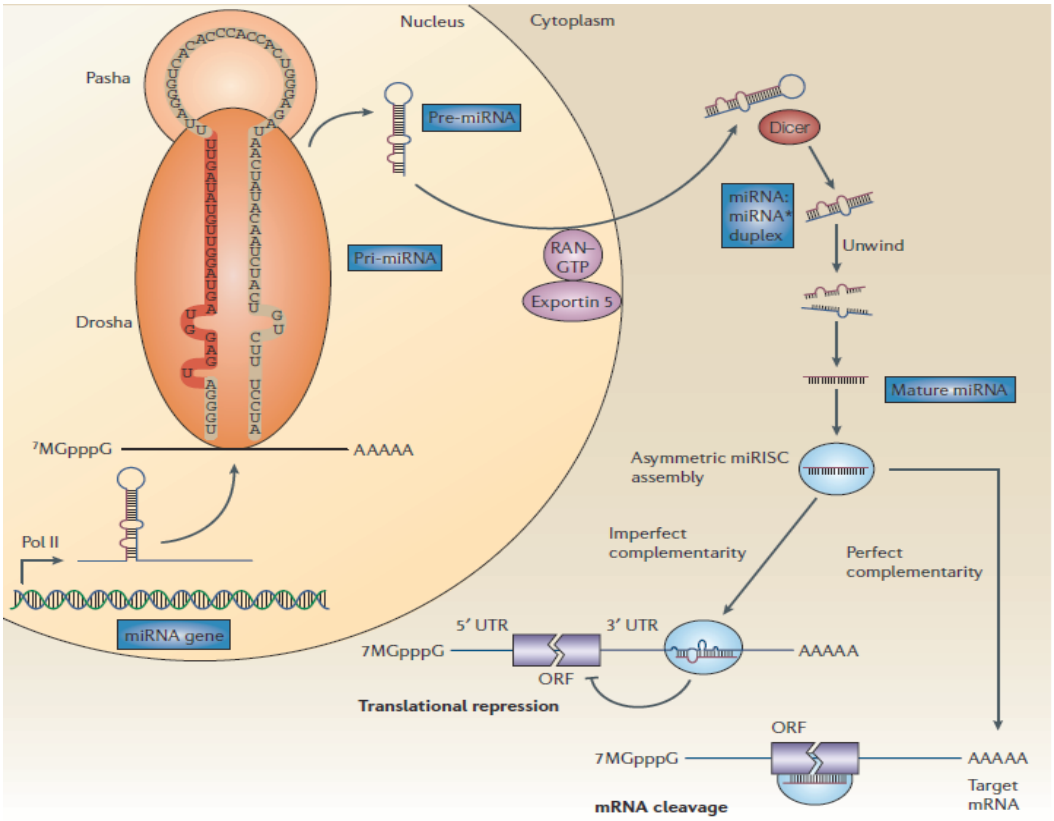

Figure 11. miRNA biogenesis. miRNA gene is transcribed by Pol II into a pri-miRNA which is processed by enzymes Drosha and Pasha into an immature miRNA called pre-miRNA. The pre-miRNA is exported into the cytoplasm by Exportin 5 where it is further processed by enzyme Dicer into a single strand mature miRNA. The mature miRNA is incorporated into the RISC complex and cleaves its target mRNAs. *Adapted from [70].*

miRNA binding is based in the perfect complementary binding of miRNA's first nucleotides (2–7 nt) to their corresponding mRNA and a needless equal binding of miRNA's flanking regions. This process allows a complex regulatory network in

which the individual miRNA may target more than 200 different mRNAs and, vice versa, a particular target could be regulated by different miRNAs [40]. Given that miRNAs can bind to multiple mRNA species, and that over 1400 human miRNAs have been identified, the miRNA repertoire of a cell can profoundly impact gene expression programs. Indeed, it has been estimated that approximately 60% of all protein-coding genes are directly targeted by miRNAs [71]. Since miRNAs can have several mRNA targets that are involved in the oncogenic process, dysregulation of miRNAs have been associated with the development of cancer. In fact, up to 50% of miRNA genes are located in cancer-related genomic locations [72].

In 2002, Calin et al. showed the first evidenced connecting miRNAs and cancer when they found that miR-15 and miR-16 are the target genes of the 13q14 deletion that is common in chronic lymphatic leukemia [73]. After this finding several works showed how miRNAs control fundamental cellular processes, such as differentiation of cells and timing of development of the organism indicating that aberrations of miRNAs are involved in various human diseases, including cancer [74]. In fact, several studies demonstrate that miRNA expression patterns serve as phenotypic signatures of different cancers and could be used as diagnostic, prognostic and therapeutic tools [75, 76]. Hence, miRNA expression-profiling studies have identified cancer-specific signatures demonstrating that miRNA signatures of cancers of different cellular origin seem to be unique underlying its potential role as disease biomarkers [77]. Changes in miRNA expression are related with its oncogenic function. Hence, it has been suggested that those miRNAs whose expression is increased in tumors may be considered as oncogenes while those who are found down-regulated are considered as tumor suppressors [78]. The oncogenic miRNAs usually promote tumor development by negatively inhibiting tumor suppressor genes or genes that control cell cycle, or apoptosis while the tumor suppressor miRNAs prevent tumor development by negatively inhibiting oncogenes or genes that control cell proliferation and differentiation.

In 2007 Porkka et al. identified for the first time a miRNA signature for PCa performing an oligonucleotide array hybridization study to assess the expression of

319 human miRNAs in PCa and found 51 miRNAs that were differentially expressed [79]. Since then, several groups have established different miRNA profiles for PCa [80-82]. The emergence of NGS technologies have offered a new approach for the identification of previously unknown miRNAs and qRT-PCR has been established the most suitable technology to validate miRNA expression-profiling results [83]. Despite this, there is no consensus in which will be the miRNA-profiling signature of PCa although the relevance of some miRNAs is already patent. Hence, the expression of several miRNAs has been found to influence androgen signaling in PCa. For instance, the expression of miR-125b, miR-21 and miR-141 has been found to be regulated by androgens [84, 85]. Moreover, other miRNAs have been correlated with different clinico-pathological parameters and progression in PCa. Several miRNAs have been associated with metastasis (miR-143 and miR-145, miR-16, miR-34a, miR-200 family) [86], Gleason score (miR-141, miR-1, miR-200 family) [87] or *T2E* translocation (miR-221) [88] in PCa. Implication of miRNAs in tumor initiation, progression and metastasis has pointed them out as potential candidates for therapeutic strategies. In this sense, different approaches have been developed to modulate the gain or loss of miRNA functions. To inhibit miRNA expression one of the approaches consisted in the introduction of anti-miRNA oligonucleotides (AMO), which are able to interact between the miRNA and its target through competitive inhibition of base-pairing [89]. Another strategy was based in the introduction of a modified mRNA carrying multiple pairing sites for endogenous miRNAs (miRNA sponge), inhibiting the function of some miRNAs through its targets [90]. Besides the already described approaches, the most frequently used strategies to down-regulate or recover the expression of miRNAs is based in the introduction of a synthetic miRNA molecule (miRNA inhibitor or miRNA mimic) which is able to interact by complementarity with the endogenous miRNA and inhibit or recover its function [91].

In all these contexts, miRNAs may be useful biomarkers as they have been found not only in cells and tissues but also in extracellular fluids such as plasma, serum, saliva and urine [92, 93]. Furthermore, their relative small size protects them from RNAse degradation and its presence in extracellular fluids allows them to

monitor physiopathological status of the patients being informative of disease progression and therapeutic response [87].

4. Therapeutics of PCa

Conventional treatment regimens for PCa basically depend on the stage of the disease. In localized low or intermediate risk PCa, radical prostatectomy or radiotherapy constitute the first options. In the case of advanced cancer, these regimens are usually followed or substituted with androgen deprivation therapy (ADT) [94]. ADT may be also employed as neoadjuvant treatment (before radical prostatectomy or radiation therapy) to reduce tumor burden or as adjuvant hormone therapy (after surgery or radiation) with the goal of providing symptomatic control of PCa for patients in whom definitive treatment with surgery or radiation is not possible or acceptable [95]. For those patients who develop metastatic or recurrent disease, palliative treatment including ADT, chemotherapy, secondary hormonal manipulation using Enzalutamide or Abiraterone, systemic radionuclides (Ra223) or immunotherapy (sipuleucel-T) has led to improved PFS [35, 39].

In 1941 Huggins and Hodges first reported the dramatic clinical effects of suppressing serum testosterone levels in men with advanced PCa [96]. From that first observation several strategies for androgen ablation have been developed and nowadays ADT is commonly accepted as first-line treatment of symptomatic metastatic PCa and there are also several evidences that prove that neoadjuvant ADT is also useful in increasing disease-specific and overall survival in men with clinically localized PCa [97, 98]. ADT could be achieved by surgical castration (orchiectomy) or suppression of luteinizing hormone-releasing hormone (LHRH) production at the level of the hypothalamus with LHRH agonists (i.e. diethylstilbestrol, DES), antiandrogens (flutamide, bicalutamide and nicalutamide) or other testosterone biosynthesis-inhibiting drugs [94, 95] (**Figure 12**).

Several studies have shown that extratesticular sources of testosterone represent an important alternative source of androgen stimulation in a significant proportion of patients with PCa. As much as 10% of baseline circulating testosterone remains in castrated men, due to peripheral conversion of adrenal steroids to testosterone.

Therefore, complete androgen blockage implicates suppressing also adrenal produced androgens [95].

Development of CRPC status is commonly driven by the use of adrenal remaining hormones or through intracrine synthesis of androgens. The 17α-hydroxylase/C17,20-lyase (CYP17) is a key enzyme for androgen and estrogen synthesis, therefore several inhibitors have been designed to modulate its activity (**Figure 12**). In this context ketoconazole and the aromatase inhibitor aminoglutethimide have been evaluated as possible agents to decrease production of adrenal steroids although they just showed a modest inhibition of CYP17 [99]. Hence, Abiraterone acetate was designed as a new potent, selective and irreversible inhibitor of CYP17. Abiraterone acetate is a CYP17 inhibitor that blocks androgen synthesis in the testes and adrenal glands in PCa. It has been shown to prolong survival in men before chemotherapy [100] and also in men with CRPC who have progressed after chemotherapy with docetaxel, and it has recently been approved by the FDA for this indication [101]. In this search of new drugs for second generation androgen ablation Enzalutamide (also known as MDV-3100) has also been developed. Enzalutamide is a nonsteroidal antiandrogen rationally designed to target AR that has recently been approved by FDA for metastatic CRPC following docetaxel [102, 103], and it has also demonstrated an increase in overall survival in the pre-chemotherapy scenario [103]. It competitively binds to the ligand-binding domain of the receptor and leads to inhibition of AR translocation to the cell nucleus, recruitment of AR cofactors and AR binding to DNA.

The current recommendation for metastatic PCa is based in the second line of new hormonal manipulations and in the treatment based on the use of the taxanes Docetaxel and Cabazitaxel [104]. The taxanes act by stabilising microtubules in the cell cytoskeleton. The binding of taxane molecules to microtubules prevents their disassembly, which leads to cell cycle arrest and apoptosis. Despite the already demonstrated efficacy and toxicity of these compounds there are different mechanisms of resistance that could be developed. For instance, Cabazitaxel was designed to address taxane resistance based on the increased transport of the drug out through up-regulation of P-glycoprotein transport molecule [104].

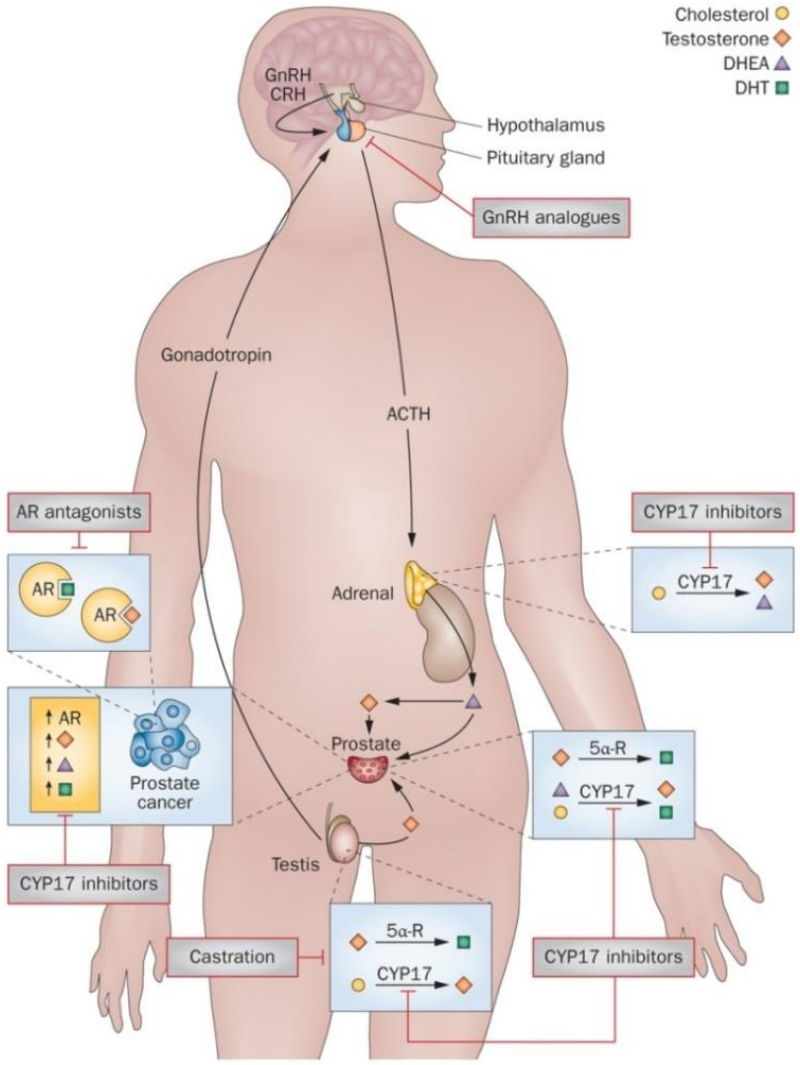

Figure 12. Androgen production and hormonal manipulations. About 90% of the androgen stimulation is due to testosterone production by the testis. Hence, one of the first hormonal maneuvers to control androgen signaling consists in the surgical castration (orchiectomy) or suppression of the gonadotropin LHRH at the hypothalamus level by the use of LHRH agonists (i.e DES, leuprolide). However 5-10% of androgens are produced by adrenal glands, therefore androgen blockage by the use of antiandrogens (i.e flutamide, bicalutamide) is needed to block the effects of adrenal androgens at the AR. Moreover upon CRPC stage, where tumors no longer respond to ADT, development of CYP17 inhibitors able to act at numerous points in the hypothalamic-pituitary-adrenal axis allows hormonal control. *Adapted from [105].*

At present there are no effective biomarkers in PCa to distinguish between indolent and aggressive disease and/or predict treatment outcome. Therefore the search for better predictors for treatment response and tumor prognosis is required to individualize PCa treatment and provide the optimal therapy with minimal side effects.

Increasingly, the development of novel targeted therapies involves defining drug-diagnostic combinations where the presence of a molecular marker identifies

patients who are most likely to respond to the new treatment. This model of developing treatment and biotype combinations in order to target patient populations with a greater chance of benefiting from treatment was first exemplified in breast cancer [23]. The *HER2+* subset of breast cancer is characterized by the expression of high concentrations of the receptor Her2 (ErbB2), which is the target of the therapeutic monoclonal antibody Trastuzumab [106], in the same sense ER+ breast tumors respond specifically to Tamoxifen [107] or aromatase inhibitors [108]. These approaches could be also translated into other tumor types if classifying them into molecular biotypes. Hence, SHIVA phase II trial (NCT01771458), a randomized multicentric trial comparing molecularly targeted therapy *vs.* conventional chemotherapy (**Figure 13**), is a proof of this concept which demonstrates that the establishment of a comprehensive tumor molecular profile is safe, feasible and compatible with clinical practice in refractory cancer patients [109].

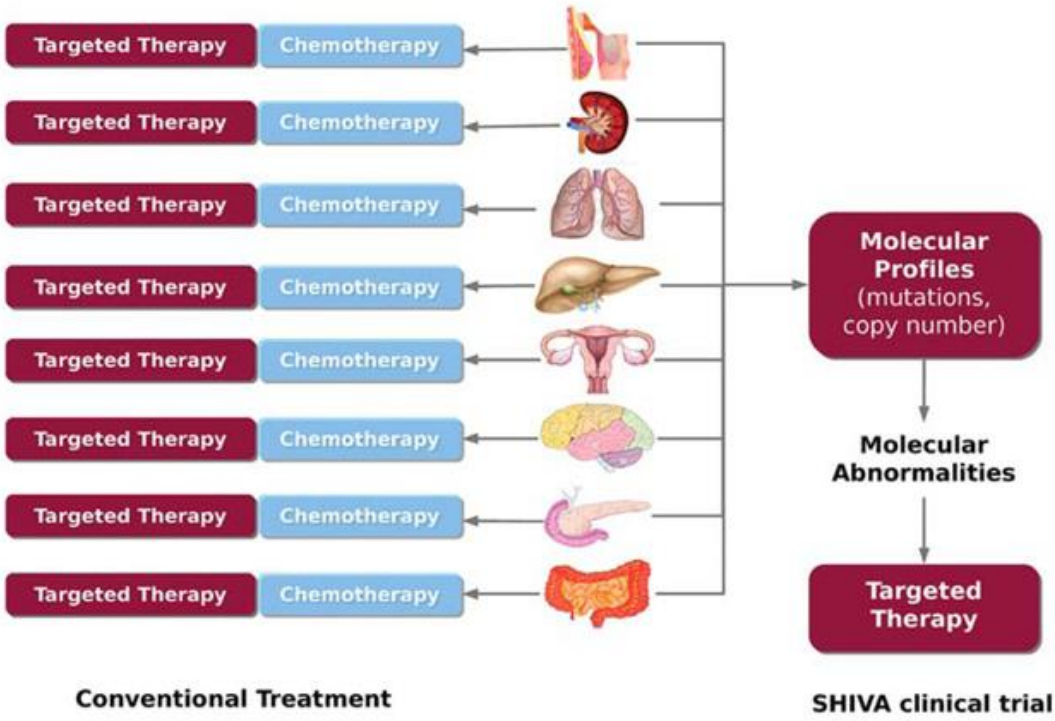

Figure 13. Molecular biotype classification *vs.* conventional treatment. Conventional treatment of tumors is based on organ classification which will dictate a specific chemotherapeutic regimen or a particular targeted therapy. However, recently the SHIVA clinical trial has changed the classical approach in cancer therapeutics basing its approach in the molecular profile present in each tumor. Patients are classified according to their molecular profile or biotype and depending on the molecular abnormalities found a particular targeted therapy is administered to those patients. *Modified from [110]*

5. Molecular profiling of different biotypes in PCa towards a precision medicine.

The emergence of the 'omics' technologies has been a revolution for the current understanding of cancer genomes. The disposal of a broad range of new tools to deeply explore the molecular heterogeneity of cancer provides new insights for the comprehensive understanding of this disease. In this sense the sequencing of the human genome in 2001 was a revolutionary step in the understanding of our genetic fingerprint [111].

To understand the molecular basis of disease a multidisciplinary approach that elucidates the genetic anomaly as well as its functional consequences is required. Hence, here we explore some of the current approaches that could be used to better elucidate PCa molecular heterogeneity.After the human sequence was completed several efforts have been made to understand functional genomics, which aims to decode dynamic aspects of the genome such as gene expression. For measuring gene expression at transcriptional level traditional techniques are available: Northern blot, PCR applications (such as quantitative real-time PCR [qPCR]) or in situ hybridization. However these techniques have the limitation of being able to study the behavior of a single gene at a time. Therefore to capture a more complete picture of the molecular state of cancer the characterization of global expression profiles or the screening of significant differences in the abundance of several mRNA is needed. Among the most powerful tools for monitoring gene expression are microarrays. Typically, a DNA array consists of rows of oligonucleotide strands, or complementary DNAs, lined up in dots on a miniature silicon chip or glass slide. These dots are labelled with a fluorescent dye and are hybridized against the public available probe sets. After hybridization, the fluorescence from each spot on the array provides a measure of the relative abundance of a given transcript and so reflects the relative expression level of the corresponding gene (**Figure 14**). Expression levels can be compared across many samples, normal and pathological, and differences in abundance could be represented as Fold-Change (FC). Moreover, clusters of genes that are regulated together can be identified in this way, whose functional relationships can uncover new aspects of cancer biology.

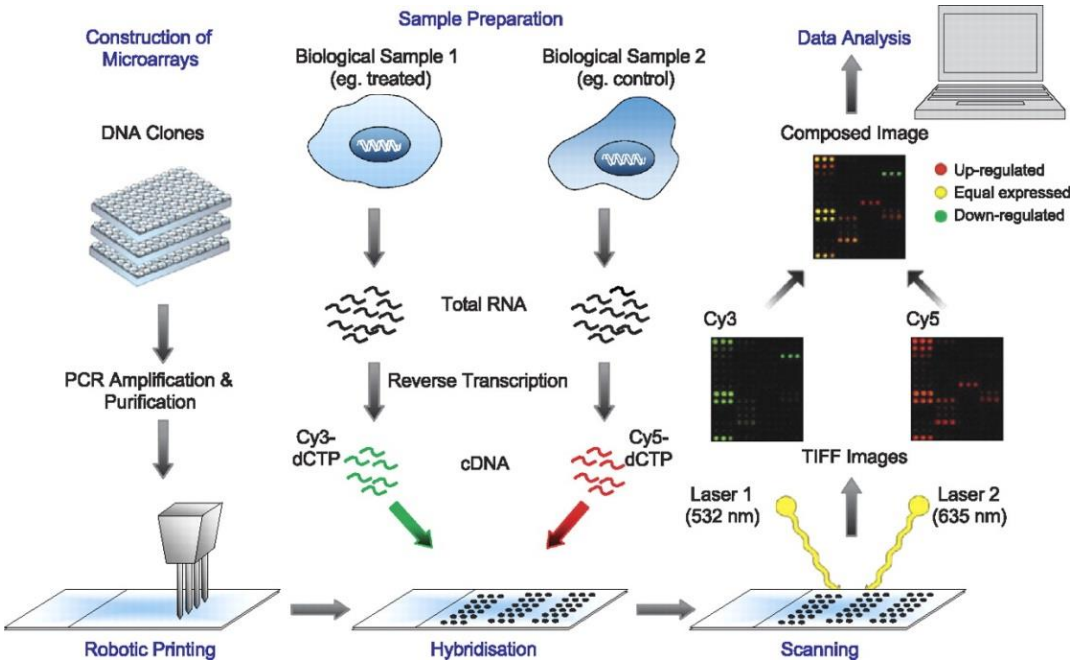

Figure 14. Overview of microarray technology. Microarray construction involves placing thousands of gene sequences in known locations on a glass slide called a gene chip. During sample preparation DNA or RNA samples from different biological specimens (i.e. normal and tumor tissues) are placed in contact with the gene chip. During the hybridization process complementary base pairing between the sample and the gene sequences on the chip produces light that is scanned and measured. Areas on the chip producing light identify genes that are up-regulated, equal expressed or down-regulated in the sample. *Adapted from (http://aniluguerra.blogspot.com.es/).*

The current knowledge of cancer genomes makes patent the clinical significance of genomic alterations in the development of the disease. Therefore, identification of cancer-associated mutations has become a standard care for cancer diagnosis and risk stratification [112]. Since 1977 when Frederick Sanger developed DNA sequencing technology, based on chain-termination method [113], laboratory and commercial sequencing applications were developed with its basis. In the past thirty years, DNA sequencing technologies and applications have undergone tremendous development and the emergence of the known as NGS has overcome the limitations in scope and low data yield of previous approaches. NGS technologies exploit these weaknesses with high-throughput capabilities, and with the ability to screen an entire genome, transcriptome and methylome in search of abnormalities and alterations [114]. Furthermore, NGS technologies allow massively parallel analysis, high throughput, and reduced cost. The NGS workflow consists of multiple steps, including library preparation and enrichment, sequencing, base calling, sequence alignment, and variant calling [112].

Roche 454 was the first commercially successful NGS system. This sequencer uses pyrosequencing technology which relies on the detection of pyrophosphate (PPi) released during nucleotide incorporation. The library DNAs with 454-specific adaptors are denatured into single strand and captured by amplification beads followed by emulsion PCR. Then on a picotiter plate, one of dNTP (dATP, dGTP, dCTP, dTTP) will complement to the bases of the template strand with the help of ATP. During this process the release of PPi will be equal to the amount of incorporated nucleotide [115] (**Figure 15**). The acceptable thresholds for data quality and depth of coverage should be determined during the assay development and validation process. The minimum depth of coverage depends on the required sensitivity of the assay, sequencing method, and type of mutations to be detected. But in any case, confirmation of variants via a second independent established technology (i.e. Sanger sequencing) is an important quality assurance step in NGS.

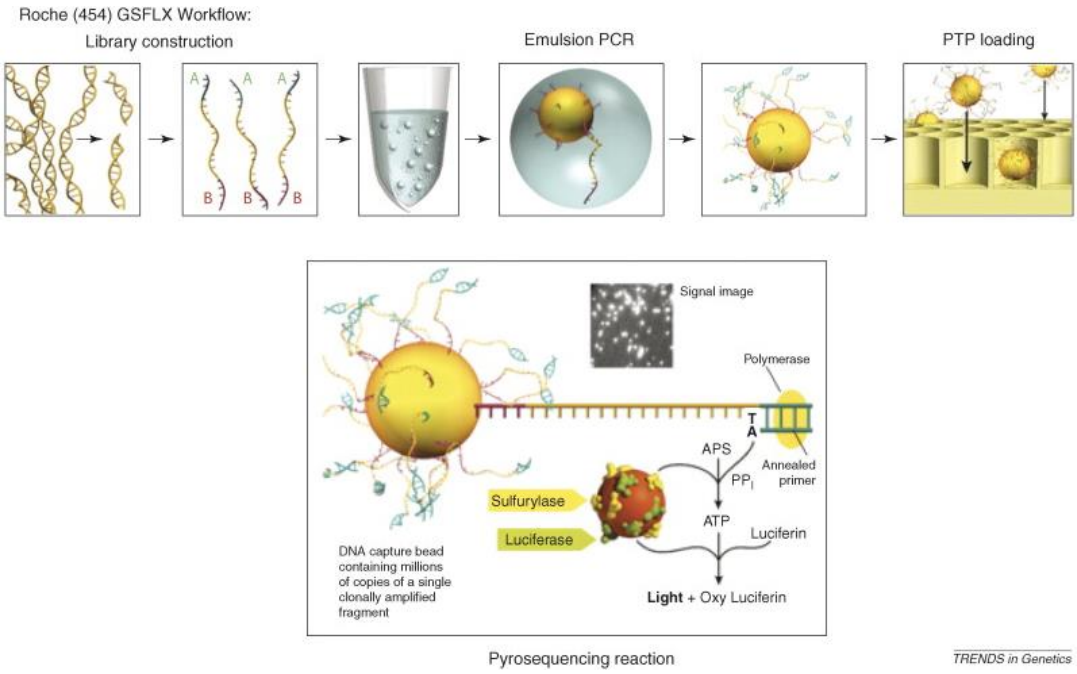

Figure 15. Roche 454 NGS workflow. Roche 454 is based on a pyrosequencing system which starts with a PCR to amplify the sequence of interest using specific primers to construct a DNA library. Once the library has been constructed an emulsion PCR is conducted in amplification beads to generate multiple copies of our DNA strand. Finally, on a picotiter plate sequencing is performed by sequencial incorporation of dNTPs that releases PPi in an amount which is proportional to the incorporated nucleotides. *Adapted from* *www.roche-applied-science.com*.

However, our phenotype is a manifestation of the proteome, the full complement of gene products, which execute the biological processes of the cell. Protein expression and function is dynamically regulated in health and dysregulation may result in disease. Therefore several low-throughput and high-throughput techniques have emerged on the forefront of proteomics research and are in various stages of clinical application. Improvements over the years have provided us with a more sensitive high-throughput gel-based technique termed as two-dimensional difference gel electrophoresis (2D-DIGE) which has been widely used to identify potential biomarkers, drug targets, or crucial mediators of disease by comparing spot intensities between diseased and normal states. 2D-DIGE separates proteins initially according to charge and then by molecular weight in the second dimension [116]. In this technique, two different protein samples (control and diseased) and one internal control (mixture of control and diseased sample in equal proportion) are labelled with any of the three fluorophores: Cy2, Cy3, or Cy5. These fluorophores have the identical charge and molecular mass but unique fluorescent properties. This allows us to discriminate them during scanning using appropriate optical filters. The labelled samples are then mixed together and separated on a single gel which is scanned with different wave lengths: 488nm (Cy2), 532nm (Cy3), and 633nm (Cy5) so each of the samples will generate its unique image (**Figure 16**). To investigate the identity of peptide and proteins mass spectrometry (MS) has been the proteomics gold standard. MS determines the molecular mass of a charged particle by measuring its mass-to-charge (m/z) ratio. Basically, a mass spectrometer consists of an ion source that converts molecules to ionized analytes, which are resolved according to m/z ratio, and a detector that registers the number of ions at respective m/z value. Finally, correlating the sequence ions generated from tandem MS data with sequence information available in protein databases, peptide sequence of unknown protein could be elucidated [117].

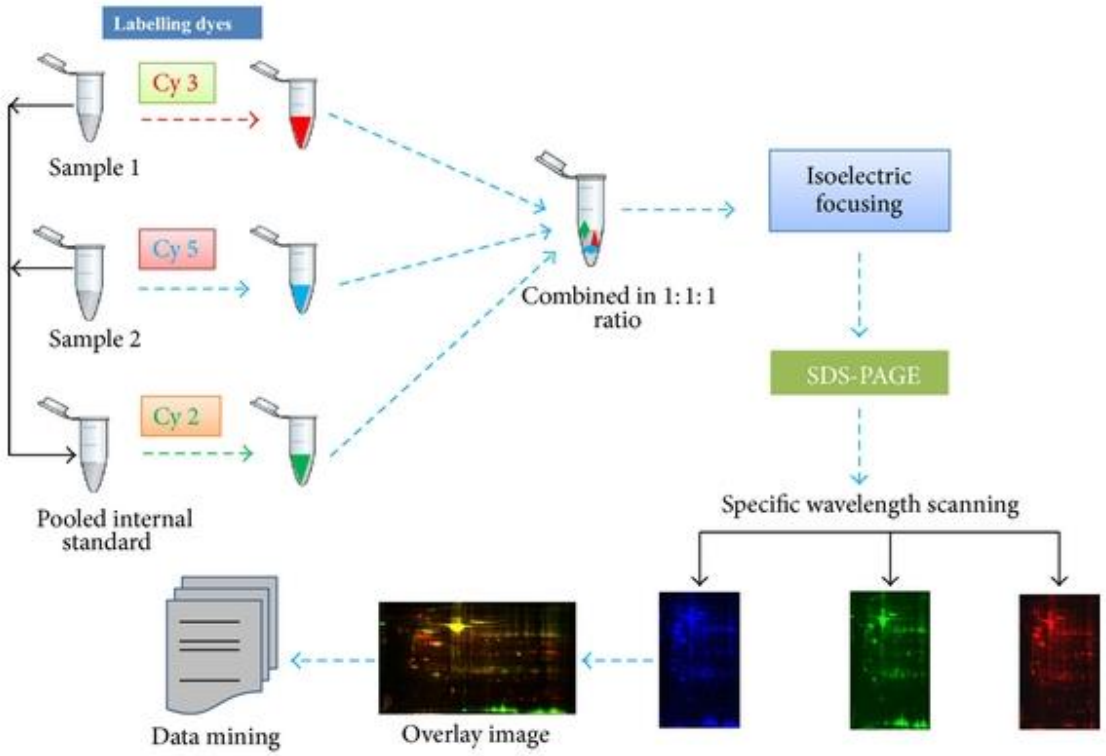

Figure 16. Scheme of a proteomic 2D-DIGE approach. Proteins are extracted from the samples and labelled with different fluorophores (Cy3 for sample 1, Cy5 for sample 2, and Cy2 for the pooled internal standard). All the samples are resolved in the same 2D gel by isoelectric focusing according to molecular weight and pH range. The protein spot pattern is detected by scanning the gel in the respective wavelength for the Cy dyes. The images are analyzed with the corresponding software to get potential candidates of interest that could be further identified by MS. *Adapted from [117].*

Hypothesis and objectives

Premises

- Per incidence and mortality, PCa constitutes a primary socio-sanitary and Public Health problem.
- Currently, the tools to orientate PCa diagnosis (PSA and DRE) are not cancer specific and present several limitations leading to biopsy-associated complications.
- A high percentage of diagnosed PCa are low-grade tumors with an indolent behavior meaning that the current diagnostic process is associated with overdiagnosis and overtreatment. On the contrary, other PCa will have a more aggressive prognostic behavior, which finally leads to disease progression and patient death.
- Nowadays molecular features do not direct the treatment of CRPC.
- This different clinical behavior is associated to characteristic PCa molecular biotypes.

Hypothesis

The comprehensive characterization of PCa molecular biotypes would optimize PCa diagnosis and patient management according to the molecular characteristics of the tumor. Hence, the inclusion in the diagnostic setting of new biomarkers identifiable by non-invasive procedures would classify patients according to optimized risk factors that could be validated within the framework of an opportunist program of early diagnosis and active surveillance. In addition, this molecular complexity would also constitute the rational for new therapeutic alternatives in the advanced PCa setting

Objectives

1. To identify miRNAs that could be used as potential biomarkers for PCa diagnosis and prognosis using a discovery approach based on miRNA microarray analysis (**Studies I and II**).
2. To perform a proteomic approach based on 2D-DIGE and MS analysis to identify new mRNA targets of miR-187 and to evaluate the potential role of these targets as novel biomarkers for PCa (**Study III**).
3. To evaluate the molecular alterations of *SPOP* gene in PCa by NGS technology to determine its role as a new prognostic and therapeutic biomarker and implications as new PCa biotype (**Study IV**).
4. To determine the relationship between *T2E* and IGF-IR in PCa and evaluate the potential implications of this cross-talk for the design of new therapeutic strategies (**Study V**).

Publications

Study I. miRNAs as biomarkers in prostate cancer

Clin Transl Oncol
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miRNAs as biomarkers in prostate cancer

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Abstract Current prostate cancer (PCa) diagnosis is based in the serum prostate-specific antigen biomarker and digital rectal examination. However, these methods are limited by a low predictive value (24–37 %) and a high risk of mistaken results. During last years, new promising biomarkers such as *Prostate Cancer Antigen 3* (PCA-3) and *TMPRSS2-ETS* fusion genes have been evaluated for their clinical use. However, the search of new biomarkers that could be used for PCa diagnosis and prognosis is still needed. Recent studies have demonstrated that the aberrant expression of microRNAs (miRNAs), small non-coding RNAs that negatively regulate gene expression, is related with the development of several cancers, including PCa. Since miRNAs serve as phenotypic signatures of different cancers, they appear as potential diagnostic, prognostic and therapeutic tools. Here, we review the current knowledge of miRNA expression patterns in PCa and their role in PCa prognosis and therapeutics.

Keywords Prostate cancer · Biomarker · MicroRNA

Introduction

Prostate cancer (PCa) is the first most common cancer in men and leads to a 10 % of cancer deaths in Europe [1]. Approximately, one in three men over the age of 50 years shows histologic evidence of PCa. However, only 10 % will be diagnosed with clinically significant PCa, implying that most PCa never progress to become life threatening. Thus far, little is known about what makes some PCas biologically aggressive and more likely to progress to metastatic and potentially lethal disease. Clinical phenotypes of PCa vary from an indolent disease requiring no treatment to one in which tumors metastasize and escape local therapy even when with early detection. Identification of candidate biomarkers for aggressive PCa is a clear need for urologists.

The current standard for the diagnosis of PCa consists of a serum test for prostate-specific antigen (PSA) and digital rectal examination (DRE) [2]. Serum PSA levels above 2.5–4 ng/ml and/or abnormalities felt during DRE may indicate the presence of PCa after a diagnostic biopsy, although the positive predictive value of these methods is only 24–37 %, respectively [2, 3]. Serum PSA levels have other limitations. Non-cancerous conditions such as prostatitis and benign prostatic hyperplasia (BPH) can cause an increase in serum PSA, resulting in a high false-positive rate relative to prostate biopsy [4]. In addition, the widespread use of the serum PSA test has led to an increase in the number of biopsies performed each year, of which many are negative for cancer [5]. Conversely, there is also a significant number of diagnosed PCa with a PSA below 4 ng/ml (estimated at 20–30 %) resulting in undiagnosed disease [6]. Although PSA is a very good marker for monitoring patients after a radical prostatectomy, its utility as a diagnostic marker is far from being optimal. Other promising biomarkers, such as *Prostate Cancer Antigen 3* (PCA3) [7] or *TMPRSS2-ETS* fusion genes [8], are being evaluated for their use in the clinical management of the PCa patients, although we are still waiting for studies with a high grade of

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evidence. Therefore, there is an urgent need for new and more specific biomarkers to improve diagnosis accuracy and to predict PCa progression. Furthermore, the lack of therapies to deal with those PCa that become resistant to castration and turn into a metastatic cancer underlines the need for developing novel therapeutic targets.

An emerging field of research in recent years has been the microRNAs (miRNAs). miRNAs are small (17–27 nt) non-coding single-stranded RNA molecules that negatively regulate gene expression by binding to imperfect complementary sites within the 3′ untranslated region (UTR) of their mRNA target at the post-transcriptional level. miRNA binding is based in the perfect complementary binding of miRNA's first nucleotides (2–7 nt) to their corresponding mRNA and a needless equal binding of miRNA's flanking regions [9]. This process allows a complex regulatory network in which the individual miRNA may target more than 200 different mRNAs and, vice versa, a particular target could be regulated by different miRNAs [10].

Regulatory pathways controlled by miRNAs have been investigated during last years and an association between altered miRNA expression and tumorogenesis has been established [11]. They have been shown to be involved in the regulation of growth, development, invasion, metastasis and prognosis of various cancers, including PCa [12]. Moreover, recent studies demonstrate that miRNA expression patterns serve as phenotypic signatures of different cancers and could be used as diagnostic, prognostic and therapeutic tools [13].

The purpose of this review is to highlight the biological implication of the miRNAs in the pathogenesis of PCa and to discuss their role as potential biomarkers in the clinical management of PCa patients.

miRNA biogenesis

Many miRNAs loci are located within cancer-associated genomic regions. These genes are transcribed by RNA Polymerase II to generate a long double-stranded RNA called pri-miRNA. The RNase enzyme III, Drosha, and the RNA-binding protein Pasha (DGCR8) binds to the pri-miRNA processing it into a shorter (~70 nucleotides) strand known as pre-miRNA. This pre-miRNA, which works as the precursor for mature miRNA synthesis, is transported to the cytoplasm by the RAN-GTP-dependent transporter exportin 5. Once in the cytoplasm, the ribonuclease Dicer processes the pre-miRNA into a miRNA:miRNA* duplex of ~22 nucleotides [14]. Mature miRNA binds to the RNA-induced silencing complex (RISC) and triggers its inhibitory function through silencing its mRNA targets (Fig. 1).

Some of the mechanisms that lead to an aberrant expression of miRNAs could be caused by epigenetic modification of miRNA gene, mutations in the precursor gene or failure in miRNA processing. For example, expression of *Dicer* was found to be up-regulated in aggressive PCa, which may be one of the mechanisms of inducing up-regulation of the miRNAs related to prostate tumors [10]. *DGCR8*, which encodes an essential cofactor for Drosha, was also up-regulated in prostate tumors. Other studies showed that reduced expression of Dicer correlates with shortened postoperative survival in lung cancer [15]. The Argonaute proteins which are crucial components of RISC complex have also been associated with tumor development. *AGO1*, *AGO3* and *AGO4* are frequently deleted in Wilms tumors where *AGO1* is notably increased in renal tumors that lack Wilms-tumor suppressor gene (*WT1*). Another Argonaute gene, *HIWI*, was found to be up-regulated in most testicular seminomas [14].

miRNAs in cancer

The first association of miRNA and tumor biology was described by down-regulation or deletion of miR-15a and miR-16-1 in B cell chronic lymphocytic leukemias [11]. Altered miRNA expression was found to be closely associated with the control of cell growth, differentiation and apoptosis. In fact, miR-15a and miR-16-1 induce apoptosis by targeting the anti-apoptotic gene *BCL2* [16].

Later, other studies reported changes of miRNA expression in several cancers. Volinia et al. [17] performed

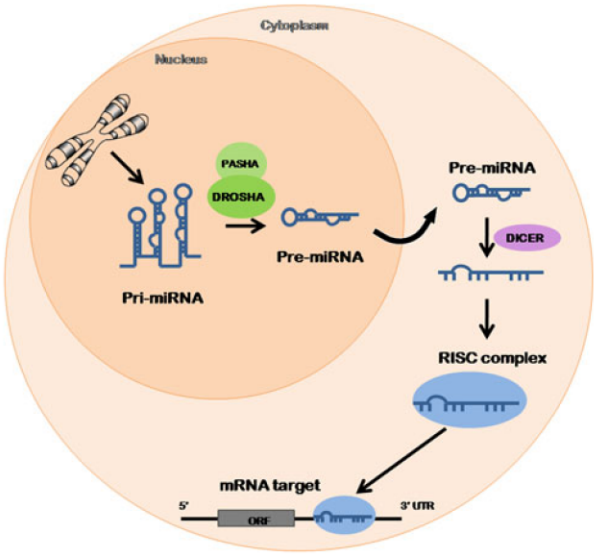

Fig. 1 miRNA biogenesis. miRNA biogenesis starts with gene translation into immature pri-miRNA, pre-processed by Drosha and Pasha into the Pre-miRNA, which is finally processed by Dicer to obtain the mature miRNA with capacity of binding to the target mRNA at 3′UTR

a large-scale miRNome analysis and identified a large portion of overexpressed miRNAs in solid tumors. They found that miR-21, miR-191 and miR-17-5p were significantly overexpressed in all the considered tumor types. However, they also validated particular miRNA signatures for each tumor: miR-125b, miR-145 and miR-21 in breast samples; and miR-103, miR-155 and miR-204 in endocrine pancreatic cancers [17]. Lu et al. [18] analyzed the expression of 217 miRNAs from 334 different human cancer samples and found that miRNA expression was different between tumors of different developmental origin, and there was also a differential expression between tumors and normal tissue. Some of the altered miRNAs found were miR-20, miR-181a, miR-15a, miR-16, miR-17-5p, miR-221, let-7a and miR-2. Most of these differentially expressed miRNAs had lower expression levels in tumors compared with normal tissues [18]. miRNA signatures have also been described in lung cancer [19], colon cancer [20], glioblastoma multiforme [21, 22], lymphomas [23], hepatocellular carcinoma [24], and other tumor types.

Functional studies suggest that those miRNA whose expression is increased in tumors may be considered as oncogenes. These oncogenic miRNAs usually promote tumor development by negatively inhibiting tumor suppressor genes or genes that control cell cycle, differentiation or apoptosis. On the other hand, some miRNAs' expression is decreased in cancerous cells. These types of miRNAs are considered tumor suppressor genes, which prevent tumor development by negatively inhibiting oncogenes or genes that control cell differentiation or apoptosis [25].

During last years, numerous miRNA expression-profiling studies have been performed to identify cancer-specific signatures, since the miRNA signatures of cancers of different cellular origin seem to be unique.

miRNAs in prostate cancer

The first miRNA expression profile in PCa was carried out by Porkka et al. [12]. They performed an oligonucleotide array hybridization method to study the expression of 319 human miRNAs in PCa and found 51 miRNAs differentially expressed in PCa [12]. Following studies confirmed some of the results achieved by these authors while others showed different expression profiles or newly identified altered miRNAs. Table 1 describes those miRNA involved in the pathogenesis of PCa.

A rapidly increasing number of platforms have been developed for miRNA expression profiling. Microarray analysis was the most common method carried out to identify tumor-specific miRNA signatures. However, the arrivals of next generation sequencing (NGS) technologies have offered a new approach in the identification of previously unknown miRNAs [18]. While miRNA array hybridization system is based in the accumulated knowledge of miRNA databases, NGS technologies allow the identification of new miRNA genes. In parallel, qRT-PCR has been established the most suitable technology to validate miRNA expression-profiling results.

Since miRNA expression profiling has been able to classify between health and tumor tissues and even between different prostate tumors, its role as potential clinical biomarkers is being investigated. miRNAs have been found to be remarkably stable in plasma and serum samples, consequently, circulating miRNAs became potential candidates for blood-based biomarkers. Michel et al. [26] showed that serum levels of miR-141 significantly discriminated patients with PCa and healthy controls. Moreover, Taylor and Gercel-Taylor demonstrated the up-regulation of miR-21, miR-141, miR-200, miR-200c, miR-200b, miR-203, miR-205 and miR-214 in circulating cancer exosomes [27]. Some other miRNAs, previously identified in cells and tissues, have also been found in extracellular fluids such as plasma serum, saliva and urine [28]. Urine is an easily available source for molecular markers, therefore, detection of miRNAs in urine of patients with PCa would represent an ideal non-invasive diagnosis approach.

miRNAs and PCa prognosis

Androgen ablation, the mainstay for management of advanced PCa, reduces symptoms in about 70–80 % of patients, but most tumors relapse within 2 years to an incurable castration resistant state, which is ultimately responsible for PCa mortality [13]. On the contrary, for early stage clinically localized disease, radical prostatectomy and radiotherapy are curative; therefore, the choice of the best treatment for a particular PCa is not trivial. For instance, serum PSA level, primary tumor stage and Gleason grade do not reliably predict outcome for individual patients, and an identification of molecular indicators of aggressiveness is still needed.

Androgen signaling has been related with miRNA expression, since some miRNAs have been found to modulate the androgen pathway and further classified prostate carcinomas according to castration resistance [12] (Table 2). For instance, the expression of miR-125b [29] (Fig. 2), miR-21 [30] and miR-141 [31] is regulated by an androgen responsive element (ARE) which controls the up-regulation of these miRNAs and consequently the inhibition of their targets. miR-331-3p is also related with regulation of androgen receptor (AR) pathway since overexpression of its target, ERBB-2, has been related with disease progression and AR signaling [32]. Other miRNAs,

Table 1 miRNAs altered in PCa

miRNA	Expression	Location	Predicted/validated target(s)	Altered function	References
miR-10a	Up-regulated	17q21.32	*HOXA1*	Gene expression, cell differentiation	[53]
miR-20a	Up-regulated	13q31.3	*E2F1-3*	Apoptosis	[17]
miR-21	Up-regulated	17q23.1	*MARCKS, PDCD4, PTEN, TPM1, SPRY2, TIMP3, RECK*	Apoptosis, castrate resistant (CR)	[30]
miR-24	Up-regulated	9q22.32/ 19p13.13	*FAF1*	Apoptosis	[39]
miR-25	Up-regulated	7q21.11	*PTEN*	Cell proliferation, cell cycle	[17, 37, 39, 52]
miR-31	Up-regulated	9p21.3	*Bcl-w, E2F6*	Apoptosis, cell cycle	[55, 56]
miR-32	Up-regulated	9q31.3	*C9orf5, Bim*	Apoptosis	[39]
miR-34b	Up-regulated	11q23.1	*CDK6, CREB, c-MYC, MET*	Cell cycle, cell proliferation	[39, 57]
miR-96	Up-regulated	7q32.2	*FOXO1, hZIPs*	Apoptosis	[55, 58, 59]
miR-106a	Up-regulated	Xq26.2	*RB1*	Cell cycle	[17, 37]
miR-125b	Up-regulated	11q24.1/ 21q21.1	*BAK1*	Apoptosis, AR, metastasis	[29, 60]
miR-141	Up-regulated	12p13.31	*Clock*	AR, metastasis	[12, 34, 61]
miR-148a	Up-regulated	7p15.2	*CAND1, MSK1*	Cell cycle, cell proliferation	[12, 62, 63]
miR-181a-1	Up-regulated	1q31.3	*RB1, RBAK*	Cell cycle, tumor progression	[39, 64, 65]
miR-182	Up-regulated	7q32.2	*FOXO1, FOXO3, BRCA1, hZIP1*	Apoptosis	[39, 55, 58, 59, 66]
miR-194	Up-regulated	1q41/11q13.1	*DNMT3a, MeCP2*	Genomic instability	[40]
miR-200a/ b	Up-regulated	1p36.33	*β-catenin, SIRT1*	EMT, cell growth	[37, 39, 67–69]
miR-200c	Up-regulated	12p13.31	*SEC23A, JAGGED1*	Cell growth, apoptosis, metastasis	[70, 71]
miR-210	Up-regulated	11p15.5	*EFNA3, MNT, HOXA1, APC, ELK3*	Hypoxia, cell proliferation, migration	[12, 72]
miR-214	Up-regulated	1q24.3	*EZH2, N-Ras, PTEN*	Cell cycle, cell proliferation	[17, 73–75]
miR-218	Up-regulated	4p15.31/5q34	*RAS, c-myc, Laminin 5 β3, THAP2, SMARCA5, and BAZ2A*	Cell proliferation, apoptosis	[76]
miR-224	Up-regulated	Xq28	*KLK1, API-5*	Apoptosis, cell proliferation, invasion	[33, 42, 64]
miR-296	Up-regulated	20q13.32	*HMGA1*	Cell proliferation, invasion	[12, 77]
miR-345	Up-regulated	14q32.2	*BAG3*	Apoptosis, invasion, metastasis	[12, 78]
miR-375	Up-regulated	2q35	*Sec23A*	Cell proliferation	[34, 37, 55]
miR-521	Up-regulated	19q13.42	*CSA*	DNA repair	[79]
miR-26a	Up and down-regulated	3p22.2	*PLAG1, EZH2*	Apoptosis, cell proliferation, invasion	[12, 17, 64]
miR-30c	Up and down-regulated	1p34.2/6q13	*BCL-9, MTA1*	Metastasis	[12, 17, 64, 80, 81]
miR-100	Up and down-regulated	11q24.1	*RAS, c-myc, Laminin 5 β3, THAP2, SMARCA5, and BAZ2A*	Cell proliferation, apoptosis	[12, 64, 76, 82]
miR-125a	Up and down-regulated	19q13.41	*ERBB2, ERBB3*	Cell proliferation, apoptosis	[12, 39, 83]
miR-195	Up and down-regulated	4p16.1	*CDK4, GLUT3, WEE1, CDK6, Bcl-2*	Cell cycle, cell proliferation, apoptosis	[12, 39, 64, 84–86]
miR-221	Up and down-regulated	Xp11.3	*p27kip1*	Cell cycle	[12, 40, 55, 87, 88]
miR-222	Up and down-regulated	Xp11.3	*p27kip1*	Cell cycle	[12, 40, 55, 87]
miR-30b	Up and down regulated	8q24.22	*GalNAc, Snail1*	Invasion, immunosuppression	[12, 37, 89]
let-7-family	Down-regulated	9q22.32	*Ras, Cdc25A, Cyclin D1*	Apoptosis, cell proliferation, cell cycle	[82]
miR-1	Down-regulated	20q13.33/ 18q11.2	*Exportin-6, Tyrosine kinase 9, PNP*	Cell proliferation, invasion	[39, 90]
miR-7	Down-regulated	9q21.32/ 15q26.1	*ERBB2*	Cell proliferation, tumor progression	[91]
miR-16	Down-regulated	13q14.2	*Bcl-2, cyclin D1 and D3, CDK1, CDK2*	Apoptosis, cell cycle, metastasis	[11, 16, 92]
miR-22	Down-regulated	17p13.3	*PTEN*	Cell proliferation, cell cycle	[12, 42, 54]

Table 1 continued

miRNA	Expression	Location	Predicted/validated target(s)	Altered function	References
miR-23a/b	Down-regulated	19p13.13/ 9q22.32	*Mitochondrial glutaminase*	Advantage in growth	[93, 94, 97]
miR-27b	Down-regulated	9q22.32	*CYP1B1, Notch1*	Hormone metabolism, cell proliferation	[12, 64]
miR-29a	Down-regulated	7q32.3	*Dkk1, Kremen2, sFRP2, B7-H3*	Cell differentiation, immune response	[12, 95, 96]
miR-34a	Down-regulated	1p36.22	*BCL-2,SIRT1,E2F3, N-MYC, MET, CDK4-6, DLL1*	Apoptosis, proliferation, survival	[39, 57, 97, 98]
miR-34c	Down-regulated	11q23.1	*CDK4, E2F3, MET, c-MYC*	Apoptosis, cell proliferation	[39, 57]
miR-92	Down-regulated	13q31.3/ Xq26.2	*Bim*	Apoptosis	[12, 17, 99]
miR-99a	Down-regulated	21q21.1	*SMARCA, SMARCD1, mTOR*	Apoptosis, cell cycle	[12, 100]
miR-101	Down-regulated	1p31.3/9p24.1	*EZH2*	Cell proliferation, invasion	[101]
miR-106b-25	Down-regulated	7q22.1	*MCM7*	Cell cycle, cell proliferation	[39, 54]
miR-107	Down-regulated	10q23.31	*Granulin*	Cell proliferation	[17, 102]
miR-126	Down-regulated	9q34.3	*CRK, Spred1, PIK3R2/p85-beta*	Cell proliferation, invasion, tumor progression	[36, 64, 103, 104]
miR-126*	Down-regulated	9q34.3	*Prostein*	Metastasis	[36, 105]
miR-128a	Down-regulated	2q21.3	*GOLM1, PHB, TROVE2, TMSB10*	Tumor progression, invasion	[39, 106, 107]
miR-143	Down-regulated	5q32	*MYO6, ERK5, KRAS*	Cell proliferation, migration, metastasis	[12, 37, 108, 109]
miR-145	Down-regulated	5q32	*MYO6, MYC, BNIP3*	Cell migration, metastasis, apoptosis	[12, 40, 64, 110, 111]
miR-146a	Down-regulated	5q34	*CXCR4, ROCK1*	CR, metastasis	[17, 112]
miR-203	Down-regulated	14q32.33	*ZEB2, Bmi, survivin, Runx2*	EMT, metastasis	[41]
miR-205	Down-regulated	1q32.2	*ErbB3, E2F1, E2F5, ZEB2, Protein Kinase Cε, IL24, IL32*	Cell cycle, cell proliferation, apoptosis,EMT	[36, 113, 114]
miR-223	Down-regulated	Xq12	*NFI-A*	Cell differentiation	[17, 115]
miR-301a	Down-regulated	17q22	*FOXF2, BBC3, PTEN, COL2A1*	Cell proliferation	[88, 116]
miR-320a	Down-regulated	8p21.3	*ETS2*	Tumor progression	[37, 117]
miR-330	Down-regulated	19q13.p32	*E2F1*	Apoptosis	[118]
miR-331-3p	Down-regulated	12q22	*ERBB2*	Cell cycle	[32]
miR-449a	Down-regulated	5q11.2	*HDAC-1*	Cell cycle, apoptosis	[39, 119]

such as miR-141, miR-143 (Fig. 3) and miR-145, have been found to be involved in cancer-related cell migration (Table 2). miR-141 is up-regulated in metastatic PCa and its expression was correlated with Gleason score [33, 34]. Loss of expression of miR-143 and miR-145 was related with development and progression of PCa [35] and metastasis [13, 36–38] and it was also related with Gleason score [38].

PCa metastasis has been also linked with the down-regulation of miR-16, miR-34a, miR-126*, miR-205, miR-146a and the up-regulation of miR-301 and miR-125b (Table 2). miR-126* inhibit the expression of prostein, which is frequently overexpressed in PCa. Interestingly, miR-126, which corresponds to the alternative miR-126* strand, was reported to be up-regulated in metastatic xenograft cell line, suggesting that strand selection mechanism could be involved in the development of metastasis [36]. miR-200 family is regulating the epithelium-mesenchymal transition (EMT) and was found down-regulated in tumor tissues [12, 39, 40]. In fact, miR-203 is progressively lost in advanced metastatic PCa showing a linkage between its expression and an antimetastatic role [41]. miR-146a is down-regulated in metastatic tumors, because it is implicated in the formation of the pre-metastatic niche [42], and in castrate resistant PCa cell lines [43].

Some other miRNAs were also related with Gleason score (miR-1, miR-31 and miR-205), tumor stage (miR-125b, miR-205 and miR-222), pT stage (miR-1), perineural invasion (PNI) status (miR-1, miR-10, miR-30c, miR-100, miR-125b and miR-224) and biochemical progression (miR-96) (Table 2).

Approximately, 50 % of PCa are characterized by the expression of the *TMPRSS2-ERG* fusion gene [44]. Although the clinicopathological significance of this alteration still remains to be elucidated, there is evidence that the status of the fusion gene defines groups of patients

Table 2 miRNAs implicated in PCa prognosis

miRNA	Expression	Prognosis parameter(s)	Target
miR-21	Up-regulated	Castrate resistant PCa (CR)	MARCKS
miR-331-3p	Down-regulated	CR	ERBB-2
miR-141	Up-regulated	CR, Gleason score	Clock
miR-146	Down-regulated	CR, metastasis	CXCR4, ROCK1
miR-125b	Up-regulated	CR, metastasis, tumor stage, perineural invasion (PNI)	BAK1
miR-96	Up-regulated	Biochemical progression, tumor recurrence	FOXO1, hZIPs
miR-1	Down-regulated	Gleason score, pT, recurrence	XPO6, PTK9, PNP
miR-143	Down-regulated	Metastasis	MYO6
miR-145	Down-regulated	Metastasis	MYO6, MYC
miR-16	Down-regulated	Metastasis	Bcl-2
miR-34a	Down-regulated	Metastasis	CD44
miR-126*	Down-regulated	Metastasis	Prostein
miR-301	Down-regulated	Metastasis	FOXF2, BBC3, PTEN, COL2A1
miR-200 family	Down-regulated	Metastasis, Gleason score, tumor stage	ZEB2, Bmi, survivin, Runx2, ErbB3, E2F1, E2F5, PKCε
miR-221	Down-regulated	Metastasis, TMPRSS2:ERG presence	p27kip
miR-10	Up-regulated	PNI	HOXA1
miR-100	Up-regulated	PNI	RAS, c-myc, Laminin 5 β3, THAP2, SMARCA5, and BAZ2A
miR-30c	Up-regulated	PNI	BCL-9, MTA1
miR-224	Up-regulated	PNI	KLK1, API-5

characterized by different prognostic factors [45] that could be taken into consideration in the clinical management of PCa patients. To date, the only association between *TMPRSS2-ERG* fusion and miRNA expression has been described in a cohort of 170 patients subjected to radical prostatectomy, in which the low expression of miR-221, a miRNA previously linked to metastasis, was significantly associated to the presence of the fusion gene [46].

miRNA therapeutics for PCa treatment

Since miRNAs were described to be involved in tumor initiation, progression and metastasis, their targeting is expected to emerge as an effective therapeutic option for cancer treatment. Different approaches are being developed to modulate the gain or loss of miRNA functions. miRNAs which act as tumor suppressors are usually down-regulated in cancer while miRNAs acting as oncogenes are commonly overexpressed; therefore, restoring its function, in the first case, or inhibiting its expression, in the second one, may become interesting therapeutic options.

To date, there is no PCa model in this field. However, it has been reported that the introduction of miR-26a using adenoassociated virus (AAV) in an animal model of hepatocellular carcinoma inhibited tumor progression [47]. In a similar way to AAV technology, cationic liposomes or polymer-based nanoparticle formulations can be developed to achieve the delivery of miRNA mimics, synthetic miRNAs which are able to restore miRNA function within the tumor cell [48].

Multiple approaches have been designed to achieve miRNAs down-regulation. One of these approaches consist in the introduction of an anti-miRNA oligonucleotide (AMO) which is able to interact between miRNA and its target through competitive inhibition of base-pairing. AMOs against miR-21 have been shown to inhibit the growth of MCM-7 cells [49]. Other study showed that intravenous administration of AMOs against miR-16, miR-122, miR-192 and miR-194 in animals offers efficient and sustained silencing [50]. Introduction of a modified mRNA to carry multiple pairing sites for endogenous miRNAs, known as miRNA sponge, was also tested to inhibit the function of some miRNAs through its real targets [51]. Recent studies down-regulate oncogene miRNAs introducing a synthetic miRNA molecule (anti-miRNA or miRNA inhibitor) which is able to interact by complementarity with the endogenous miRNA and inhibit its function. In another study, several small organic molecules

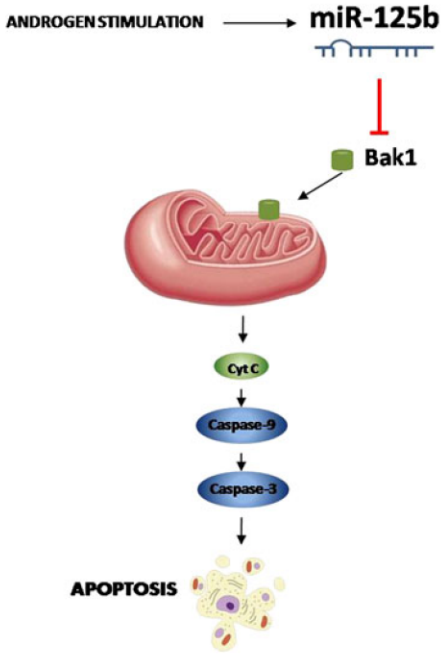

Fig. 2 miR-125b function. miR-125b is overexpressed in prostate tumors and seems to play a role in castrate-resistant PCa growth. Androgen stimulation induces an increased miR-125b expression which leads to a suppression of the expression of the pro-apoptotic protein Bak1, which is necessary for the apoptotic cascade. Cyt C, cytochrome c

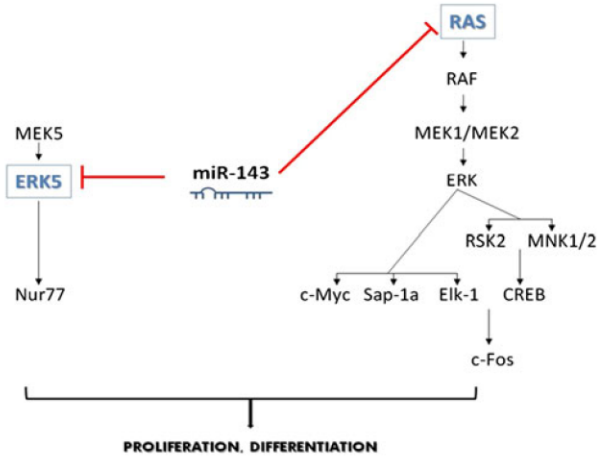

Fig. 3 miR-143 function. miR-143 is down-regulated in PCa and is inversely correlated with cell proliferation. Down-regulation of miR-143 induces an increased expression of its target ERK5, which is implicated in the regulation of cell proliferation. ERK5 overexpression is associated with metastasis, cell proliferation, motility and invasion. Moreover, miR-143 plays an important role in PCa proliferation by suppressing *KRAS* and subsequent inactivation of MAPK pathway

were also screened to find a potential inhibitor of miRNA function. Azobenzene, for example, was found to block miR-21 function acting as a potential inhibitor of miRNA expression [52]. Therefore, miRNA-based therapeutics offer promising results for cancer treatment although they are still far away from clinical application. Nevertheless, there is already a phase I clinical trial for antisense-mediated blocking of liver-specific miR-122 in non-human primates, which resulted in reduced cholesterol synthesis and improved fatty acid metabolism [48].

Hence, we can conclude stating that there is no agreement in which would be the miRNA-profiling signature of PCa. However, it is patent the relevance of some miRNAs (Table 1) that appear strongly up- or down-regulated in prostate tumors and could even classify PCa regarding tumor stage, castration resistance or invasion capacity. These miRNAs represent potential factors for PCa diagnosis and prognosis and promising therapeutic tools. Nevertheless, further studies should be performed to obtain a better knowledge of the particular function and relation with PCa development of these high-potential biomarkers.

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Conflict of interest None.

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Study II. Identification of miR-187 and miR-182 as Biomarkers of Early Diagnosis and Prognosis in Patients with Prostate Cancer Treated with Radical Prostatectomy

Identification of miR-187 and miR-182 as Biomarkers of Early Diagnosis and Prognosis in Patients with Prostate Cancer Treated with Radical Prostatectomy

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Abbreviations and Acronyms

BPFS = biochemical PFS
DRE = digital rectal examination
FDR = false discovery rate
FFPE = formalin fixed, paraffin embedded
PCA3 = prostate cancer antigen 3
PCR = polymerase chain reaction
PFS = progression-free survival
PSA = prostate specific antigen
qRT-PCR = quantitative reverse transcriptase-PCR
sRNA = small RNA

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Purpose: miRNAs are noncoding RNAs that negatively regulate target mRNA gene expression. Aberrant miRNA expression is associated with prostate cancer pathogenesis. We identified miRNAs as potential biomarkers for prostate cancer diagnosis and prognosis.

Materials and Methods: Total RNA was obtained from 10 normal prostate and 50 prostate cancer samples, and analyzed using the GeneChip® miRNA 2.0 Array. At a median followup of 92 months (range 2 to 189) an independent cohort of 273 paraffin embedded prostate cancer samples was used for validation by quantitative reverse transcriptase-polymerase chain reaction. Another 92 urine samples from patients undergoing prostate biopsy were evaluated for these miRNAs.

Results: miR-182 and 187, the miRNAs most differentially expressed between normal and tumor tissue, were selected for further validation. miR-187 inversely correlated with cT ($p = 0.125$) and pT ($p = 0.0002$) stages, Gleason score ($p = 0.003$) and *TMPRSS2-ERG* status ($p = 0.003$). The log rank test showed associations of miR-182 with biochemical ($p = 0.026$) and clinical ($p = 0.043$) progression-free survival, as also noted on multivariate analysis. A significant independent improvement in the definition of risk of progression was achieved by combining miR-182 expression with Gleason score ($p < 0.0001$). miR-187 detection in urine provided an independent predictive value for positive biopsy. A prediction model including serum prostate specific antigen, urine PCA3 and miR-187 provided 88.6% sensitivity and 50% specificity (AUC 0.711, $p = 0.001$).

Conclusions: Results show that miR-182 and 187 are promising biomarkers for prostate cancer prognosis to identify patients at risk for progression and for diagnosis to improve the predictive capability of existing biomarkers.

Key Words: prostatic neoplasms, biological markers, microRNAs, diagnosis, prognosis

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PROSTATE cancer, the most common cancer in men, causes 10% of cancer deaths in Europe. Approximately 1 of 3 men older than 50 years shows histological evidence of prostate cancer but only 10% are diagnosed with clinically significant prostate cancer.[1] Clinical phenotypes of prostate cancer vary from indolent disease requiring no treatment to tumors that metastasize and escape local therapy even with early detection.

The current standard for prostate cancer screening consists of a PSA blood test and DRE,[2] although the positive predictive value of these methods is only 24% and 37%, respectively.[2,3] PSA is the single most significant predictive factor to identify men at increased risk for prostate cancer to date[4] but it is not cancer specific and it is commonly increased in benign conditions.[1,5,6] Thus, PSA screening leads to prostate cancer over diagnosis and overtreatment.

Few biomarkers are currently validated for prostate cancer diagnosis. A recent FDA (Food and Drug Administration) clinical grade urine based assay for the noncoding transcript *PCA3*, which is over expressed in greater than 95% of prostate cancers, was useful when combined with serum PSA for prostate cancer detection.[7] Another potential biomarker is the specific *TMPRSS2* and *ERG* rearrangement at 21q22, which is 100% indicative of prostate cancer.[1] However, because it is present in only approximately 50% of prostate cancers, additional clinically robust biomarkers that can differentiate indolent from aggressive prostate cancer are needed.[1]

miRNAs are a class of small noncoding RNA consisting of 19 to 22 nucleotides involved in various biological processes, including development, differentiation, apoptosis and cell proliferation. miRNAs regulate the expression of more than 60% of protein coding genes.[8] miRNAs can function as tumor suppressor genes or oncogenes and they also contribute to the initiation and development of various types of cancer, including prostate cancer.[8] Specific miRNA patterns in body fluids, such as the serum or urine of patients with prostate cancer, suggest a promising role for these molecules as surrogate markers. To date most miRNA studies of prostate cancer have involved relatively small patient cohorts, limiting the validity and clinical application of potential miRNA biomarkers or signatures.[9]

We identified miR-182 and 187 as the most differentially expressed miRNAs based on the miRNA expression pattern in clinical prostate cancer and normal prostate specimens. We describe their clinicopathological significance in a large retrospective series of 273 prostate cancer cases. We also evaluated their potential as biomarkers for early detection of prostate cancer in patient urine as an alternative to prostate biopsy.

MATERIALS AND METHODS

Clinical Prostate Specimens

Fresh tissue. All study patients provided written informed consent before tissue collection. The study was approved by the institutional ethics committee. Ten fresh frozen normal prostatic tissues and 50 prostate cancer specimens were obtained from the biobanks of the Fundación Instituto Valenciano de Oncología, Hospital Clínico Universitario de Valencia and Hospital Universitario Central de Asturias (table 1).

FFPE tissue. FFPE blocks corresponding to 273 patients with prostate cancer were retrieved from the archives of the Fundación Instituto Valenciano de Oncología biobank according to certain criteria, including specimens obtained at radical retropubic prostatectomy from 1996 and 2002 and no previous treatment for prostate cancer, including androgen deprivation therapy or chemotherapy preoperatively. Table 1 lists patient characteristics. Gleason score was uniformly evaluated by the same pathologist (AC). Ten FFPE samples of normal prostate tissue from patients undergoing radical cystectomy were analyzed as controls. *TMPRSS2-ERG* gene fusion status was determined as previously described.[10] Followup in the retrospective series was 92 months (range 2 to 189). Radiological tests such as computerized tomography or bone scan were done when clinically indicated according to EAU (European Association of Urology) guidelines. During followup 122 patients (45%) had biochemical progression and 74 (27%) had clinical progression.

Urine Samples

Total urine samples were obtained after DRE in an independent cohort of 92 men who underwent PCA3

Table 1. *Clinicopathological features of analyzed series*

	No. Fresh Frozen (%)	No. Retrospective (%)
Age:*	Not available	
Less than 68		131 (48)
68 or Greater		141 (52)
PSA (ng/ml):	Not available	
Less than 10		157 (58)
10–20		74 (27)
Greater than 20		42 (15)
Specimen Gleason score:		
6 or Less	15 (30)	107 (39)
7	25 (50)	134 (49)
8–10	10 (20)	32 (12)
cT:	Not available	
cT2b or less		251 (92.3)
cT3a or greater		21 (7.7)
pT:		
pT2 or less	35 (71)	136 (50)
pT3 or greater	14 (29)	136 (50)
pN:†	Not available	
pN0		239 (95)
pN1 or greater		12 (5)
Perineural invasion:	Not available	
Neg		125 (49)
Pos		129 (51)
TMPRSS2-ERG status:[11]		
Neg	15 (37)	118 (43)
Pos	26 (63)	155 (57)

*Median 68 years (range 49 to 84).
† Lymphadenectomy limited to obturator fossa in most patients at study inclusion.

evaluation before needle biopsy. All biopsies were performed with a minimum of 12 cores (if initial biopsy) according to EAU guidelines. Total urine samples were obtained from 92 patients after DRE and before needle biopsy. We used 2 ml to analyze the *PCA3* score with the PROGENSA® PCA3 Assay. The remaining urine was centrifuged and the pellet was reconstituted in 350 µl TRIzol® reagent for RNA extraction.

RNA Isolation

RNA was isolated using the mirVana™ miRNA Isolation Kit for fresh frozen urine samples and the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE tissues according to manufacturer specifications.

miRNA

Microarrays. To identify differential miRNA expression in normal prostate vs prostate cancer we analyzed 60 fresh frozen prostate tissues using the GeneChip miRNA 2.0 Array. For each sample 500 ng total RNA with an average RIN (RNA integrity number) of 6.7 (range 3.1 to 9.1) was tagged with a PolyA tail and biotin using the FlashTag® Biotin HSR RNA Labeling Kit for GeneChip miRNA arrays. Microarray hybridization was performed at 48C and 60 rpm for 16 hours using the GeneChip Hybridization, Wash and Stain Kit. Results were analyzed using GeneChip Command Console® Software, version 3.0 and miRNA QCTool software (Affymetrix®).

qRT-PCR for validation. Select miRNAs differentially expressed in microarray studies were confirmed by qRT-PCR. Total RNA (100 ng) with a 260/280 nm absorbance ratio of 1.5 to 2 was reverse transcribed using the TaqMan® miRNA Reverse Transcription Kit and miRNA specific stem-loop primers (Applied Biosystems®) according to manufacturer instructions. Two µl of this cDNA were amplified in triplicate by qRT-PCR in a final volume of 10 µl per reaction in a 7500 Fast Real-Time Thermocycler using miRNA assays (Applied Biosystems). RNU44 and RNU48 served as housekeeping genes for miRNA expression normalization (supplementary table 1, http://jurology.com/). The relative expression of miRNAs was determined with the mean value of control samples as the calibrator according to the $2^{-\Delta\Delta Ct}$ method.

Urine miR-182 and 187 Potential Diagnostic Use

Urine samples with an RNA concentration of at least 10 ng/µl and a 260/280 nm absorbance ratio of 1.3 to 2 were selected for study. Reverse transcription was performed in parallel with the TaqMan miRNA Reverse Transcription Kit and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) to obtain cDNA from the miRNA and total RNA of each sample. Before PCR we pre-amplified miR cDNA and cDNA using TaqMan PreAmp Master Mix. qRT-PCR was done using specific miRNA and mRNA assays (supplementary table 1, http://jurology.com/).

Statistical Analysis

Microarray data were processed using bioinformatics tools (Partek® Genomics Suite™) such as array outlier control (normal unscaled SE), robust multi-array average normalization, principal components analysis and hierarchical clusters. We applied statistical corrections, including the p value and multiple variant correction tests such as the Bonferroni and FDR tests set at 5%. The association of miRNA-182 and 187 with clinicopathological parameters (categorical) was assessed using the chi-square test to determine homogeneity and the linear trend for ordinal variables ($p < 0.05$). The impact of biological factors on BPFS and clinical PFS was determined by the Kaplan-Meier proportional risk log rank test. Biochemical progression was defined as PSA 0.4 ng/ml or greater during followup. Clinical progression was defined as local (prostatic fossa), regional (lymph nodes) or distant (metastasis) progression. Univariate predictors of PFS were entered into a Cox proportional hazards model using stepwise selection to identify independent predictors of outcome, considering the 95% CI. The predictive ability of various markers to detect prostate cancer in urine samples was assessed in adjusted univariate logistic regression models. All variables significant at $p < 0.1$ on univariate analysis were entered into a multivariate logistic regression model and assessed using the Hosmer-Lemeshow test. Discrimination was evaluated by calculating the ROC AUC. The capacity of the resulting model to predict the biopsy result was compared with that of PSA using the chi-square test and the ROC AUC of each model was compared using the DeLong test. A cross-validation study of the same subjects was performed by comparing the observed diagnosis on biopsy with that predicted by the full model. The 2-sided test was used with $p \leq 0.05$ considered statistically significant. Statistical analysis was done with SPSS®, version 20.0 and Epidat 4.0 (Consellería de Sanidade, Servizo Galego de Saúde, Xunta de Galicia, Galicia, Spain).

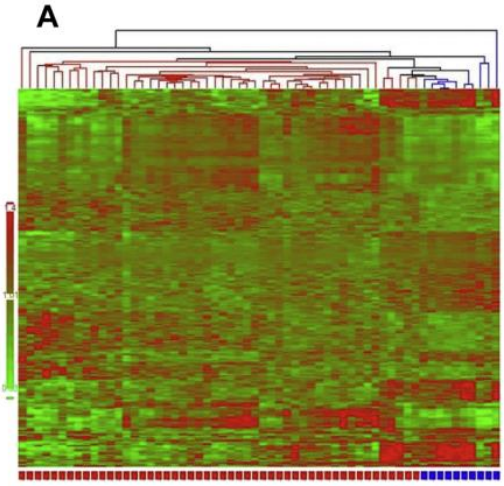
RESULTS

miRNA

Expression profiling in prostate cancer vs normal prostate. Normal and prostate cancer differential miRNA expression profiles were evaluated in fresh frozen samples and miRNA microarrays (fig. 1, *A*), as shown in GEO (Gene Expression Omnibus) database Accession No. GSE45604 (http://www.ncbi.nlm.nih.gov/geo/). A total of 11 sRNAs were differentially expressed (Bonferroni test $p < 0.05$). Expression was increased in prostate cancer for 5 sRNAs, including U78_x (3.8-fold), SNORD78 (3.6-fold), U78_s (3.7-fold), miR-182 (4.7-fold) and U17b (2.7-fold). It was decreased in the remaining 6 sRNAs, including miR-224 (4.05-fold), miR-34a* (3-fold), miR-221* (3.3-fold), miR-187 (12-fold), miR-221 (2.2-fold) and miR-34c-3p (1.7-fold) (fig. 1, *B*).

The supplementary results and supplementary fig. 1 (http://jurology.com/) show the relationship between miRNAs and clinicopathological variables. Notably, miR-182 and miR-187 were also differentially expressed according to Gleason score, pT and *TMPRSS2-ERG* (supplementary fig. 1, http://jurology.com/).

A



B

Probeset ID	p-value	Bonferroni p-value	FDR p-value	Fold Change
hsa-miR-224	1.48E-07	0.0006	0.0003	-4.05
hsa-miR-34a-star	1.66E-07	0.0007	0.0003	-3.00
U78_X	2.39E-07	0.001	0.0003	3.38
hsa-miR-221*	1.12E-06	0.005	0.001	-3.35
ENSG00000212378_s	1.43E-06	0.006	0.001	3.63
U78_s	1.55E-06	0.007	0.001	3.78
hsa-miR-182	1.63E-06	0.007	0.001	4.70
Has-miR-187	4.94E-06	0.022	0.002	-12.08
U17b	5.96E-06	0.027	0.002	2.81
hsa-miR-221	6.16E-06	0.028	0.002	-2.25
hsa-miR-34c-3p	9.16E-06	0.041	0.003	-1.79

Figure 1. Prostate cancer miRNA expression profile. *A*, hierarchical cluster of miRNA expression signals of prostate cancer (red rectangles) and normal tissue (blue rectangles) in samples grouped in clusters by expression profile similarity measured by Euclidean distance. Probe hybridization intensity was equivalent to sRNA expression. Green areas indicate low expression. Red areas indicate high expression. *B*, relationship of prostate cancer and normal tissue of 11 statistically differentially expressed sRNAs. Multivariate Bonferroni and FDR tests were used to decrease number of false-negative results (0.01% and 1%, respectively). Probeset identification (*ID*) indicates name of each sRNA in miRNA Sanger database. Fold change indicates increase or loss of expression of each sRNA in tumor vs normal tissue.

Microarray data validation by qRT-PCR analysis and prognostic value. The most significantly regulated miRNAs in our series, miR-182 (4.7-fold) and miR-187 (−12-fold), were selected for further analysis. Expression of these 2 miRNAs was confirmed in the same cohort of samples as the microarray experiments and in an independent, retrospective cohort of 273 primary tumors (supplementary figs. 2 and 3, http://jurology.com/). We also assessed the relationship of the 2 miRNAs to clinicopathological characteristics and patient outcome. miR-187 expression was even more decreased in advanced prostate cancer cases (pT3 or greater $p = 0.0002$, cT3a or greater $p = 0.125$ and Gleason score 7 or greater $p = 0.003$, supplementary table 2 and supplementary fig. 4, http://jurology.com/). Consistent with microarray data *TMPRSS2-ERG* inversely correlated with miR-187 expression ($p = 0.003$, supplementary fig. 4, http://jurology.com/).

From the prognostic point of view we observed a significant association of miR-182 expression with BPFS and PFS (table 2 and supplementary fig. 5, http://jurology.com/). Cox proportional hazard multivariable analysis revealed that higher miR-182 expression independently conferred a worse prognosis for BPFS and PFS (table 2).

Considering that miR-182 was significantly associated with Gleason score (microarray data) and prognosis we tested whether miR-182 expression would reveal different behavior in each Gleason score category, thus, proposing a new variable combining Gleason score with miR-182 expression using the median as the cutoff. This combination was highly significant for BPFS and PFS ($p < 0.000001$ and 0.00002, respectively, fig. 2). It was a significantly independent predictor of worse outcome for BPFS but not for PFS ($p < 0.000001$, supplementary table 3, http://jurology.com/).

Urine Sample Biomarker Analysis

A total of 92 urine samples were analyzed for the expression of *PCA3*, *TMPRSS2-ERG*, *GOLPH2*, *SPINK1*, miR-182 and miR-187. Patients were classified according to biopsy (45 negative and 47 positive). Supplementary table 4 (http://jurology.com/) lists the collected variables.

Table 3 shows the univariate logistic regression model used to evaluate the predictive capability of these biomarkers for prostate cancer in diagnostic biopsies. At a 10% significance level only PSA, *PCA3* and miR-187 were significantly associated with positive biopsy. By adjusting a multivariate model including PSA, PCA3 and miR-187 we found a significant reduction in the Akaike information criterion of the combined model compared to the simplest model (117.91 vs 122.08, $p = 0.017$, supplementary figs. 6 to 8, http://jurology.com/).

ROC curves were generated for the PSA and the combined (PSA, *PCA3* and miR-187) models (fig. 3). For the full model the optimal cutoff to predict positive biopsy was 0.388 or greater, achieving a diagnostic test with 69.3% accuracy (95% CI 59.1–79.5), 88.6% sensitivity (95% CI 78.1–99.2), 50% specificity (95% CI 34.1–65.9), 63.9% positive

Table 2. *BPFS and PFS log rank test and Cox regression*

		BPFS				PFS			
	No. Pts	No. Events (%)	Univariate p Value	HR (95% CI)	Multivariate p Value	No. Events (%)	Univariate p Value	HR (95% CI)	Multivariate p Value
Specimen Gleason score:			<0.0001		<0.0001		<0.0001		0.001
2–6	107	32 (58.2)		1		15 (78.4)		1	
7	134	65 (29.7)		4 (2–7.5)	<0.0001	46 (56.4)		4 (2–9)	<0.0001
Greater than 7	32	25 (11.7)		2 (1–3.5)	0.003	14		1.5 (1–3)	0.156
PSA (ng/ml):			<0.0001		<0.0001	—	0.024	—	Not significant
Less than 10	157	55 (49)		1		34 (69.2)			
10–20	74	36 (40.3)		3 (2–4.5)	<0.0001	24 (52.2)			
Greater than 20	42	31 (23.3)		2.5 (1.5–4)	0.0002	17 (54.2)			
cT:			<0.0001		0.027	—	0.029	—	Not significant
cT2b or less	251	107 (43.3)		1		67 (62.6)			
cT3a or greater	21	16 (14.5)		2 (1–3.5)		8 (58.6)			
pT:			<0.0001	—	Not significant		0.001	—	Not significant
pT2 or less	136	41 (58.9)				24 (79.3)			
pT3 or greater	136	80 (23)				50 (46.6)			
pN:	—	—	<0.0001		Not significant		0.213		Not significant
pN0	239	104 (43.4)				66 (62.7)			
pN1 or greater	12	11 (8.3)				5 (50.9)			
Margins:			<0.0001		0.001		<0.0001		0.027
Neg	135	38 (56.7)		1		23 (77.9)		1	
Pos	137	84 (21.7)		2 (1.5–3)		52 (38)		2 (1–3)	
TMPRSS2-ERG:			0.737	—	Not significant	—	0.664	—	Not significant
Neg	118	54 (42.1)				31 (65.7)			
Pos	155	68 (38.2)				44 (58.5)			
miR-182:			0.02		0.032		0.04		0.043
Low	68	24 (55.1)		1		12 (67.3)		1	
Medium	135	59 (43.5)		2 (1–3.5)	0.009	40 (60.8)		2.5 (1–5)	0.013
High	69	38 (39.1)		1.5 (1–2)	0.147	23 (57.8)		1 (0.5–2)	0.387
miR-187:			0.331	—	Not significant		0.608	—	Not significant
Low	137	65 (41.7)				40 (55.7)			
High	135	57 (37)				35 (69.2)			

predictive value (95% CI 51.1–76.8) and 81.5% negative predictive value (95% CI 65.0–98.0). This model was cross-validated and for the same cutoff of 0.388 an accuracy of 62.5% was achieved with 81.8% sensitivity and 43.2% specificity.

DISCUSSION

One of the main challenges of prostate cancer management is to distinguish between indolent tumors, which can be controlled by active surveillance, and tumors with aggressive behavior that require more radical treatment strategies. Prognostic factors such as serum PSA and Gleason score in biopsy samples are not sufficiently accurate to predict high risk in the diagnostic or prognostic context.[3,6,11,12] Thus, in the last few years a growing number of groups have considered the potential role of miRNAs as biomarkers of human tumors. Specifically, several groups identified miRNAs

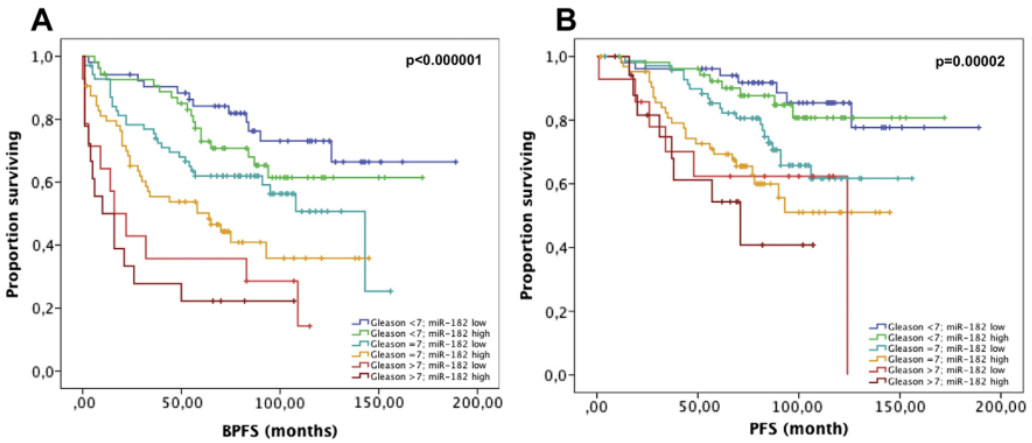


Figure 2. Kaplan-Meier plots and survival table show prognostic impact of combining Gleason score with miR-182 expression. miR-182 expression significantly differentiated 2 patient groups with different behavior for each Gleason score. *A*, BPFS. *B*, PFS.

Table 3. *Predictive model adjustment for prostate cancer positive biopsy*

	Univariate		Multivariate	
	OR (95% CI)	p Value	OR (95% CI)	p Value
PSA	1.080 (0.985–1.184)	0.099	1.087 (0.986–1.199)	0.092
PCA3	1.013 (1.002–1.024)	0.025	1.011 (1.000–1.022)	0.054
miR-182	0.976 (0.857–1.112)	0.717	–	–
miR-187	0.859 (0.736–1.001)	0.052	0.858 (0.729–1.009)	0.064
SPINK1	0.940 (0.828–1.067)	0.336	–	–
GOLPH2	1.054 (0.881–1.260)	0.566	–	–
TMPRSS2-ERG:		0.189	–	–
Neg (baseline)	–			
Pos	1.760 (0.757–4.093)			

that are differentially expressed in prostate cancer,[8] including some associated with androgen signaling,[13] prognosis and metastasis.[14,15] However, many of these studies require validation since findings are often inconsistent among series.

A comprehensive study was performed to identify and validate miRNAs that may be useful in the prostate cancer clinical setting, combining a discovery phase using high throughput technology with retrospective and prospective cohorts of samples in different clinical contexts. Our evaluation of 60 fresh frozen tissues revealed 5 significantly down-regulated miRNAs (miR-187, 224, 34a*, 221 and 34c) and 1 over expressed miRNA (miR-182).

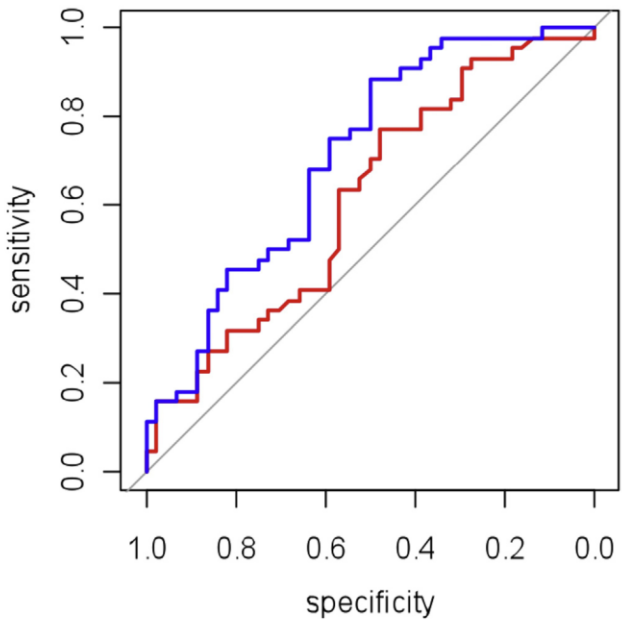


Figure 3. ROC curves of PSA (red curve) and combined biomarker model of PSA, PCA3 and miR-187 (blue curve) to predict prostate cancer in urine samples (AUC 0.615, 95% CI 0.496–0.733, p = 0.064 and 0.711, 95% CI 0.603–0.819, p = 0.001, respectively). Combined model discriminated probability of positive prostate cancer biopsy significantly better than PSA alone.

Several groups have now investigated aberrant expression of miRNAs based on expression signatures in prostate cancer samples.[8,16,17] Interestingly, our miRNA expression data were highly consistent with those of Fuse et al, who found that miR-187, 224, 34 and 221 were under expressed in prostate cancer.[16] Some of these miRNAs, such as the miR-34 and 221 families, were extensively studied in different tumors[18] and also described as associated with some prostate cancer features.[19,20] For example, miR-34a and 221 are linked to metastasis[15] and miR-224 is associated with perineural invasion.[21]

Given the high fold change in differential expression between prostate cancer and normal prostate, we selected miR-182 (4.7-fold) and miR-187 (−12-fold) for further validation. A large independent series of patients treated with radical prostatectomy who had long followup was analyzed and confirmed the up-regulation and down-regulation of miR-182 and 187, respectively. Up-regulation of miR-182 was previously reported in prostate cancer[22] and other tumors,[23,24] while miR-187 was recently found to be lost in prostate cancer[16] and ovarian carcinoma[25] but over expressed in breast cancer progression.[26] Therefore, our results are in agreement with those of previous studies. To our knowledge we report for the first time the association of miR-187 but not miR-182 with clinicopathological parameters such as pT and Gleason score in a large series of cases.

At least 50% of prostate cancers harbor the *TMPRSS2-ERG* rearrangement, which defines patient groups characterized by different biological and clinical behaviors.[1,27] Our microarray series showed that only miR-187 (−3.2), miR-182 (1.9) and miR-183 (1.9) were differentially expressed according to patient *TMPRSS2-ERG* status. To date the only association between *TMPRSS2-ERG* fusion and miRNA expression was found for miR-221.[28] Our results show that miR-187 expression inversely correlates with the fusion gene in the microarrays and the retrospective series. To our knowledge from the prognostic point of view no association between miR-187 and BPFS or PFS has been reported to date and from our analysis it could be concluded that miR-187 is not associated with prognosis.

In contrast to previous studies[29] and our microarray series, we found no statistically significant association between miR-182 expression and clinicopathological parameters. However, a robust and independent correlation between miR-182 expression and the prostate cancer prognosis for BPFS and PFS was demonstrated (table 2). This association of miR-182 expression with prognosis was also described for other tumors such as glioma[23]

and colorectal cancer.[24] Interestingly, our analysis shows agreement between our microarray data and the direct association found by Tsuchiyama et al between miR-182 and Gleason score[29] as well as the independent behavior of these variables on multivariate analysis in our retrospective series. As a result, a combined variable between Gleason score and miR-182 levels was obtained that perfectly discriminates different patient groups according to the risk of progression. In this respect patients with Gleason score less than 7 and low miR-182 expression were at lowest risk for progression compared to patients with Gleason score greater than 7 and miR-182 over expression (fig. 2). This classification could be used to better distinguish patients who are suitable candidates for active surveillance alone from those who need more aggressive treatment.

Another clinical setting in which miRNA stability may provide an advantage is cancer diagnosis in extracellular body fluids such as plasma, serum, saliva or urine.[9] In prostate cancer the usefulness of urine based testing for *PCA3* expression (PCA3-PROGENSA® assay) was already documented in a large series.[7] Moreover, Laxman et al reported the usefulness of generating a multiplexed, urine based diagnostic test combining several biomarkers (PSA, *PCA3*, *GOLPH2*, *SPINK1* and *TMPRSS2-ERG*) for prostate cancer.[30] In our study and to reproduce the results of Laxman et al we also used a series of 92 urine samples from patients undergoing needle biopsy, incorporating the analysis of miR-182 and 187. As a result, we established a prediction model including serum PSA, urine *PCA3* and miR-187 with significantly greater accuracy than PSA alone. This predictive model achieved 88.6% sensitivity and 50% specificity with 69.3% diagnostic precision, suggesting that miR-187 could be a promising biomarker for early diagnosis of PCa. In conclusion, our results provide preliminary evidence of 2 miRNAs with a role in prostate cancer pathogenesis that have potential as biomarkers in the context of prognosis to identify patients at risk for progression and in the diagnostic setting to improve the predictive capability of existing biomarkers.

ACKNOWLEDGMENTS

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Supplementary Results

miRNA profile according to clinicopathological parameters

Some of the miRNAs identified in the microarray study were also differentially expressed according to the Gleason score of the primary tumor samples. We found that miR-224, miR-34a* and miR-34c-3p showed a statistically significant expression profile when a Bonferroni test was applied. While these three miRNAs together with miR-221, miR-221*, miR-182 and miR-187 were differentially expressed only upon False Discovery Rate (FDR) analysis (Figure 1SA).

To study some miRNAs were associated with the pathological (pT) stage of the tumor we also performed a miRNA expression profile according to the pT status of the samples. It was observed that some miRNAs, such as miR-297 (1.8 fold) and miR-555 (1.2 fold), were able to distinguish between different pathological stages (pT2 vs pT3) and also that other miRNAs could discriminate between normal tissue and a particular pathological stage (pT2 or pT3). Therefore, the loss of expression of some miRNAs such as miR-34a* (3.2 fold), miR-224 (3.9 fold), miR-187 (16.2), miR-34c-3p (1.9 fold), miR-221 (3.2 fold) and miR-145 (1.4 fold) could be considered as signatures of a lower pathological stage (pT2). While the over-expression of other miRNAs like miR-182 (5.7 fold) and down-regulation of miR-224 (3.7 fold) and miR-221* (3.5 fold) where statistically differentially expressed in pT3 samples when compared with normal tissue (Figure 1SB).

We further studied the association between miRNA expression and the presence of the translocation *TMPRSS2-ERG* in primary PCa. Although no miRNAs were differentially expressed according to the multivariate statistical analysis tests (Bonferroni and FDR), we found that the miRNAs with the highest fold change upon the presence of the fusion gene ($p<0.05$) corresponded to the down-regulated miR-187 (3.2 fold) and the up-regulated miRNA cluster composed of miR-182 (1.9 fold) and miR-183 (1.9 fold) (Figure 1SC).

Supplementary Tables

Supplementary Table 1. Details of the qRT-PCR assays employed in the study.

Assay name	Gene name	Assay Id	Gene Id	Amplicon size
hsa-miR-182	Hsa-miR-182-5p	002334	MI0000272	24
mmu-miR-187	Hsa-miR-187-3p	001193	MI0000274	22
RNU44*	RNU44	001094	NR_002750	60
RNU48*	RNU48	001006	NR_002745	57
B2M	Beta-2-microglobulin	Hs99999907_m1	Hs.534255	75
KLK3 (PSA)	Kallikrein-related peptidase 3	Hs04185002_m1	Hs.171995	81
TMPRSS2-ERG fusion	FUSION	Hs03063375_ft	-	106
GOLM1 (GOLPH2)	Golgi-membrane-protein-1	Hs00213061_m1	Hs.494337	88
SPINK1	Serine peptidase inhibitor, Kazal type 1	Hs00162154_m1	Hs.407856	85

Supplementary Table 2. Association between the miR-182 and miR-187 expression and the clinicopathological parameters.

Parameters	n	miR-182 (mean ± standard deviation)	p*	miRNA-187 (mean ± standard deviation)	p*
Age (years)					
<68	**132**	3.80 ± 5.71	0.320	0.27 ± 0.53	0.643
≥ 68	141	3.64 ± 3.68		0.36 ± 0.75	
PSA					
<10ng/ml	157	3.74 ± 5.54	0.137	0.31 ± 0.62	0.227
10-20ng/ml	74	3.74 ± 2.72		0.30 ± 0.73	
>20ng/ml	42	3.63 ± 4.44		0.33 ± 0.65	
Gleason-sp					
< 7	107	3.28 ± 2.64	0.720	0.43 ± 0.81	**0.003**
≥ 7	166	4.01 ± 5.69		0.24 ± 0.51	
cT					
≤ cT2	250	3.69 ± 4.83	0.129	0.33 ± 0.67	0.125
> cT2	21	4.12 ± 3.86		0.17 ± 0.25	
pT					
≤pT2b	136	3.46 ± 3.06	0.880	0.36 ± 0.57	**0.0002**
≥pT3a	136	3.99 ± 5.98		0.28 ± 0.73	
Margins					
No	135	3.32 ± 3.27	0.174	0.37 ± 0.75	0.247
Yes	137	4.11 ± 5.84		0.27 ± 0.54	
pN					
No	239	3.61 ± 4.76	0.297	0.33 ± 0.69	0.515
Yes	12	5.96 ± 7.00		0.24 ± 0.23	
TMPRSS2-ERG					
Negative	118	4.04 ± 6.08	0.944	0.41 ± 0.72	**0.003**
Positive	155	3.94 ± 3.42		0.25 ± 0.59	

*U Mann-Whitney and Kurskal-Wallis test for variables with two and more than two categories respectively.

Supplementary Table 3. Log-rank and Cox regression tests for BPFS and PFS including a combined variable between Gleason score and miR-182 expression.

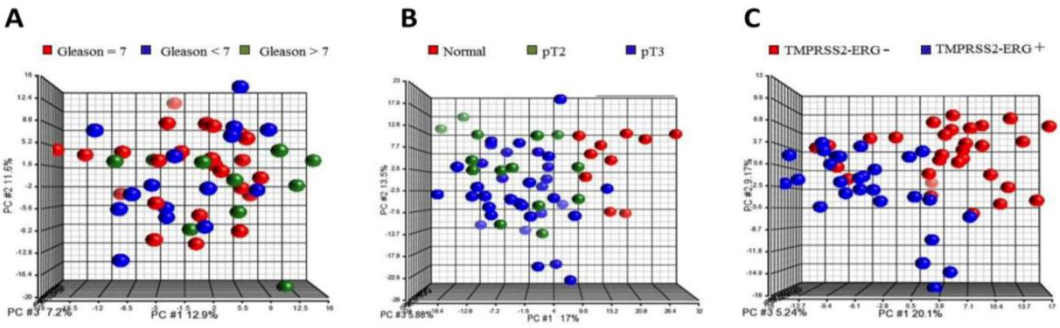
		Biochemical progression						Clinical progression					
Parameters	**n**	**Events**	**%BPFS**	**p-Univariate**	**HR**	**95% CI**	**p-Multivariate**	**Events**	**%Clinical PFS**	**p-Univariante**	**HR**	**95% CI**	**p-Multivariate**
Gleason-sp				<0.0001			NS			<0.0001			0.001
2-6	107	32	58.2					15	78.4		1		
7	134	65	29.7					46	56.4		4.2	2-8.8	0.0002
>7	32	25	11.7					14	-		1.5	0.8-2.8	0.156
PSA				<0.0001			<0.000001			0.024			NS
≤10ng/ml	157	55	49		1			34	69.2				
10-20ng/ml	74	36	40.3		3.1	1.9-5	<0.00001	24	52.2				
>20ng/ml	42	31	23.3		2.7	1.6-4.5	0.0001	17	54.2				
cT				<0.0001			NS			0.029			NS
≤cT2b	251	107	43.3					67	62.6				
≥cT3a	21	16	14.5					8	58.6				
pT				<0.0001			0.023			0.001			NS
≤pT2	136	41	58.9		1			24	79.3				
≥pT3	136	80	23		1.7	1-2.6		50	46.6				
pN				<0.0001			NS			0.213			NS
pN0	239	104	43.4					66	62.7				
pN≥1	12	11	8.3					5	50.9				
Margins				<0.0001			0.031			<0.0001			0.027
Negative	135	38	56.7		1			23	77.9		1		
Positive	137	84	21.7		1.7	1-2.7		52	38		1.7	1-2.9	
miR-182				0.02			NS			0.04			0.043
Low	68	24	55.1					12	67.3		1		
Medium	135	59	43.5					40	60.8		2.4	1.2-4.9	0.013
High	69	38	39.1					23	57.8		1.2	0.7-2.1	0.795
Gleason and miR-182				<0.000001			<0.000001			0.00002			NS
Gl <7; miR-182 low	52	13	66.4		1			7	77.7				
Gl <7; miR-182 high	54	18	61.7		8.7	3.7-20.8	<0.000001	8	80.7				
Gl = 7; miR-182 low	70	30	25.4		4.4	2-9.4	0.0001	21	61.7				
Gl = 7; miR-182 high	64	36	35.8		4.5	2.3-9	0.000001	25	51.0				
Gl >7; miR-182 low	14	11	14.3		2.3	1.2-4.5	0.011	6	0.0				
Gl >7; miR-182 high	18	14	22.2		2.5	1.1-5.8	0.028	8	40.8				

SP, specimen; BPFS, biochemical progression free survival; PFS, progression free survival; NS, Not significant; Gl, Gleason.

Supplementary Table 4. Description of biomarker expression in urine samples.

	Total	Negative Biopsy	Positive Biopsy
N	92 (100%)	45 (48.9%)	47 (51.1%)
PSA			
N Valid	92 (100%)	45 (100%)	47 (51.1%)
Mean (SD)	7.15 (5.53)	6.13 (3.86)	8.12 (6.66)
Median (IR)	5.5 (4.1-7.9)	5.1 (3.5-7.4)	5.9 (4.4-9.1)
PCA3			
N Valid	89 (96.7%)	44 (97.8%)	45 (95.7%)
Mean (SD)	62.7 (45.3)	51.3 (36.1)	73.8 (50.8)
Median (IR)	52 (37-74)	50 (26-63)	57 (38-99)
TMPRSS2-ERG			
Absence	55 (59.8%)	30 (66.7%)	25 (53.2%)
Presence	37 (40.2%)	15 (33.3%)	37 (46.8%)
GOLPH2			
N Valid	85 (92.4%)	41 (91.1%)	44 (93.6%)
Mean (SD)	-2.10 (2.41)	-2.25 (2.35)	-1.95 (2.48)
Median (IR)	-2.3 (-3.9--0.3)	-2.6 (-4.0--0.7)	-2.0 (-3.4-0.2)
SPINK1			
N Valid	86 (93.5%)	40 (88.9%)	46 (97.9%)
Mean (SD)	-1.22 (3.47)	-0.83 (3.17)	-1.56 (3.71)
Median (IR)	-1.2 (-3.6-1.1)	-0.9 (-3.5-1.2)	-1.8 (-3.6-1.1)
miR-182			
N Valid	88 (95.7%)	43 (95.6%)	45 (95.7%)
Mean (SD)	10.05 (3.23)	10.18 (3.55)	9.93 (2.93)
Median (IR)	10.3 (8.1-12.1)	10.3 (8.1-12.6)	10.1 (8.1-11.6)
miR-187			
N Valid	91 (98.9%)	45 (100%)	46 (97.9%)
Mean (SD)	6.30 (2.87)	6.90 (2.99)	5.72 (2.64)
Median (IR)	6.8 (4.2-8.4)	7.6 (5.5-8.8)	6.2 (4.0-7.8)

Supplementary Figures

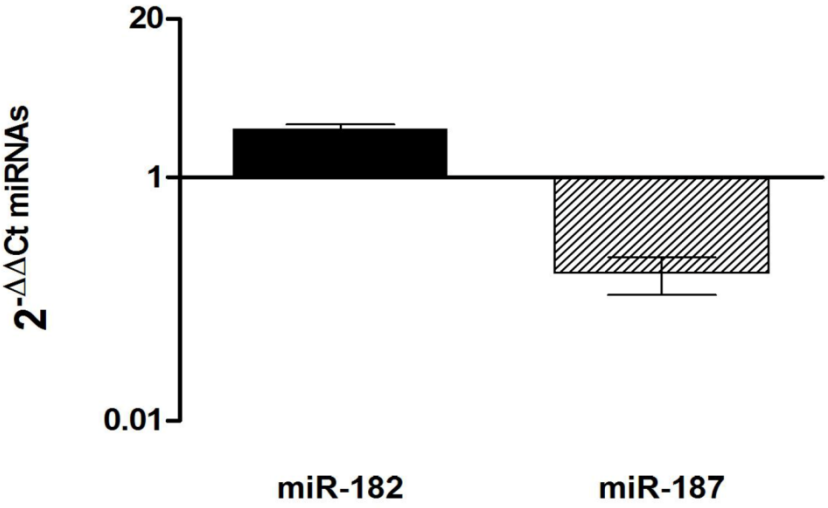


Gleason <7 vs. 7 vs. >7			
Probeset ID	p-value	Bonferroni p-value	FDR p-value
hsa-miR-224	<0.0001	0.01	0.008
hsa-miR-34a*	<0.0001	0.02	0.008
hsa-miR-34c-3p	<0.0001	0.06	0.008
hsa-miR-221	<0.0001	0.06	0.012
hsa-miR-221*	<0.0001	0.08	0.012
hsa-miR-182	<0.0001	0.08	0.012
Has-miR-187	<0.0001	0.23	0.023

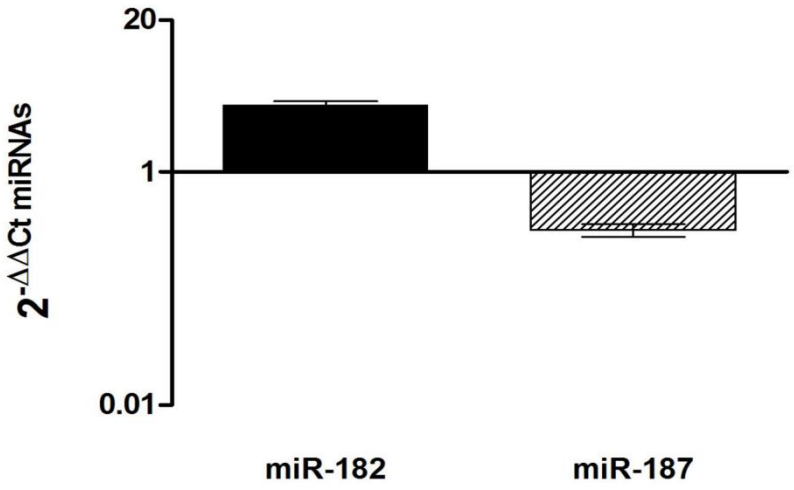
pT3 vs. pT2	
Probeset ID	Bonferroni p-value
hsa-miR-297	0.035
hsa-miR-555	0.038
pT2 vs. N	
Probeset ID	Bonferroni p-value
hsa-miR-34*	0.0006
hsa-miR-224	0.003
hsa-mir-187	0.005
hsa-miR-34c-3p	0.022
hsa-miR-221*	0.038
hsa-miR-145	0.043
pT3 vs N	
Probeset ID	Bonferroni p-value
hsa-miR-182	0.012
hsa-miR-224	0.039
hsa-miR-221*	0.046

TMPRSS2-ERG positive vs. negative		
Probeset ID	p-value	Fold Change
hsa-miR-187	0.0090542	-3.2
hsa-miR-182	0.0123571	1.9
hsa-miR-183	0.029737	1.9

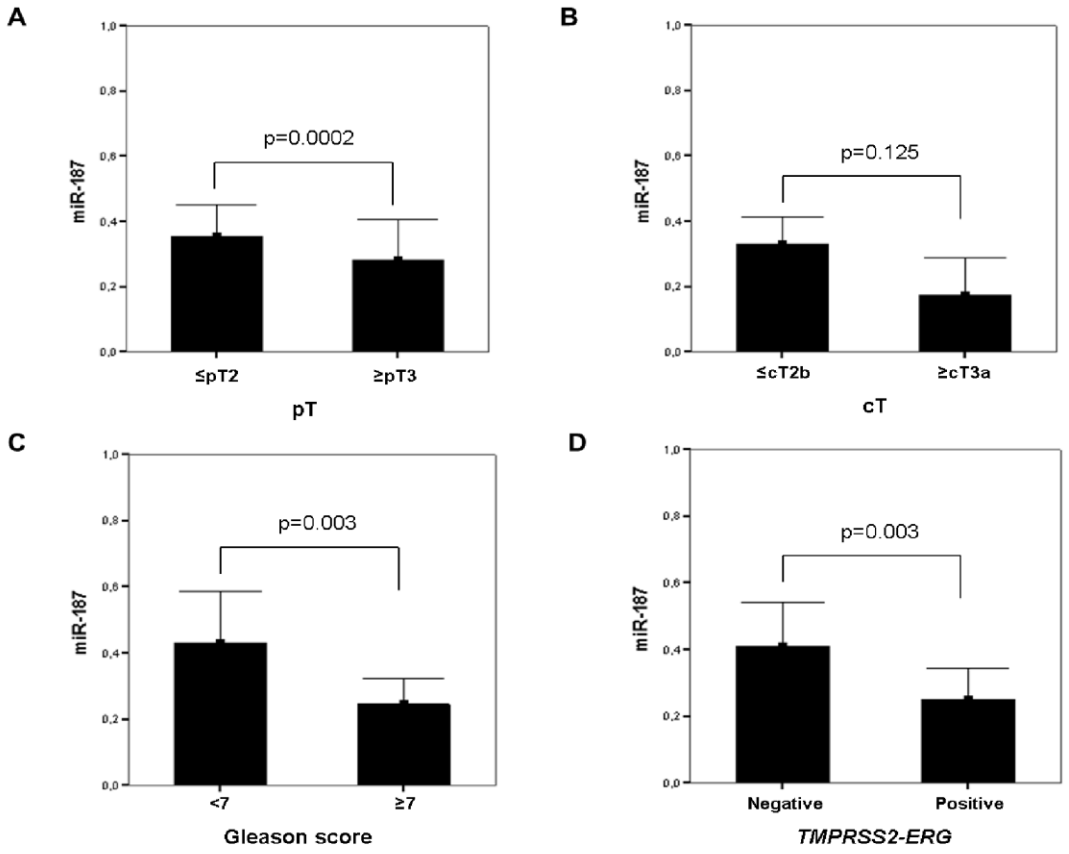
Supplementary Figure 1. Principal component analysis (PCA) of miRNA profile according to clinicopathological parameters. A) miRNA profile and Gleason scores. Samples are represented in different colors (red, blue and green) depending on their Gleason score (7, <7 or >7 respectively). The table below the graph shows the list of sequences statistically significant in the comparison between the different Gleason scores. B) miRNA profile and different pathological stage (pT). Samples are represented in different colors (red, blue and green) depending on their pT (normal, pT2 or pT3 respectively). Tables below the graph show the list of sequences statistically significant in the comparison between the different pT stages. C) miRNA profile and TMPRSS2-ERG gene fusion status. Samples are represented in blue or red depending on the presence or absence, respectively, of the translocation. The miRNAs differentially expressed in the comparison between these two populations are indicated in the table below the graph.



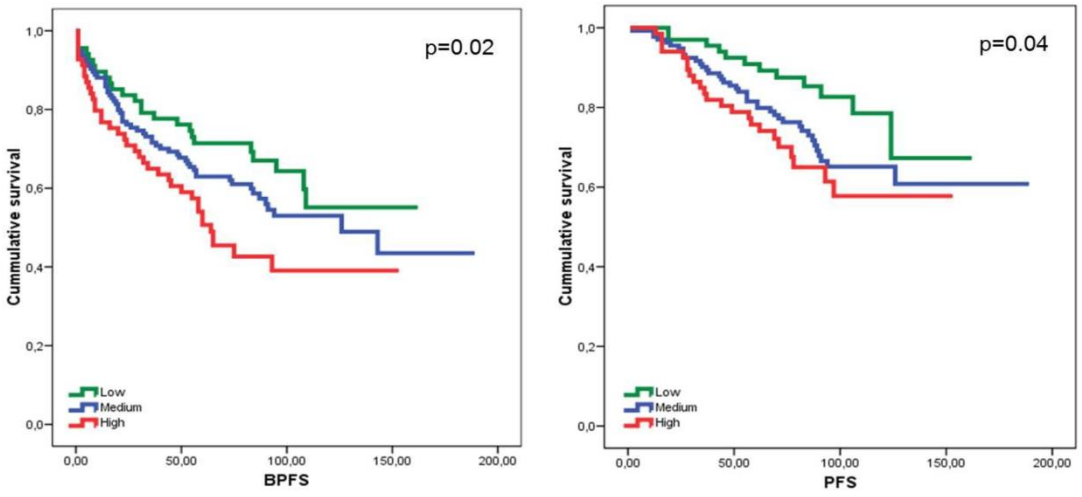
Supplementary Figure 2. miRNA microarray validation by real- time PCR. The differential expression of miR-182 and miR-187 between PCa and normal tissues observed in the microarray analysis was validated by qRT-PCR. A cohort of 60 fresh-tissue samples, equal to that used for microarray analysis, was employed to determine the miRNA expression. The up-regulation (2.5 fold) of miR-182 and down-regulation of miR-187 (-6.1) was confirmed.



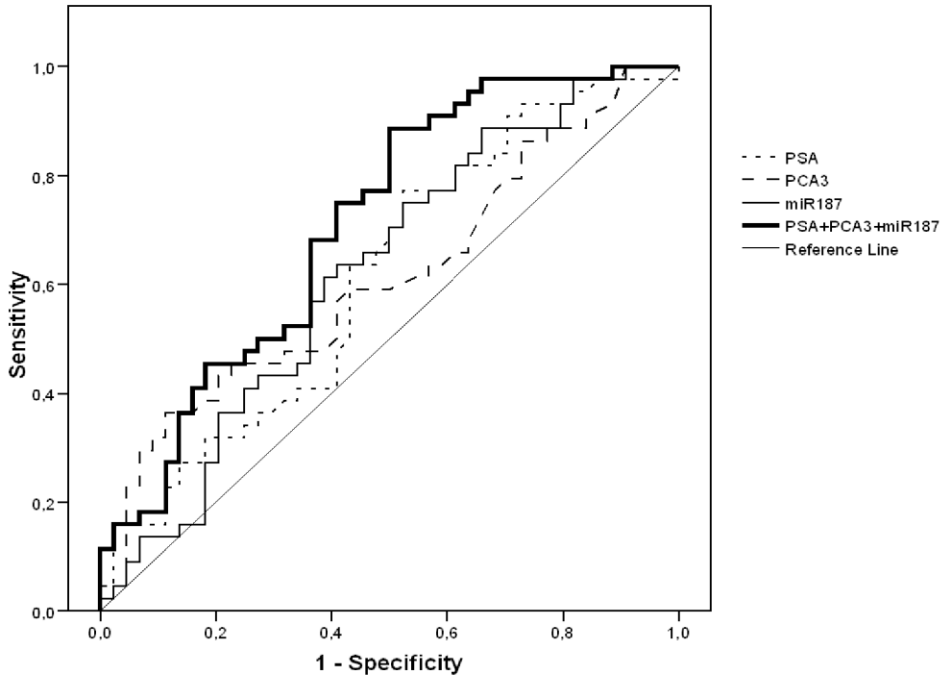
Supplementary Figure 3. Biological validation of the differential expression of miR-182 and miR-187 in a retrospective cohort of samples. The differential expression of miR-182 and miR-187 was further confirmed in an independent cohort of FFPE samples (n=273) with more than 5 years of follow-up. We observed the up-regulation of miR-182 (3.7 fold) and down-regulation of miR-187 (-3.2 fold) in prostate tumors when compared with normal prostate tissue.



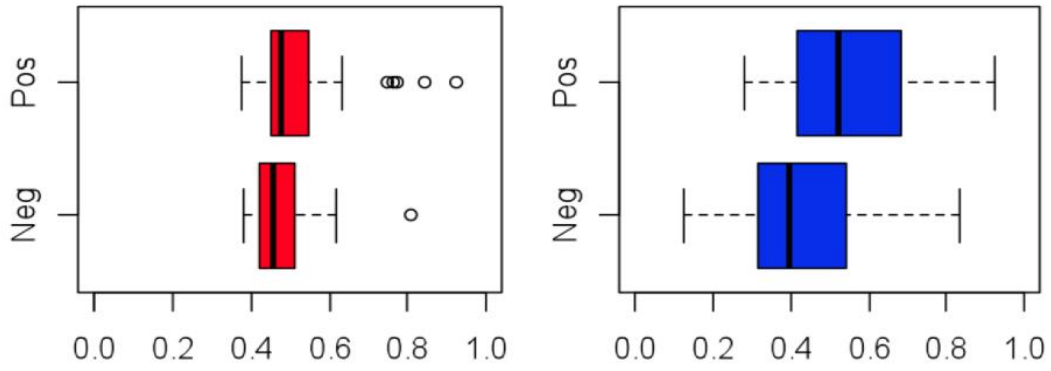
Supplementary Figure 4. Association between miR-187 expression and clinicopathological parameters. A statistically significant correlation between the expression of miR-187 in PCa and different clinicopathological parameters was observed. miR-187 expression was inversely correlated with: A) pT (p=0.0002); B) cT (p=0.125); C) Gleason score (p=0.003); and D) TMPRSS2-ERG (p=0.005) status.



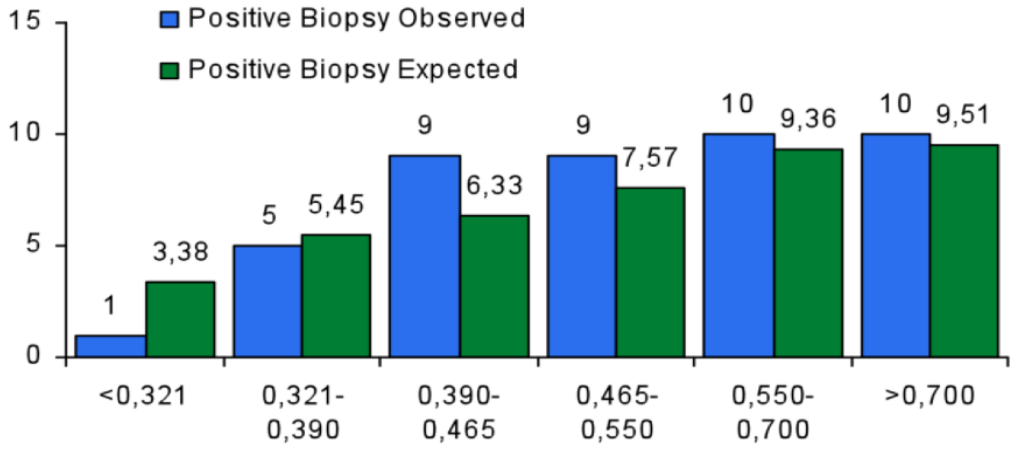
Supplementary Figure 5. Prognostic value of miR-182 expression. Kaplan-Meier plots and log rank tests for biochemical and progression free survival (BPFS and PFS respectively) of the retrospective cohort of samples (n=273) indicates that a higher expression of miR-182 is significantly associated with a worse prognosis for both biochemical (p=0.02) and clinical (p=0.04) progression free survival.



Supplementary Figure 6. Comparison of single biomarkers with a combined model for predicting PCa in urine samples. ROC curves from PSA, PCA-3, miR-187 and combined model (PSA, PCA3 and miR-187) for predicting PCa in urine samples. The areas under the curve are 0.615 (95%CI 0.496-0.733; p=0.064), 0.612 (95%CI 0.494-0.730; p=0.07), 0.622 (95%CI 0.505-0.740; p=0.048) and 0.711 (95% CI 0.603-0.819; p=0.001) respectively. The combined model is able to discriminate the probability of a positive PCa biopsy significantly better than PSA, PCA-3 and miR-187 alone.



Supplementary Figure 7. Probabilities of prediction of positive biopsies of the analyzed regression models. Estimated probabilities for both regression models: only with PSA (red) and with PSA, *PCA3* and miR-187 (blue). As can be appreciated in the figure, the full model discriminates better patients according to the result of the biopsy.



Supplementary Figure 8. Calibration of the combined model for predicting PCa in urine samples. Applying the Hosmer-Lemeshow test it can be appreciated that the whole model (PSA, *PCA3* and miR-187) is correctly calibrated (p=0.292). In this figure we can examine the observed (blue) and expected (green) positive biopsies for each sextile of risk.

Study III. miR-187 targets the androgen-regulated gene ALDH1A3 in prostate cancer.

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RESEARCH ARTICLE

MiR-187 Targets the Androgen-Regulated Gene *ALDH1A3* in Prostate Cancer

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Abstract

miRNAs are predicted to control the activity of approximately 60% of all protein-coding genes participating in the regulation of several cellular processes and diseases, including cancer. Recently, we have demonstrated that miR-187 is significantly downregulated in prostate cancer (PCa) and here we propose a proteomic approach to identify its potential targets. For this purpose, PC-3 cells were transiently transfected with miR-187 precursor and miRNA mimic negative control. Proteins were analyzed by a two-dimensional difference gel electrophoresis (2D-DIGE) and defined as differentially regulated if the observed fold change was ±1.06. Then, MALDI-TOF MS analysis was performed after protein digestion and low abundance proteins were identified by LC–MS/MS. Peptides were identified by searching against the Expasy SWISS PROT database, and target validation was performed both in vitro by western blot and qRT-PCR and in clinical samples by qRT-PCR, immunohistochemistry and ELISA. DIGE analysis showed 9 differentially expressed spots ($p<0.05$) and 7 showed a down-regulated expression upon miR-187 re-introduction. Among these targets we identified aldehyde dehydrogenase 1A3 (ALDH1A3). ALDH1A3 expression was significantly downregulated in PC3, LNCaP and DU-145 cells after miR-187 re-introduction. Supporting these data, the expression of ALDH1A3 was found significantly ($p<0.0001$) up-regulated in PCa samples and inversely correlated ($p<0.0001$) with miR-187 expression, its expression being directly associated with Gleason score ($p = 0.05$). The expression of ALDH1A3 was measured in urine samples to evaluate the predictive capability of this biomarker for the presence of PCa and, at a signification level of 10%, PSA and also ALDH1A3 were significantly associated with a positive biopsy of PCa. In conclusion, our data illustrate for the first time the role of ALDH1A3 as a miR-187 target in PCa and provide insights in the utility of using this protein as a new biomarker for PCa.

and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer death in men [1]. Approximately one in three men over the age of 50 years shows histological evidence of PCa. However, only 10% of these will be correctly diagnosed with clinically significant PCa [2]. PSA levels combined with digital rectal examination (DRE) are the main criteria for PCa diagnosis, but often lead to over diagnosis and overtreatment [3]. Consequently, the identification of new biomarkers, able to improve the diagnosis and detection of potentially aggressive PCa, are needed to better support clinical decisions.

miRNAs are a class of small non-coding RNA molecules consisting of 19–22 nucleotides; they are involved in a variety of biological processes, including development, differentiation, apoptosis and cell proliferation. miRNAs regulate gene expression through translational repression and mRNA cleavage of more than 60% of protein coding genes [4, 5]. Several studies suggest that an individual miRNA can regulate hundreds of targets [6] and can function either as a tumor suppressor or oncogene, depending on the target genes [7], as well as contributing to the initiation and development of various types of cancer, including PCa [5].

Using miRNA microarray analysis (NCBI Gene Expression Omnibus database accession number GSE45604), our group identified miR-187 as a tumor suppressor miRNA in PCa [8]. Although its utility has been demonstrated in the diagnostic setting, to date no experimentally confirmed targets for miR-187 have been identified in PCa. Most computational algorithms predict miRNA targets based primarily on sequence complementarities between the 5' end of the mature miRNA and the 3'-untranslated region (3'-UTR) of target mRNAs; however, these algorithms yield relatively high rates of both false positives and false negatives [6]. Moreover, it is known that more than 25% of experimentally validated targets cannot be predicted by any of the most common miRNA target prediction software [9]. Therefore, gene expression and proteomic screening approaches are urgently needed to experimentally identify miRNA targets.

This study used a proteomic approach based on two-dimensional gel electrophoresis (2D-DIGE) followed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis and, for the first time, identified *ALDH1A3* as a miR-187 target in PCa. In addition, the potential utility of ALDH1A3 as a tumor biomarker was evaluated.

Material and Methods

2.1. Clinical prostate specimens

Formalin-fixed and paraffin-embedded (FFPE) blocks corresponding to 195 PCa patients were retrieved from the archives of the Biobank of the *Fundación Instituto Valenciano de Oncología* accomplishing the following inclusion criteria: specimens obtained from radical retropubic prostatectomies between 1996 and 2002 and no history of previous treatment for PCa (including androgen deprivation therapy or chemotherapy prior to surgery). All patients gave written informed consent for tissue donation for research purposes before tissue collection, and the study was approved by the Ethics Committee of the Fundación Instituto Valenciano de Oncología (ref. number. 2010–19). Exclusion criteria included any previous treatment or presence of other tumors together with the unacceptance of donation consent. The clinical data were reviewed from the clinical records and stored in a PCa-specific database. Patient characteristics and demographics are shown in Table 1. Gleason score was uniformly assessed by the same pathologist (AC). For comparative and calibration purposes, 8 samples of normal prostate tissue

Table 1. Demographics and main clinical and pathological features of the analyzed series.

Parameters	Retrospective series	
	n	%
PSA		
<10ng/ml	109	**55.8**
10–20ng/ml	54	**27.7**
>20ng/ml	32	**16.5**
Gleason-sp		
≤6	66	**33.8**
7	106	**54.4**
8–10	23	**11.8**
cT		
<cT2b	177	**90.8**
cT2b	18	**9.2**
pT		
<pT2	91	**46.7**
≥pT3	104	**53.3**
pN*		
pN0	169	**93.9**
pN≥1	11	**6.1**

SP, specimen; cT, clinical stage; pT, pathological stage; PSA, prostatic specific antigen; pN, pathologic stage with respect to lymph node status; NA, not available.

* Lymphadenectomy was limited to the obturator fossa in most of the cases at the inclusion period.

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obtained from patients undergoing radical cystectomies without pathological evidence of prostatic disease were also analyzed.

Ten fresh tissue samples from histologically confirmed PCa were retrieved from the archives of the biobank from Hospital Clínico Universitario de Valencia (INCLIVA) for validation purposes. Total urine samples were obtained after DRE and immediately prior to diagnostic needle biopsy from an independent cohort of 123 men with suspicion of PCa, from whom 63 lead to a positive biopsy.

2.2. Cell lines and miRNA transfection

PC-3, LNCaP, DU-145, and 22RV1 were cultured in RPMI 1640 (GIBCO, Invitrogen, Life Technologies, CA, USA) while vCaP PCa-derived cells were cultured in DMEM (ATCC, Middlesex, UK) medium, with 10% fetal bovine serum, 100U/ml Penincillin and 0.1ug/ml Streptomycine at standard cell culture conditions (37°C in 5% CO_2 in a humidified incubator). miR-187, which has previously been found to be downregulated in PCa [8], was analyzed in these cell lines by qRT-PCR as described below.

PC-3, LNCaP and DU-145 cells were transiently transfected, using siPORT NeoFX Transfection Agent (Applied Biosystems, Life Technologies, California, USA), with 40nM precursors of miR-187 (hsa-miR-187-3p miRNA mimic) and miRNA mimic negative control 1#, according to the manufacturer's protocol (Applied Biosystems, Life Technologies, California, USA). Cells were harvested 72 h after transfection and cell viability was measured to evaluate its toxicity using the CellTiter 96 AQueous nonradioactive cell proliferation assay (Promega, Wisconsin, USA) in accordance with the manufacturer's instructions.

2.3. Identification of target genes for miR-187

Proteins from the miR-187 mimic versus miRNA mimic negative control 1# transfected PC-3 cells were analyzed by two-dimensional difference gel electrophoresis (2D-DIGE). Briefly, proteins of the two compared groups were precipitated using the 2-D Clean-Up kit (GE Healthcare, Piscatawey, NJ, USA). Samples were then resuspended in DIGE staining buffer DIGE (7M urea, 2 M tiourea, 4% CHAPS, 20 mM Tris) and quantified using Bradford protein assay (BioRad, Hercules, CA, USA). Samples were labelled with 400 pmol/50 μg of protein with the CyDye DIGE Fluor fluorophors Cy3 and Cy5 (GE Healthcare, Piscatawey, NJ, USA) as recommended by the manufacturer. A pool containing equal amounts of all samples was also prepared and labelled with Cy2 to be used as an internal standard on all gels to aid image matching and cross-gel statistical analysis. Six biological repeats of each transfected sample were performed and six gels were generated in total. Protein separation was performed by bidimensional electrophoresis. In the first dimension, proteins were separated according to their isoelectric point on immobilised 24 cm linear pH gradient (IPG) strips (GE Healthcare, Piscatawey, NJ, USA), rehydrated in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG Buffer, 50 mM DTT) overnight. In the second dimension, the proteins were separated according to the molecular weight in 25 cm x 21 cm x 1 mm 12.5% acrylamide gels. The gels were scanned with a Typhoon 9400 Variable Mode Imager (GE Healthcare, Piscatawey, NJ, USA) and the subsequent gel images were imported into the DeCyder Differential Analysis Software. Proteins were defined as differentially regulated if the observed fold change was ±1.06 ($p < 0.05$) between miRNA mimic negative control 1# transfected PC-3 and miRNA-187 mimic transfected PC-3. Protein digestion was performed with sequencing grade trypsin (Promega, Wisconsin, USA) as described elsewhere [10]. MALDI-TOF MS analysis was then performed using 0.5 μL of digestion mixture spotted onto the MALDI target plate. After air-drying the droplets at room temperature, 0.5 μL of matrix [5 mg/mL CHCA (Sigma, St.Louis, MO, USA) in 0.1% TFA-ACN/H2O (1:1, v/v)] was added and allowed to air-dry at room temperature. One known sample was processed identically as quality control. The resulting fractions were analyzed in a 4700 Proteomics Analyzer (ABSciex, Framingham, MA, USA) in positive reflectron mode (2000 shots each position). Low abundance proteins were identified by LC–MS/MS using a trap column (NanoLC Column, 3μ C18-CL, 75 μ m x 15cm, Eksigen, Dublin, CA, USA) through an isocratic flux of 0.1% TFA at 2 μ L/min during 10 min. Once peptides were concentrated into the pre-column they were eluted into the analytical column (LC Column, 3 μ C18-CL, 75um x 25cm, Eksigen, Dublin, CA, USA) to separate. Peptides were finally eluted using a 5a 40% B gradient over 30 min, into a nanoESI qQTOF mass spectrometer (5600 TripleTOF, ABSciex, Framingham, MA, USA). The information from the MS and MS/MS was analyzed with the Paragon algorithm, Protein Pilot Software (ABSciex, Framingham, MA, USA). The peptides were identified using the information in the tandem mass spectra by searching against the Expasy SWISS PROT database.

2.4. Western Blotting of ALDH1A3

For in vitro validation purposes, transfected PC-3, LNCaP and DU-145 cells with miR-187 mimic or miRNA mimic negative control (72 h) were harvested, rinsed with PBS and then lysed with ice-cold lysis buffer (50 mM TrisHCl pH = 8, 150 mM NaCl, 0.02% NaN_3, 0.1%, Sodium dodecyl sulphate (SDS), 1% Nonidet P-40 (NP40), 0.5% deoxycholic acid (DOC), Protease inhibitor cocktail tablets (1 ×). Cell lysates were centrifuged at 10,000 rpm for 10 min at 4°C, the supernatant was then mixed with 5 × SDS sample buffer, boiled for 5 min and separated through 12% SDS-PAGE gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes by electrophoretic transfer. The membranes were blocked in 5%

skimmed milk for 1 h, rinsed and incubated overnight at 4°C with the primary antibodies: ALDH1A3 (Novus Biologicals, Littleton, CO, USA; 1:500 dilution) and β-actin (Millipore, Billerica, MA, USA; 1:200000 dilution). Excess antibody was then removed by washing the membrane in PBS/0.1% Tween 20, and the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies: goat anti-mouse IgG or donkey anti-rabbit IgG (1:10000) (GE Healthcare, Piscatawey, NJ, USA). After washes in PBS/0.1% Tween 20, immune-detection was performed using the enhanced chemiluminescent (ECL) Western blotting detection system (Euroclone, Milano, Italy), according to the manufacturer's instructions.

2.5. miRNA target reporter assay

PC-3 miR-187 mimic or Negative Control cells were transfected with 3'UTR Go Clone Reporter (50ng) (Switchgear Genomics, La Hulpe, Belgium) containing the 3'UTR sequence of ALDH1A3 cloned downstream of the RenSP luciferase reporter or the empty vector containing only the luciferase reporter using Dharmafect 2 reagent (Dharmacon, GE Healthcare,Piscatawey, NJ, USA). The cells were lysed and reporter activity was measured 24 h post transfection using LightSwitch Luciferase Assay Reagent (Switchgear Genomics).

2.6. RNA isolation and qRT-PCR

Isolation of RNA from both cell lines and clinical specimens was carried out using mirVana miRNA Isolation Kit (Ambion, Life Technologies, CA, USA). Total RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied BioSystems, Life Technologies, CA, USA) and High Capacity cDNA Reverse Transcription Kit (Applied BioSystems, Life Technologies, CA, USA) according to the manufacturer's indications. Then, 2 μl of this cDNA, corresponding to 96 PCa tumor samples, was amplified by real-time PCR in a final volume of 10 μl per reaction on an ABI 7500-fast thermocycler using mRNA assays (Applied Biosystems, Life Technologies, CA, USA). For miRNA evaluation, 1.33 μl of miRcDNA was amplified in a final volume of 20 μl. miRNA assays for RU44(001094), RU48 (001006) and miR-187 (001193), and mRNA assay for *ALDH1A3* (Hs00167476_m1) were used (Applied Biosystems, Life Technologies, CA, USA). All reactions were performed in triplicate. The relative expression of the mRNAs or miRNA was determined using the mean value of the control samples as calibrator and following the $2^{-\Delta\Delta Ct}$ method [11]. For cell line evaluations, miR-187 expression was analyzed by qRT-PCR using a universal human RNA pool (Cat# 740000 Stratagene, La Jolla, CA) as normalization control.

2.7. Immunohistochemistry of ALDH1A3

The same FFPE PCa blocks used for RNA analysis were incorporated in 11 tissue microarrays (TMA). Two or three representative areas (1 mm in diameter) of each tumor were selected for TMA production by first examining the hematoxylin & eosin-stained prostatectomy tumor slide and then sampling the tissue from the corresponding paraffin blocks. A tissue microarray instrument (Beecher Instruments, Sun Prairie, WI) was used for TMA assembly. From the TMA blocks, 3-μm-thick sections were subjected to immunohistochemical staining using rabbit anti-human ALDH1A3 polyclonal-Ab (Novus Biologicals, Littleton, CO, USA; 1:50 dilution). Human prostate tissue was used as positive control as recommended by the manufacturer. The percentage of ALDH1A3-positive cells and the cytoplasmic staining intensity were scored semiquantitatively, forming four groups (from 0 to 3). Cases were scored as low expressors when the staining intensity was between 0 and 1, and high expressors when the intensity was 2 and above.

2.8. ALDH1A3 ELISA

Urine samples from 123 patients with suspicion of PCa were centrifuged at 1000 x g to remove the debris. The urine supernatant was used to estimate the ALDH1A3 protein level by using a quantitative human sandwich ELISA kit (Blue Gene Biotech Co Ltd, Shanghai, China). The standard reference was between 0 and 50 ng/ml, with intra and inter-assay CV less than 10%. The optical density (O.D) was determined at 450nm using a Victor Multilabel Plate Reader (PerkinElmer Life Sciences, Massachusetts, USA).

2.9. Statistical analysis

To study the prognostic value of the *ALDH1A3* gene we used binary variables reflecting the positive status of measures. The association between *ALDH1A3* expression and clinicopathological parameters (categorical) was assessed using Spearman with significance considered at 5%. The impact of biological factors on BPFS and clinical PFS was determined by the Kaplan-Meier proportional risk log rank test [12]. Biochemical progression was defined as serum PSA greater than 0.4 ng/ml during follow-up and clinical progression was defined as local (prostatic fossa), regional (lymph nodes) or distant (metastasis) progression. BPFS and clinical PFS were considered individually from the date of surgery to the date of the event. Statistical analysis was done with SPSS, version 20.0. The Student's t test aplying FDR, p-value, PCA and hierarchical clustering were applied to the samples of the proteomic study. All results are given as mean ± SEM (GraphPad Prism 4.0 Software, Graph Pad Software, Inc.)

Results

3.1. miRNA expression in PCa cell lines

Analysis of miRNA levels by qRT-PCR confirmed decreased expression of miR-187 in PCa cell lines: PC-3, LNCaP, DU-145 and 22RV1 (Fig 1A). In PC-3 the miR-187 expression level was

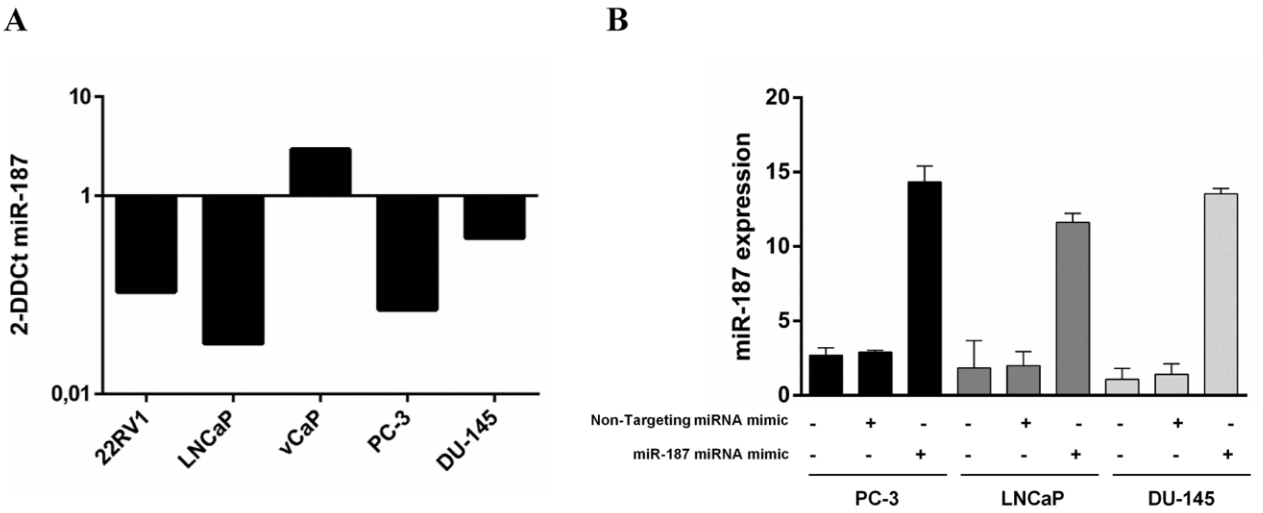

Fig 1. miRNA expression profile in PCa cell lines and miR-187 re-introduction. A) Expression of miR-187 was analyzed by qRT-PCR using a universal human RNA pool as calibrator to normalize the relative expression of the analyzed miRNAs following the $2^{-\Delta\Delta Ct}$ method. As can be appreciated, all cell lines but vCaP showed down-regulation of miR-187. B) miR-187 miRNA mimic and miRNA mimic negative control were transfected into PC-3, LNCaP and DU-145 cells for 72h. The re-introduction of miR-187 in PC-3, LNCaP and DU-145 was confirmed by real-time PCR. The histogram shows the increase in the miR-187 mRNA level in the miR-187 miRNA mimic transfected cells when compared with cells transfected with the miRNA mimic negative control and non-transfected cells.

doi:10.1371/journal.pone.0125576.g001

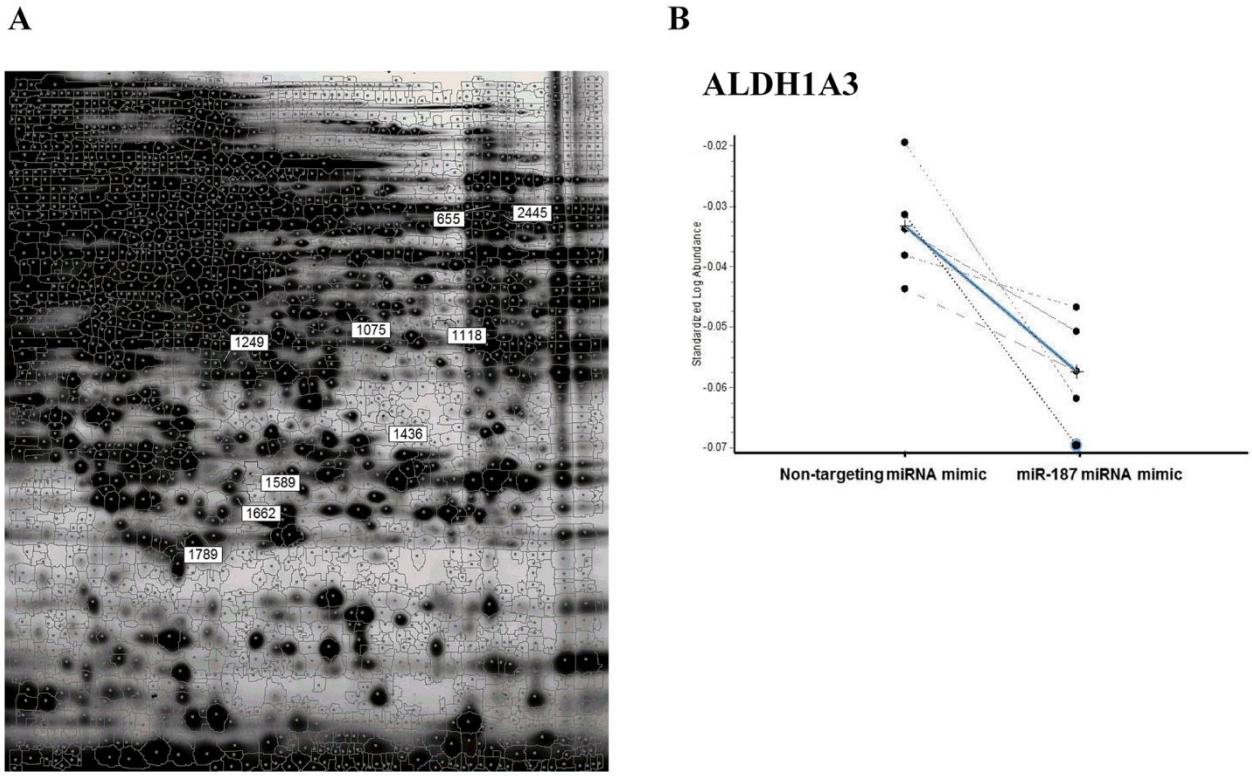

Fig 2. Identification of miR-187 putative targets by 2D-DIGE and LC-MS/MS. PC-3 cells were transfected either with miRNA mimic negative control or miR-187 miRNA mimic, harvested after 72h, and protein lysates were labeled with Cy3 or Cy5 (miR-187 and control) and Cy2 for the internal standard. A) 2D-DIGE gel image obtained at pH 3–10 and 12,5% SDS-polyacrilamide. The numbers refer to the identification given to the spots differentially expressed. Spot 655 was further identified by LC-MS/MS as ALDH1A3. B) Comparison of the expression of one of the spots (655 or ALDH1A3), in the six gels analyzed, between the cells transfected with miR-187 or with the negative control. The average fold change between the two conditions was -1.06 with a p-value of 0.003. ALDH1A3, aldehyde dehydrogenase family 1 member A3

doi:10.1371/journal.pone.0125576.g002

equivalent to that observed previously in PCa patients [8], and for this reason this cell line was chosen for the subsequent analysis.

3.2. Identification of ALDH1A3 as putative miR-187 target

In order to identify potential targets of the miR-187 a series of proteomic analysis and validation experiments was performed. miR-187 miRNA mimic was reintroduced in PC-3, LNCaP and DU-145 and demonstrated that miR-187 expression was recovered in PC-3, LNCaP and DU-145 cells (Fig 1B). A global proteomic approach using DIGE and LC-MS/MS was conducted with samples from PC-3 cells transfected with a negative control and PC-3 transfected with miR-187 miRNA mimic. Cells were harvested 72h after transfection, lysed and separated by bidimensional electrophoresis. After separating the protein extracts and fluorescence scanning, 9 differentially expressed spots were detected (Fig 2A). These 9 protein spots ($p<0.05$) displayed at least 1.06 fold regulation over six independent experiments. Seven out of these 9 spots showed a down-regulated expression upon miR-187 re-introduction, which was consistent with the expected inhibitory effect of the miRNA on most of its targets (S1 Table). Among these targets aldehyde dehydrogenase 1A3 (ALDH1A3) was identified (Fig 2B).

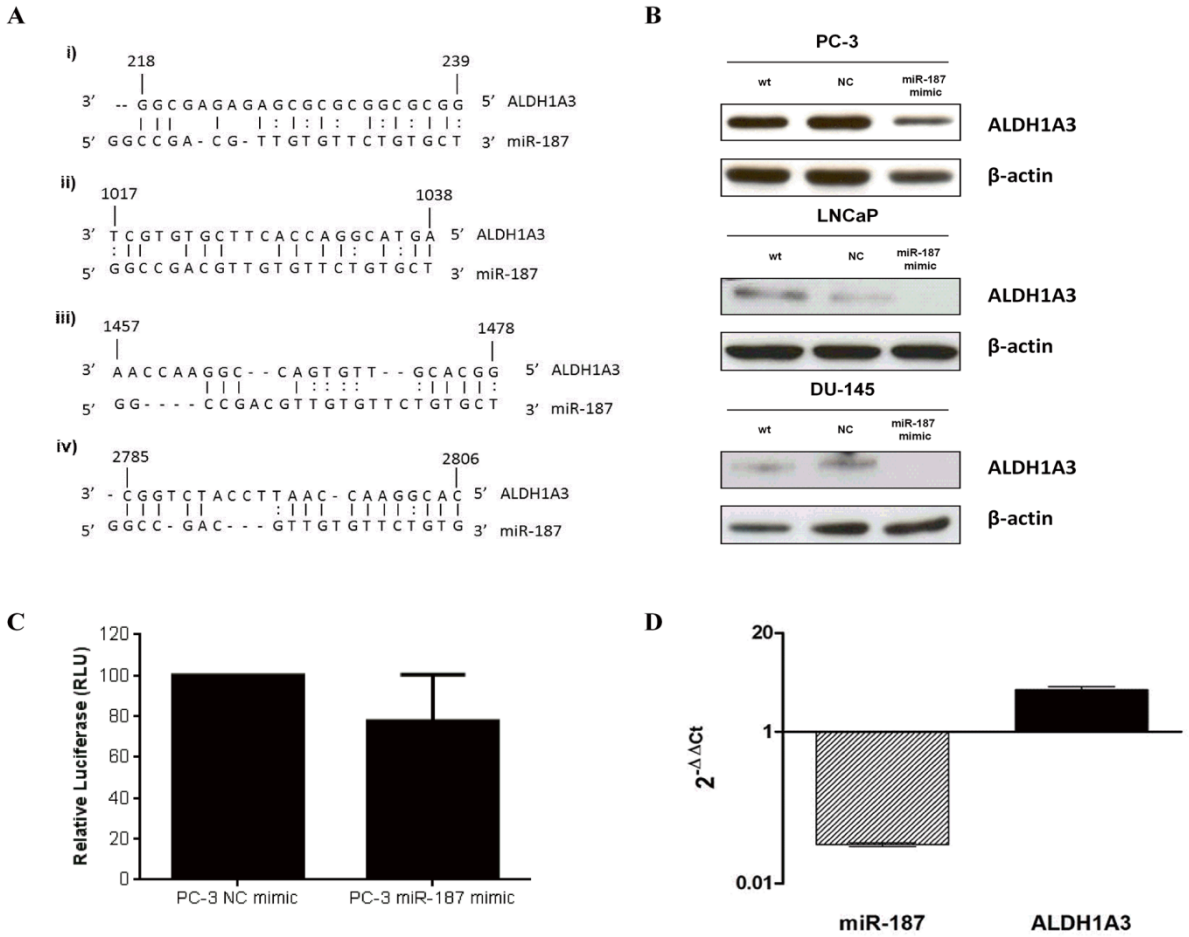

Fig 3. Validation of ALDH1A3 as target of miR-187. A) RNA22 predicted mRNA-miRNA heteroduplexes. RNA22 v1.0 software predicted three different miR-187 binding sites within ALDH1A3 mRNA sequence. Putative miR-187 binding sites were found in ALDH1A3 5'UTR region (i), coding region (CDS) (ii and iii) and 3'UTR (iv). All of them accomplish the criteria of a base pair minimum value of 14 and a maximum folding energy of -25. B) Western Blot analysis shows a reduction in the expression of the protein ALDH1A3 upon the re-introduction of miR-187 (miR-187 miRNA mimic transfected cells), confirming the inhibitory effect of the miRNA through its putative target. Cells were transfected with miR-187 miRNA mimic or negative control and harvested after 72h. Total cell extracts were prepared and electrophoresed by SDS-PAGE, followed by immunoblotting with anti-ALDH1A3 antibody. To ensure equal protein loading the membrane was immunoblotted with anti β-actin antibody. C) Luciferase reporter assay performed with firefly luciferase under control of the ALDH1A3 3′ UTR confirm the inhibition of ALDH1A3 mRNA expression (20% decrease of luciferase signal) upon miR-187 re-introduction. Data are presented relative to the vector control assigned a value of 100. The mean ± s.e.m. of three independent experiments is shown. D) Overexpression of ALDH1A3 mRNA was confirmed in a cohort of human prostate tumors (n = 96). There was an inverse correlation (not statistically significant) between the down-regulation of miR-187 found in these samples and the up-regulation of ALDH1A3.

doi:10.1371/journal.pone.0125576.g003

3.3. Validation of *ALDH1A3* as putative miR-187 target

To demonstrate that *ALDH1A3* is a target of miR-187, bioinformatics target screening; using the most common miRNA target prediction software (Targetscan, Pictar and miRanda) was performed. However, none of these programs matched the 3'-UTR region of *ALDH1A3* with the miR-187 sequence. Nevertheless, applying the RNA22 tool, which does not rely upon cross-species conservation, is resilient to noise and allows G:U pairing of target mRNA to miRNA seed sequence [13], confirmed a match between *ALDH1A3* and the miR-187 seed sequence (Fig 3A).

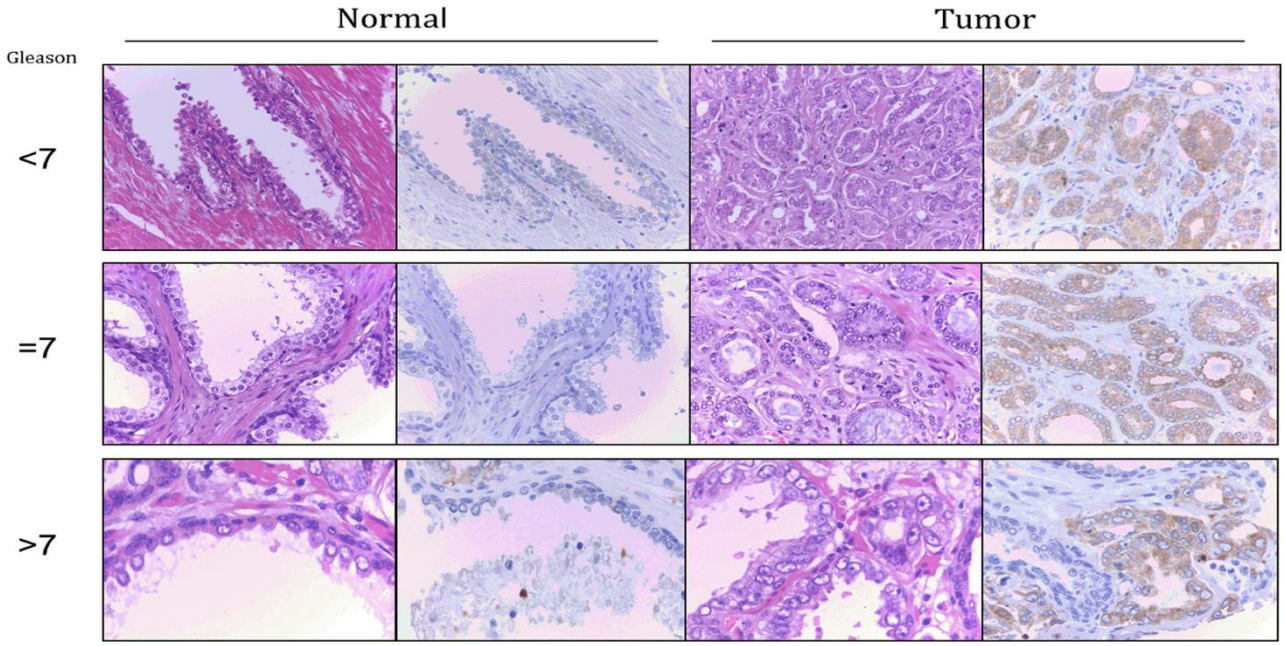

Fig 4. ALDH1A3 immunohistochemistry. Immunohistochemical staining for ALDH1A3 is significantly higher ($p<0.0001$) in tumor samples (right) than in normal controls (left). Staining between different tumor grades (Gleason score lower than 7, equal to or higher than 7) was compared finding a direct correlation between Gleason score and ALDH1A3 expression ($p = 0.05$). Immunohistochemical staining is shown for ALDH1A3 (right column) in each sample together with the hematoxylin & eosin-stained tissue (left column).

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To validate these findings, we confirmed the strong down-regulation of ALDH1A3 upon miR-187 re-introduction by western blot analysis in PC-3, LnCaP and DU-145 cells (Fig 3B).

To further confirm the role of *ALDH1A3* as a miR-187 target a luciferase reporter plasmid containing the 3'UTR sequence of *ALDH1A3* was cloned into PC-3 cells transfected with miR-187 mimic. Enhanced expression of miR-187 (PC-3 miR-187 mimic) significantly reduced reporter activity of 3'UTR *ALDH1A3* constructs to about 20% compared with the control (PC-3 NC mimic) (Fig 3C).

qRT-PCR data showed a higher expression of *ALDH1A3* (1.2 fold) when compared to the samples re-expressing miR-187 (data not shown). Furthermore, we compared the expression of *ALDH1A3* mRNA expression with the down-regulation of miR-187 in two independent cohorts of primary PCa tumors from FFPE ($n = 96$) (Fig 3D) and fresh tissue ($n = 10$) (S1 Fig). However, no correlation between *ALDH1A3* and clinicopathological parameters was found, and then as expected, *ALDH1A3* did not constitute a prognostic indicator for either BPFS ($p = 0.773$) or PFS ($p = 0.430$) in this series.

ALDH1A3 protein expression was further evaluated by immunohistochemistry (IHC) in the 195 cases included in the TMA (Fig 4). We found that ALDH1A3 was significantly ($p<0.0001$) up-regulated in PCa samples (average intensity = 1.42) when compared with normal prostate tissue (average intensity = 0.12). Moreover, in order to investigate the role of *ALDH1A3* as a miR-187 target, we studied the correlation with miR-187 expression. Interestingly, we found that miR-187 expression was significantly inversely correlated ($p<0.0001$) with ALDH1A3 protein expression. We further studied the correlation of *ALDH1A3* with clinicopathological parameters and prognosis. Although ALDH1A3 expression did not constitute a prognostic indicator, we found a statistically significant direct correlation with Gleason score

($p = 0.05$). Hence, 37% of cases with a Gleason score <7 showed high ALDH1A3 intensity of staining compared with 56% of PCa with Gleason≥ 7.

To further explore the role of *ALDH1A3* in the diagnostic setting we performed an ELISA assay to measure ALDH1A3 expression in urine (n = 123). A univariate logistic regression model was performed to evaluate the predictive capability of this biomarker, together with PSA, for the presence of PCa in diagnostic biopsies. At a significance level of 10%, PSA and ALDH1A3 were both significantly associated with a positive biopsy of PCa (Fig 5).

Discussion

miRNAs are predicted to control the activity of approximately 60% of all protein-coding genes, and have been shown to participate in the regulation of several cellular processes. By base pairing to mRNAs, microRNAs mediate translational repression or mRNA degradation [4]. Having previously demonstrated the role of miR-187 in PCa progression and diagnosis [8], we decided to further investigate potential targets of this miRNA that could be also of interest as biomarkers. For this purpose we reintroduced miR-187 precursor in PC-3 cells and performed a proteomic approach.

Sequences recognized by miRNA seeds are found in many genes, which makes it difficult to identify physiologically relevant miRNA–target relationships from sequence analysis alone [14]. Moreover, previous results obtained by Yang et al. [6] and Schramedei et al. [15] confirmed that less than 10% of proteins identified by a proteomic approach were predicted by commonly used algorithms such as Pictar, Targetscan and miRanda. In line with these findings, none of the proteins identified by proteomic screening in the present study were predicted *in silico* by the above mentioned algorithms. Nevertheless, using the RNA22 tool it was possible to find a match between miR-187 and our potential target in the *ALDH1A3* coding region (CDS). This algorithm is different from previously reported methods in that it does not use a cross-species sequence conservation filter, thus allowing the discovery of microRNA binding sites that may not be present in closely related species [13]. Moreover, RNA22 takes into account the hypothesis that, in addition to 3'UTRs, numerous binding sites are likely to exist in 5'UTRs and CDSs allowing the identification of previously unidentified miRNA/mRNA heteroduplexes.

In this study, 9 putative targets of miR-187 were identified by 2D-DIGE and MS analysis (S1 Table). From these we selected aldehyde dehydrogenase 1A3 (*ALDH1A3*) for further evaluation because it has been described to be regulated by androgens [16]. Western blot analysis, qRT-PCR and IHC confirmed the direct regulation of *ALDH1A3* by miR-187. First, the inverse correlation between ALDH1A3 and miR-187 was confirmed by recovering miR-187 expression in PC-3, LNCaP and DU-145 cells, which led to a down-regulation of ALDH1A3 protein levels. Second, the inhibitory effect of miR-187 on ALDH1A3 expression was further confirmed by a luciferase reporter assay that showed a decrease in ALDH1A3 expression ($\sim 20\%$ reduction in luciferase signal) upon miR-187 mimic transfection. Third, the inhibition of *ALDH1A3* was observed when analysing a cohort of PCa human patient samples, both fresh and FFPE tissues. In these cohorts, the strong down-regulation of miR-187 was accompanied by an increased *ALDH1A3* mRNA expression. Forth, the role of *ALDH1A3* as miR-187 target was confirmed by IHC analysis. Hence, ALDH1A3 was found to be up-regulated in prostate tumors and the expression of this protein is inversely correlated with the expression of the miRNA. In addition, the potential role of *ALDH1A3* as candidate prognostic biomarker for PCa was evaluated, although in the cohort of samples analysed it did not provide any additional information. Nevertheless, the association of *ALDH1A3* expression with Gleason score provides evidence of an increase in *ALDH1A3* expression with tumor staging. We have previously postulated that loss

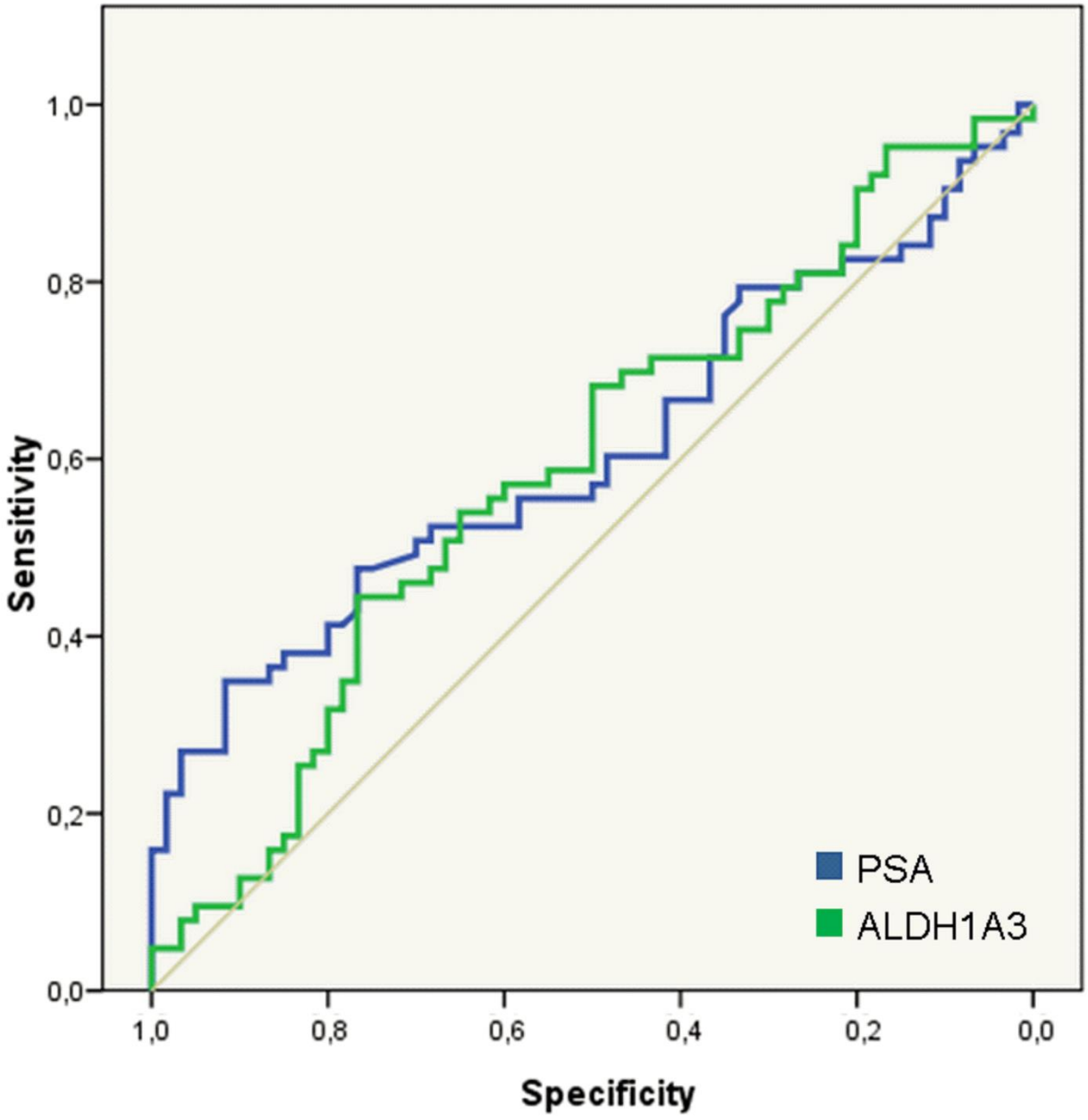

Fig 5. Diagnostic role of ALDH1A3 in urine samples. ROC curves from PSA and ALDH1A3 for predicting PCa in urine samples. The areas under the curve are 0.610 (95%CI 0.509–0.710; p = 0.036 and 0.591 (95% CI 0.490–0.692; p = 0.083) respectively. At a significance level of 10%, both PSA and ALDH1A3 were significantly associated with a positive biopsy of PCa.

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of miR-187 during PCa progression could indicate a role as tumor suppressor [8]. Additionally, ALDH1A3 was found to cooperate with PSA in the prediction of the biopsy result. Apart from its association with the presence of the tumor in IHC of FFPE slides, we were able to measure ALDH1A3 in urine samples, finding a positive association with tumor appearance. In this

context, the identification of *ALDH1A3* as a miR-187 target and its up-regulation in PCa indicates its potential role as an oncogene with an implication in PCa development.

ALDH1A3 is a member of the human aldehyde dehydrogenase family that includes different subtypes *ALDH1A1*, *ALDH1A2*, *ALDH1A6*, etc. that catalyze the oxidation of retinal to retinoic acid (RA)[17], which is required for normal prostate development [18]. The implication of these enzymes in RA synthesis causes them to function as key enzymes in pathways associated with cell proliferation, differentiation and survival. *ALDH1A3* has been found to play a role as a predictor of metastasis in breast cancer [19]. ALDH1A isozymes, mainly ALDH1A1 and ALDH1A3, have been also described as markers of cancer stem cells in different tumors and key determinants for the survival and drug resistance of cancer cells [19, 20]. In agreement with the association with stemness of ALDH1A3, miR-187 has been recently identified as an miRNA that specifically characterizes human embryonic stem cells and induces pluripotent stem cells [21]. Therefore both genes, miRNA and target, seem to regulate pluripotent cell characteristics which are related with a more undifferentiated and aggressive tumor phenotype. In this regard, recent results show that high ALDH activity can be also used to isolate human prostate cancer cells with significantly enhanced tumorigenicity and metastatic behavior [22]. Thus, using a FACS sorting kit such as ALDEFLUOR, which classifies cells according to ALDH activity, might be a useful tool for the stratification of prostate cancer patients at risk of developing metastatic disease.

It is recognized that a single miRNA can modulate several genes [4, 5] and probably the effects of the restoration of miR-187 are broader than those observed in a single gene. Nevertheless, our data illustrate for the first time the role of ALDH1A3 as a miR-187 target in PCa and provide insights into the utility of including this protein as a new biomarker for PCa.

Supporting Information

S1 Fig. ALDH1A3 expression in an independent cohort of PCa fresh tissue. With the aim of performing a validation in an independent set of PCa patients, overexpression of ALDH1A3 mRNA was confirmed in a cohort of human fresh prostate tumors (n = 10). There was an inverse correlation ($p<0.0001$) between the down-regulation of miR-187 found in these samples and the up-regulation of ALDH1A3.
(TIF)

S1 Table. Putative miR-187 predicted targets.
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: ICS KS MJV JALG. Performed the experiments: ICS EM AA CM. Analyzed the data: ICS EM AA CM AC JRB JALG. Contributed reagents/materials/analysis tools: MJV KS AC JRB JALG. Wrote the paper: ICS EM AA CM JALG.

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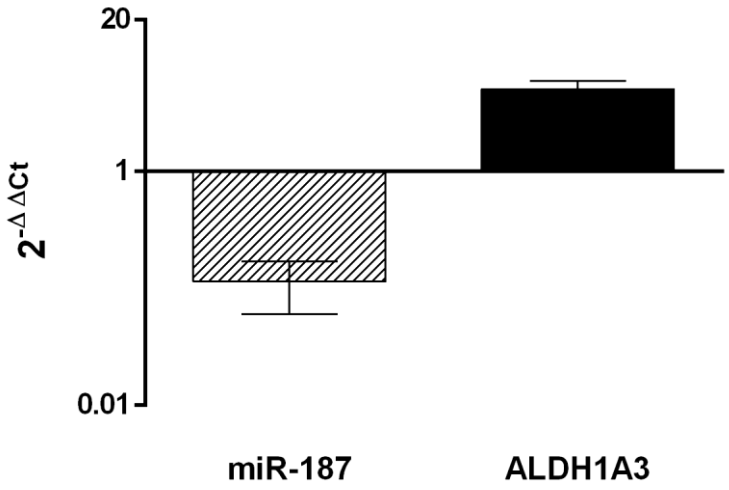

Figure 1S. ALDH1A3 expression in an independent cohort of PCa fresh tissue. With the aim of performing a validation in an independent set of PCa patients, overexpression of ALDH1A3 mRNA was confirmed in a cohort of human fresh prostate tumors (n=10). There was an inverse correlation ($p<0.0001$) between the down-regulation of miR-187 found in these samples and the up-regulation of ALDH1A3.

Table 1S. Putative miR-187 predicted targets

Spot	Fold-change	P-value	Putative target
655	-1,06	0,003	**ALDH1A3**
1075	1,08	0,0059	SPB5, MTNA, CAPG
1436	-1,23	0,0081	SPB5, CAPZB
1118	-1,14	0,026	GIPC1, HORN, GALK1,
1249	-1,13	0,033	HMOX2, LEG7
1789	1,27	0,034	Unknown
2445	-1,15	0,046	PHGDH, ACTG, ALDOA
1662	1,06	0,05	ITPA, LEG7
1589	-1,08	0,052	Unknown

Study IV. Clinico-pathological significance of the molecular alterations of the *SPOP* gene in prostate cancer.

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Valencia, April 2015

To whom it may concern,

As senior author of the publication entitled "**Clinico-pathological significance of the molecular alterations of the *SPOP* gene in prostate cancer**", I certify that Irene Casanova Salas has substantially contributed to the development of this study and shares first co-authorship with María García Flores. In this study we have performed a comprehensive approach for studying *SPOP* mutations and expression in a large cohort of tumors and we have determined its clinico-pathological significance as prognostic and therapeutic biomarker in prostate cancer (PCa). We have evaluated *SPOP* mRNA expression and the presence of mutations in a large cohort of PCa patients with a long follow-up. With this study we have demonstrated the role of *SPOP* as tumor suppressor in PCa and its association with clinico-pathological parameters and prognosis.

The contribution of the first authors to the publication could be summarized in:

- ICS was responsible of the experimental design, sample preparation and analysis of expression. She performed also the statistical analysis and manuscript preparation and editing.

- MGF was responsible of sample preparation, analysis of expression and mutational evaluation. She was also involved in manuscript editing.

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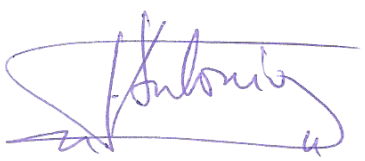

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Head of Laboratory

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Original Research

Clinico-pathological significance of the molecular alterations of the *SPOP* gene in prostate cancer

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KEYWORDS
Speckle-type POZ protein (SPOP)
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Biomarker
Biotype
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Abstract ***Aims:*** Speckle-type POZ protein (SPOP) is an E3 ubiquitin ligase adaptor recently described to be mutated in prostate cancer (PCa). Hence, studying the gene expression profile and the presence of SPOP mutations in PCa and understanding its clinico-pathological significance as prognostic and therapeutic biomarker are important to further understand its role in PCa development.

Patients and methods: A cohort of 265 paraffin-embedded PCa samples from patients with more than 5 years of follow-up and treated with radical prostatectomy were collected at our institution for SPOP evaluation. RT-qPCR analysis was performed for expression studies while mutations were assessed by next generation sequencing. Relationship with prognosis was analysed using log-rank analysis and multivariable Cox regression.

Results: SPOP was found to be strongly down-regulated in PCa (median = 0.24; range = 0.04–9.98) and its expression was associated with both, biochemical ($p = 0.003$) and clinical progression free survival ($p = 0.023$), the very low SPOP expression levels being associated to the worst prognosis. Multivariate analysis demonstrated that low levels of SPOP independently predicted a worse prognosis for both, biochemical (Hazard ratio (HR) = 0.5; confidence interval (CI) 95% [0.4–0.9], $p = 0.011$) and clinical progression (HR = 0.6; IC 95% [0.4–1], $p = 0.046$). SPOP mutations were found in 10% of TMPRSS2-ERG

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(T2E)-negative cases. Log-rank tests showed that mutations were significantly associated with biochemical progression free survival (BPFS) ($p = 0.009$) and also were significant in the multivariable analysis (HR = 3.4; IC 95% [1.5–7.6], $p = 0.004$).
Conclusions: The present study demonstrates that prognosis varies depending on SPOP expression and mutational status, hence, defining a new biotype of PCa associated with a worse prognosis.

1. Introduction

The emergence of new technologies and large-scale sequencing studies of cancer genomes confirm that cancer arises as a result of mutations in cancer cells [1]. Clinically, prostate cancer (PCa) is a heterogeneous disease diagnosed in one in six men and leading to a 10% of cancer deaths in Europe [2]. Clinical phenotypes of PCa vary from an indolent disease, requiring active surveillance, to one in which tumours metastasise and escape local therapy even when with early detection. This variable behaviour observed, is a consequence of the molecular heterogeneity found in PCa [3]. Most common genetic alterations in PCa come from structural genomic changes such as deletions, amplifications and translocations. Deletion of the tumour suppressor gene *PTEN,* amplification of the *androgen receptor* (AR) or rearrangement between *TMPRSS2* and the ETS-family of transcription factors are some well-established alterations in this disease. In contrast, the presence of point mutations is less common, mainly affecting *AR*, *PTEN* and *AKT* [4]. However, a recent study has demonstrated that the most common non-synonymous mutation in PCa involves speckle-type POZ protein (SPOP) [5].

SPOP gene encodes for the substrate-recognition component of a Cullin3-based E3-ubiquitin ligase (Cul3) and it is found to be expressed in several tissues including prostate [6]. *SPOP* is located in the 17q21 locus which has been described to be a region with a high allelic imbalance in primary tumours [7]. Structurally, SPOP consists of an N-terminal MATH domain that recruits substrate proteins and a C-terminal BTB (Bric-a-brac/Tamtrack/Broad complex) domain that interacts with Cul3. Recurrent mutations in *SPOP* occur in 6–12% of PCa and are exclusively found in the substrate-binding cleft (MATH domain) of the protein [5].

Previous works revealed that *SPOP* mutations appeared exclusively in tumours negative for *ERG* rearrangement, supporting the idea of a new molecular subtype in PCa [5]. In the present study we confirm the presence of *SPOP* mutations in a large cohort of *TMPRSS2-ERG* (T2E) negative tumours. Moreover, we demonstrated the loss of expression of *SPOP* in a retrospective series of 265 PCa, and described for the first time its clinico-pathological significance and its role as tumour suppressor in PCa primary tumours.

2. Material and methods

2.1. Prostate specimens

Formalin fixed (neutral buffered formalin, pH 7.2, 4% formaldehyde, 16 h; following the standards of the Department of Pathology of our institution) and formalin fixed paraffin-embedded (FFPE) tissue samples from PCa patients were retrieved from the archives of the Biobank of the *Fundación Instituto Valenciano de Oncología*, according to the following criteria: specimens obtained from radical retropubic prostatectomies from 1996 to 2002 and no history of previous treatment for PCa (including androgen deprivation therapy or chemotherapy prior to surgery). We identified 265 cases that met these criteria. Written informed consent for tissue donation for research purposes was obtained from all patients prior to tissue collection, and the study was approved by our Institutional Ethics Committee (ref. number:2010-19). Patient characteristics and demographics are shown in Table 1. Gleason score was uniformly assessed by the same pathologist Ana Calatrava (AC), who also certified high-density cancer areas in haematoxylin and eosin stained slides to ensure a purity of at least 75% of cancer cells. For comparative and calibration purposes, we also analysed 10 samples of normal prostate tissue obtained from patients undergoing radical cystectomies without pathological evidence of prostatic disease. *T2E* gene fusion status was determined by reverse transcription polymerase chain reaction (RT-PCR) and fluorescent in situ hybridisation (FISH) as already described [8] and quantitative RT-PCR (qRT-PCR). Follow-up of the retrospective series ranged from 2 to 189 months (median 96 months). Biochemical progression was defined as serum PSA greater than 0.4 ng/ml during follow-up and clinical progression was defined as local (prostatic fossa), regional (lymph nodes) or distant (metastasis) progression. During this follow-up 120 patients (45.3%) reported a biochemical progression, of which 73 (27.5%) had clinical progression.

2.2. RNA isolation and qRT-PCR

Isolation of RNA, from three sections of 10 μm FFPE samples, was carried out using RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Life

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Table 1
Demographics and main clinical and pathological features of the analysed series.

Parameters	Retrospective series	
	n	Frequncy (%)
Age		
⩽55	15	**5.7**
56–65	81	**30.6**
66–75	140	**52.8**
>75	29	**10.8**
PSA		
<10 ng/ml	153	**57.7**
10–20 ng/ml	73	**27.5**
>20 ng/ml	39	**14.7**
Gleason-sp		
⩽6	107	**40.4**
7	127	**47.9**
8–10	31	**11.7**
cT		
⩽cT2c	246	**92.8**
⩾cT3a	19	**7.2**
pT		
⩽pT2	132	**49.8**
⩾pT3	133	**50.2**
pN*		
pN0	232	**87.5**
pN ⩾ 1	12	4.5
pNx	21	**8**
Margins		
Negative	133	**50.2**
Positive	132	**49.8**
TMPRSS2-ERG status		
Negative	90	**34**
Positive	175	**66**

SP, specimen; cT, clinical stage; pT, pathological stage; PSA, prostatic specific antigen; pN, pathologic stage with respect to lymph node status.
* Lymphadenectomy was limited to the obturator fossa in most of the cases at the inclusion period.

Technologies, CA, United States of America (USA)) following the providers' specifications.

Total RNA (100 ng) with a 260/280 nm absorbance ratio of 1.5–2 was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, CA, USA) according to the manufacturer's indications. cDNA was amplified by real-time PCR on an ABI 7500-fast thermocycler using SPOP assay (Hs00737433_m1), T2E (Hs03063375_ft) and B2M (Hs99999907_m1) assay as housekeeping gene (Applied Biosystems) All reactions were performed in duplicate. The relative expression of *SPOP* (RQ) was determined using the mean value of the control samples as calibrator and following the $2^{-\Delta\Delta Ct}$ method [9].

2.3. DNA isolation

DNA was isolated from five sections of 5 μm FFPE cases using kitQIAamp® DNA Investigator kit (Qiagen, Hilden, Germany) after applying the Deparaffinization Solution (Qiagen) as indicated by the manufacturer. The concentration of 100 ng of DNA with a 260/280 nm absorbance ratio of 1.5–2 was assessed.

2.4. Next generation sequencing

Sequencing analysis of exons 5, 6 and 7 of *SPOP* was carried out using the 454 GS-Junior® next-generation sequencer platform (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Hence, 60 ng of genomic DNA was amplified using *SPOP* specific primers (Table 1S). These primers were modified with a universal sequencing tail and multiple identifiers nucleotide sequences (Integrated DNA Technologies Inc, Skokie, IL, USA). Amplicons were purified and quantified, and an emulsion PCR was performed according to the manufacturer's protocol (Roche). A sequencing reaction was performed and the results obtained were analysed using Amplicon Variant Analyser software (Roche). In all samples analysed, at least 100 reads of the sequenced exons of *SPOP* were obtained. We required a minimum of 10% of the obtained reads covering a site for mutation validation.

2.5. Sanger sequencing

DNA fragments of 298, 312 and 228 bp corresponding respectively to exons 5, 6 and 7 of *SPOP* were amplified by PCR using the primers described above. The PCR products were purified using the ExcelaPure® 96-Well UF PCR plates (EdgeBio, Gaithersburg, MD, USA) and checked on 1.5% agarose gel electrophoresis. Sequencing was performed by applying the same primers and using the BigDye® terminator sequencing kit version 3.1 (Applied Biosystems) on a 3130XL genetic analyser (Applied Biosystems). The sequencing results were interpreted with Sequencing Analysis software version 5.2 (Applied Biosystems) using the reference data from Ensemble database (www.ensemble.org) (Chromosome 17: 47,661,867-47,741,217, Gene: SPOP ENSG00001 21067 and Transcript: SPOP-202 ENST00000393331).

2.6. Statistical analysis

SPOP gene prognostic value was assessed by means of binary variables reflecting the positive status of measures. The association between *SPOP* expression and clinico-pathological parameters (categorical) was assessed using the Spearman correlation with significance considered at 5%. The impact of biological factors on biochemical (biochemical progression free survival (BPFS)) and clinical (progression free survival (PFS)) progression-free survival was determined by the Kaplan–Meier proportional risk log rank test. BPFS and PFS were considered individually from the date of surgery to the date of the event. Univariate predictors of BPFS and PFS were entered into a Cox proportional hazards model

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using stepwise selection to identify independent predictors of outcome, considering the 95% CI [10].

3. Results

3.1. Expression profile of SPOP in PCa primary tumours

SPOP gene was found to be down-regulated [relative quantities (RQ) < 1] in almost all the studied samples (93.5% of the cases) when compared with normal prostate tissue (Fig. 1). *SPOP* expression was classified in 'High' and 'Low' expression depending on if the obtained value (RQ) was above or below the first quartile respectively. For the whole series, the association between *SPOP* expression with clinico-pathological characteristics and follow-up was analysed. *SPOP* expression was inversely correlated with Gleason score($r = -0.124$; $p = 0.045$), while no other statistically significant correlations were found with the other clinicopathological parameters. Log-rank analysis for both, BPFS and PFS showed a significant association between prognosis and *SPOP* expression (Table 2, Fig. 2A and B). Moreover, the Cox proportional hazard multivariate analysis exhibited that low levels of *SPOP* independently predicted a worse prognosis for both biochemical and clinical progression (Table 2).

Additionally, up-regulation of *SPOP* (RQ > 1) was observed in 17 cases (6.5%) corresponding to a series of tumours with a low-grade Gleason score and lower risk of progression (Fig. 2C and D). The time to progression after log-rank tests showed a better prognostic behaviour, in tumours with an up-regulated *SPOP* expression, although it was not statistically significant.

Since *SPOP* mutations have been described to be mutually exclusive with the presence of the T2E translocation, our series of patients was divided depending on the status of the fusion gene. Then, we assessed if the correlations of *SPOP* expression with clinico-pathological parameters and prognosis were dependent on the T2E status. Interestingly, we observed that the loss of *SPOP* expression was conferring the worst prognosis for PFS in the group of patients negative for the T2E (Fig. 2F). In fact when we compared the Kaplan–Meier analysis for the clinical progression between the whole cohort ($n = 265$) (Fig. 2B) and those non-expressing T2E ($n = 90$) (Fig. 2F) we observed that the prognosis value of *SPOP* expression was statistically more significant and discriminant in the subgroup of patients without the fusion gene. Moreover, in the T2E-negative group of patients SPOP was also significant in the Cox proportional hazard multivariable analysis (hazard ratio (HR): 0.3. IC 95% [0.1–0.7], $p = 0.011$).

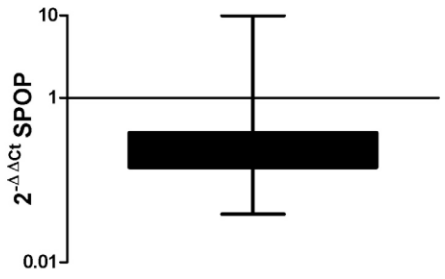

Fig. 1. Speckle-type POZ protein (SPOP) expression profile in prostate cancer (PCa). The differential expression (RQ) of SPOP between PCa and normal tissues was analysed in 265 FFPE tumour samples by qRT-PCR following the $2^{-\Delta\Delta Ct}$ method. SPOP was found to be down-regulated (RQ < 1) in 93.5% of the cases, while 17 cases (6.5%) showed an up-regulated SPOP (RQ > 1) (median = 0.24; range = 0.04–9.98).

3.2. Mutation profile of SPOP in T2E negative PCa tumours

The evaluation of the presence of mutations in the gene *SPOP* was performed in 90 cases corresponding to those negative for T2E. Mutations were detected in nine cases (10%) following the established criteria of sensitivity and they were located in exons 5 or 6. These mutations were present in high percentages, from 7.41% to 82.76% (median = 10%), and eight were confirmed by Sanger sequencing (Table 3). No association between *SPOP* mutations and clinico-pathological characteristics was found. However, log-rank tests showed that *SPOP* mutations were significantly associated with worse BPFS ($p = 0.009$) (Fig. 3) and also constituted an independent variable for poor prognosis after the Cox proportional hazard multivariable analysis (HR = 3.4; IC 95% [1.5–7.6], $p = 0.004$).

No association between *SPOP* mutations and expression level was found. Nevertheless, we found that *SPOP* expression was lost in all cases with mutations (Table 3).

4. Discussion

Recent studies have identified *SPOP* as the gene most commonly affected by somatic point mutations in PCa [5,11], however little is known about SPOP knockdown in PCa and its prognostic implications. In this study a comprehensive approach was conducted for studying *SPOP* mutations and expression in a large cohort of tumours. Hence, for the first time the loss of expression of *SPOP* in a retrospective series of 265 PCa, as well as, the clinico-pathological significance and its role as tumour suppressor in PCa were demonstrated.

Previous studies assessing smaller cohorts of patients found a down-regulation of *SPOP* gene [5] or protein [12] in PCa but, none of them showed any association with neither clinico-pathological parameters nor prognosis. The present study evidences the loss of *SPOP* expression in PCa. Furthermore, it demonstrates that *SPOP* expression is inversely associated with tumour aggressiveness and prognosis. Our findings have showed that the expression of *SPOP* is inversely correlated with Gleason score and furthermore provides independent

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Table 2
Log-rank and Cox regression tests for biochemical progression free survival (BPFS) and progression free survival (PFS).

Parameters	*n*	Biochemical progression						Clinical progression					
		Events	%BPFS	*p*-Univariate	Hazard ratio (HR)	95% CI	*p*-Multivariate	Events	%Clinical PFS	*p*-Univariate	HR	95% Confidence interval (CI)	*p*-Multivariate
Age													
⩽55	15	4	73.3	0.282			NS	3	79.4	0.360			NS
56–65	81	43	26					29	50.4				
66–75	140	60	45.8					36	68.4				
>75	29	13	54.2					5	60.6				
Gleason-sp				<0.0001			<0.0001			<0.0001			0.004
2–6	107	34	57.1		1			17	76.9		1		
7	127	62	29.5		3.4	1.8–6.2	<0.0001	42	57.5		3.5	1.7–7.4	0.001
>7	31	24	12.1		2.2	1.3–3.7	0.002	14	–		1.7	1–3.1	0.097
PSA				<0.0001			0.005			0.059			NS
⩽10 ng/ml	153	56	47.9		1			34	69				
10–20 ng/ml	73	36	39.8		2.2	1.4–3.6	0.001	24	53.4				
>20 ng/ml	39	28	25.6		1.6	1–2.7	0.064	15	55.7				
cT				<0.0001			0.013			0.007			0.014
⩽cT2c	246	106	43.1		1			65	63.5		1		
⩾cT3a	19	14	17.6		2.2	1.2–4		8	53.7		2.5	1.2–5.4	
pT				<0.0001			0.039			0.001			NS
⩽pT2	132	41	58.5		1			24	78.3				
⩾pT3	133	79	22.7		1.6	1–2.5		49	47.9				
pN				<0.0001			0.007			0.267			NS
pN0	232	104	41.1		1			65	61.9				
pN⩾1	12	11	8.3		2.4	1.3–4.4		5	50.9				
Margins				<0.0001			0.046			<0.0001			0.019
Negative	133	38	56.9		1			23	77.3		1		
Positive	132	82	21.1		1.6	1–2.5		50	38.5		1.9	1.1–3.2	
TMPRSS2-ERG				0.223			NS			0.906			NS
Negative	90	46	34.3					25	64.3				
Positive	175	74	44.7					48	61.2				
SPOP				0.003			0.005			0.023			0.045
Low	66	38	36		1			23	58.9		1		
High	198	81	43.8		0.5	0.4–0.8		50	64		0.6	0.4–1	

SP, specimen; BPFS, biochemical progression free survival; PFS, progression free survival; NS, Not significant.

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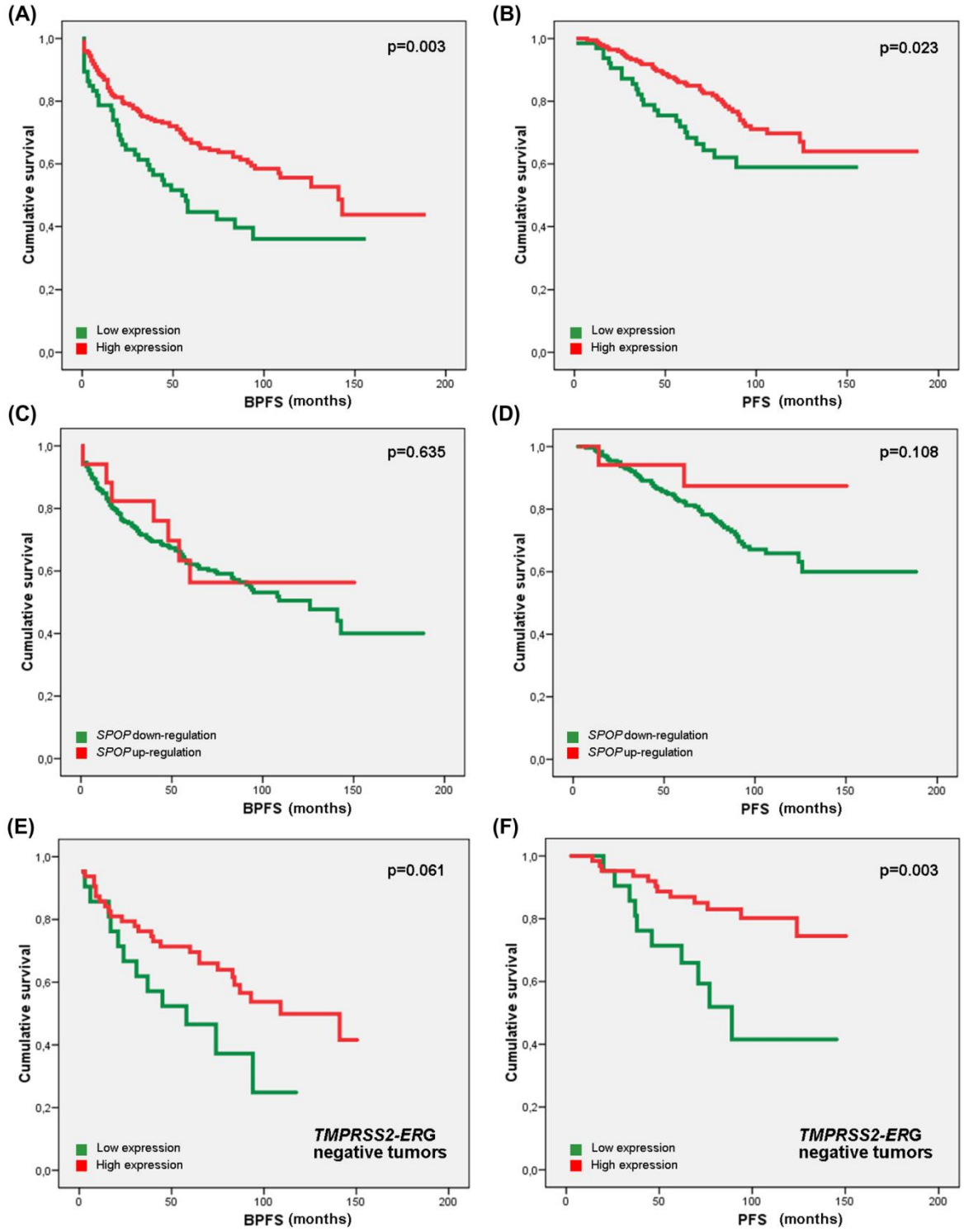

Fig. 2. Kaplan–Meier plots showing the prognostic impact of Speckle-type POZ protein (SPOP) expression. (A) A cohort of 265 prostate cancer (PCa) samples was divided, according to the first quartile of SPOP expression, in high and low expressors. As can be appreciated SPOP expression differentiates two groups with different behaviour for biochemical progression free survival (BPFS) (B) and progression free survival (PFS). (C) The total series was divided into two groups depending if SPOP was found up or down-regulated when compared with normal tissue (RQ). Prognosis behaviour was compared between the two groups for both BPFS (D) and PFS. Although it was not statistically significant, the Kaplan–Meier plot shows a better prognosis for those tumours with an up-regulated SPOP. (E) Log-rank analysis was also performed in the subgroup of patients not harbouring the rearrangement TMPRSS2-ERG (T2E) ($n = 90$). The time to progression showed a better prognostic behaviour in the tumours with a high expression of SPOP for both BPFS (F) and PFS. Furthermore, the different behaviour between high and low expressors was more evident in these T2E-negative tumours with a greater statistical significance.

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Table 3
Description of mutations identified in *SPOP* gene.

Cases	*SPOP* mutation	Exon	Sanger sequencing	*SPOP* expression (RQ)	Mutation type	Gleason score	pT
1	p.F104V, c.310T>G	5	Confirmed	0.12896	–	<7	⩽pT2
2	p.F133L, c.399C>G	6	Confirmed	0.10850	COSM95272 [5,11,20,21]	>7	⩾pT3
3	p.D130N, c.388G>A	6	Confirmed	0.15308	COSM1290717 [19]	7	⩾pT3
4	p.F102S, c.305T>C	5	Confirmed	0.17485	SNP_rs193920894	7	⩾pT3
5	p.D153N, c.457G>A	6	Confirmed	0.45923	–	7	⩽pT2
6	p.Q120Stop, c.358C>T	6	Confirmed	0.21981	–	<7	⩾pT3
7	p.F133L, c.399C>G	6	Confirmed	0.06766	COSM95272 [5,11,20,21]	7	⩾pT3
8	p.W131G, c.391T>G	6	Confirmed	0.15194	COSM242641 [5,21]	7	⩽pT2
9	p.F133V, c.397T>G	6	Not confirmed	0.81737	COSM219965 [16]	>7	⩾pT3

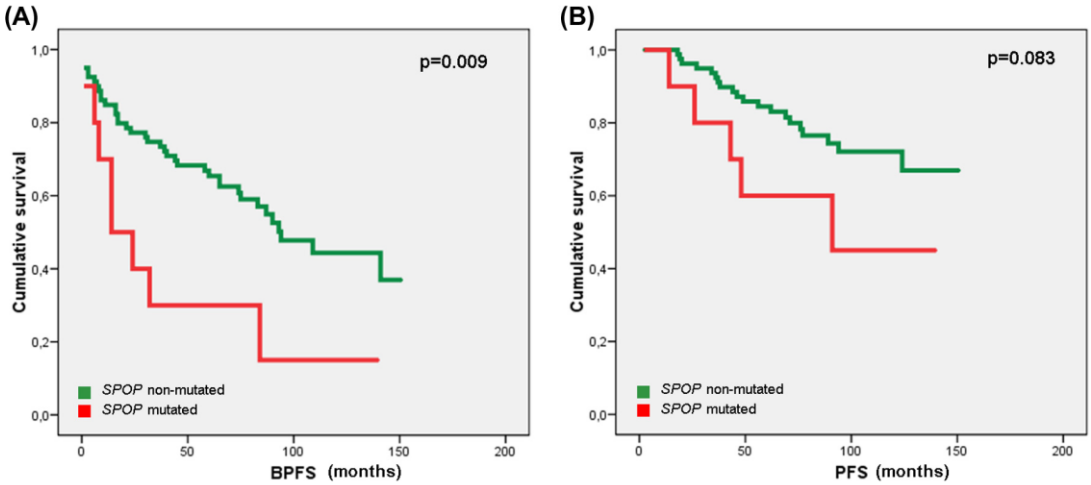

Fig. 3. Kaplan–Meier plots showing the prognostic impact of Speckle-type POZ protein (SPOP) mutations. (A) As can be appreciated, the presence of mutations in SPOP significantly differentiates two groups of patients with different prognosis behaviour. Prostate cancer (PCa) samples with mutations in SPOP experience a shorter time to progression for both biochemical progression free survival (BPFS) (B) and progression free survival (PFS).

prognostic information for both BPFS and PFS. Interestingly, a small group of patients from our series (17 cases) had a different behaviour since they showed an up-regulation of *SPOP* gene (RQ > 1) when compared with normal prostate leading to a less aggressive pattern characterised by a lower Gleason score and risk of progression. In this sense, earlier studies have already demonstrated the interaction of *SPOP* with critical oncogenes, such as SRC-3 [13] mediating its ubiquitination. Hence, *SPOP* mutations or loss of expression would lead to an accumulation of SRC-3 and possibly other oncogenes. These data, together with our latest results, confirm the potential role of *SPOP* as tumour suppressor in PCa.

T2E has already been described to define a subgroup of PCa tumours with different clinico-pathological characteristics and prognostic behaviour [14]. Moreover, certain lesions have been found to be mutually exclusive with the presence or absence of ETS rearrangements [15]. *SPOP* mutants, for instance, have only been found in those T2E-negative tumours [5,16]. According to these findings, the status of T2E has also been taken into account for our prognosis analyses finding a strong association between *SPOP* expression and prognosis in the group of patients not harbouring the translocation. In our series, the mutational analysis has been performed in this subgroup of patients finding a mutation rate of 10%, which is in line with previous studies [5,17]. Some of these mutations have been previously described in other works [5,17–21], however this is the first time that mutations p.F104V, p.D153N and p.Q120Stop were reported. Furthermore, the association between *SPOP* mutations and expression was studied and despite no statistical significance was found all the mutated cases showed a down-regulation of *SPOP*. Hence, other mechanisms leading to SPOP down-regulation should exist. Genomic loss of SPOP gene locus in chromosome 17q21 has already been described and could explain part of this loss of expression [7]. Moreover, other alterations such as miRNAs post-transcriptional modulation could be leading to this down-regulation; miR-145 has recently been described to regulate *SPOP* expression [22].

Association between *SPOP* mutations, clinico-pathological characteristics and patient outcome has only been evaluated in a multi-institutional study where no

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significant association was found [17]. Nevertheless, our study shows for the first time that the presence of mutations in *SPOP* leads to a worse prognosis; hence, selecting patients with time to biochemical progression lower (median time 19 months) compared with those with no *SPOP* mutations (median time 69 months). Two main reasons could explain this discrepancy with the former study. First, there is a difference regarding patient cohorts. Although the sample size in the Blattner's study is larger than ours, it represents demographically different cohorts making it highly heterogeneous. On the contrary, our series of patients have been treated and monitored at the same institution. Second, the proportion of low Gleason score PCa (score <7) in our study is significantly higher than in Blattner's study (40% versus 16% respectively) indicating that the high proportion of high Gleason score PCa in Blattner's study could mask the prognostic effect of *SPOP* mutations. This fact becomes important when comparing patients with or without *SPOP* mutations since the non-mutated ones harbour a median time to biochemical progression significantly much larger (69 months versus 19 months of mutated), representing those patients with a low risk PCa and a lower Gleason score. In fact, the median time to biochemical progression for *SPOP* mutated cases in our series is similar to that reported in Blattner's study (15 months).

Our finding of a significant association between *SPOP* mutations and clinical outcome opens the possibility of using this genetic characteristic as subrogate marker of poor prognosis that could be used in different clinical contexts. These detected mutations would select patients with a known worse prognosis that could be candidates for radical treatments such as surgery or radiotherapy. In addition, once the mutation is characterised, a high sensitive genetic test could be designed (qRT-PCR, digital PCR) in order to detect these alterations in body fluids such as urine or plasma that can be useful for monitoring patient outcome.

5. Conclusion

In conclusion, molecular alterations in *SPOP* gene are defining a new subtype in PCa apparently exclusive with ETS rearrangements. Besides, in the context of prognosis the study of *SPOP* mutations or expression allows the identification of patients at different risk of progression and subjected to different therapeutic options.

Conflict of interest statement

None declared.

Acknowledgements

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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.ejca.2014.08.009.

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Study V. ERG deregulation induces IGF-IR expression in prostate cancer cells and affects sensitivity to anti-IGF-IR agents

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Valencia, April 2015

To whom it may concern,

I certify that Irene Casanova Salas has contributed to the publication entitled "**ERG deregulation induces IGF-IR expression in prostate cancer cells and affects sensitivity to anti-IGF-IR agents**". In this work we have assessed the association between the fusion gene *TMPRSS2-ERG (T2E)* and the receptor IGF-I (IGF-IR) in prostate cancer (PCa) both *in vitro* and in patient samples. We have evaluated also how this relationship affects to the sensitivity to IGF-1R inhibitory agents.

This publication has been the result of a collaborative project between our institution and the Experimental and Oncology Laboratory, headed by Dr. Katia Scotlandi, at the Rizzoli Institute in Bologna (Italy). Hence, the reason why ICS is not first authoring this publication is due to the fact that the main part of this work has been performed in Dr. Scotlandi's laboratory. Nevertheless, ICS has been implicated in both cell lines and patient studies. In fact, as a result of her findings we have established a new collaboration with Dra. María Jesús Vicent head of the Laboratory of Therapeutic Polymers of the Centro de Investigación Príncipe Felipe (Valencia, Spain) with whom we are developing a new polymer conjugate with therapeutic potential to target the AR-T2E-IGF-IR axis in prostate cancer .

Jose Antonio López-Guerrero, PhD
Head of Laboratory

ERG deregulation induces IGF-1R expression in prostate cancer cells and affects sensitivity to anti-IGF-1R agents

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ABSTRACT

Identifying patients who may benefit from targeted therapy is an urgent clinical issue in prostate cancer (PCa). We investigated the molecular relationship between *TMPRSS2-ERG* (T2E) fusion gene and insulin-like growth factor receptor (IGF-1R) to optimize the use of IGF-1R inhibitors.

IGF-1R was analyzed in cell lines and in radical prostatectomy specimens in relation to T2E status. ERG binding to *IGF-1R* promoter was evaluated by chromatin immunoprecipitation (ChIP). Sensitivity to anti-IGF-1R agents was evaluated alone or in combination with anti-androgen abiraterone acetate *in vitro* at basal levels or upon ERG modulation.

IGF-1R analysis performed in PCa cells or clinical samples showed that T2E expression correlated with higher IGF-1R expression at mRNA and protein levels. Genetic modulation of ERG directly affected IGF-1R protein levels *in vitro*. ChIP analysis showed that ERG binds *IGF-1R* promoter and that promoter occupancy is higher in T2E-positive cells. IGF-1R inhibition was more effective in cell lines expressing the fusion gene and combination of IGF-1R inhibitors with abiraterone acetate produced synergistic effects in T2E-expressing cells.

Here, we provide the rationale for use of T2E fusion gene to select PCa patients for anti-IGF-1R treatments. The combination of anti-IGF-1R-HAbs with an anti-androgen therapy is strongly advocated for patients expressing T2E.

INTRODUCTION

Chromosomal translocations are genetic lesions that are produced by illegitimate recombination events between two non-homologous chromosomes or within the same chromosome and that result in chimeric genes [1]. Although fusion genes have been considered exclusive mutations of lymphomas, leukemias and sarcomas, several tumor-specific rearrangements have been recently identified in carcinomas. In particular, in 2005, a chromosomal rearrangement leading to the fusion of the androgen-regulated gene *TMPRSS2* and one of the *ETS* genes, predominantly *ERG*, was described as being expressed in 40–70% of prostate cancers (PCas) from a radical prostatectomy series [2]. PCa is one of the most commonly diagnosed cancers in adult men, accounting for 10% of cancer deaths in Europe [3]. PCa progression is accompanied by genetic mutations, including *TMPRSS2-ERG* (T2E) rearrangement, which is considered an early event because it is found in localized disease more frequently than in high-grade prostatic intraepithelial neoplasia (PIN) [4].

Because *TMPRSS2* contributes only untranslated sequences, the fusion gene results in the overproduction of a truncated ERG protein (tERG) [2, 5]. ERG shares with other ETS transcription factors the same DNA-binding domain that recognizes the 5′-GGAA/T-3′ motif. ETS proteins are considered proto-oncogenes because they control the expression of target genes involved in cell proliferation, apoptosis and invasion [6]. Studies exploring the functional significance of truncated ERG protein are controversial but suggest that ETS activation promotes epithelial-mesenchymal transition (EMT) and invasiveness [5, 7, 8]. Nevertheless, T2E has been reported as insufficient to induce a transformed phenotype but instead to cooperate with other mutations [9]. We analyzed the impact of T2E on the insulin-like growth factor (IGF) system. The IGF system is composed of three receptors [insulin receptor (IR), IGF-1 receptor (IGF-1R) and mannose 6-phosphate receptor (M6P/IGF-2R)], three ligands (insulin, IGF-1, IGF-2), and six known types of circulating IGF-binding proteins (IGFBP1–6) that modulate the bioavailability and bioactivity of the IGFs [10, 11]. The role of the IGF system and particularly IGF-1R in human cancer has been widely documented [11]. In the prostate, IGF-1R plays a critical role in normal gland growth and development, as well as in cancer initiation and progression [12]. Epidemiologic studies have associated circulating IGF-1 levels with risk of developing disease [13–15]. However, numerous experimental and clinical studies have produced controversial evidence, suggesting a need for further studies. Indeed, although the intensity of IGF-1R immunostaining has generally been reported to increase from benign prostatic hyperplasia (BPH) to PIN to carcinoma [16], several studies have not confirmed this linear relationship and have reported that reduced IGF-1R is associated with hyperplasia and proliferation or metastatic lesions [17, 18]. Despite this variation may be due to technical factors, clinical studies evaluating the prognostic role of IGF-1R expression have also provided controversial results, reporting either positive or negative associations between receptor expression levels and patient outcome [19, 20]. In addition, phase II studies using IGF-1R inhibitors have failed to demonstrate efficacy in castration-resistant PCa (CRPC) patients [21, 22], putatively due to incomplete pathway blockade, onset of resistance mechanisms or lack of a suitable patients selection. A better understanding of the molecular determinants of aberrant IGF-1R expression in prostate tumors is thus required to define subgroups of patients who may benefit from anti-IGF-1R therapies. In this study, we demonstrated that T2E directly binds the *IGF-1R* gene promoter, thus affecting its expression and treatment sensitivity in PCa.

RESULTS

tERG directly binds to the *IGF-1R* promoter in prostate cells and modulates IGF-1R expression

A panel of five prostate cancer cell lines, VCaP, DU-145, PC-3, LNCaP and 22RV1, characterized by different expression levels of the androgen receptor (AR) and T2E gene fusion, and non-malignant RWPE-1 prostate cells (Supplementary Figure S1) was analyzed for the expression of different components of the IGF system. No IGF-1 or IGF-2 expression was found in the cell lines (data not shown), confirming the paracrine activation of the pathway in this tumor. IR expression is generally higher in PCa cell lines with respect to normal cells (Figure 1). This difference is particularly evident at the protein level and does not appear to reflect a regulation at the transcriptional level. In contrast, IGF-1R expression is generally low in malignant cells, with the only notable exception of VCaP cells, which express the T2E fusion gene. These data were confirmed at the mRNA and protein levels, thus supporting regulation at the transcriptional level for IGF-1R expression (Figure 1).

To better understand the role of tERG in IGF-1R modulation, IGF-1R protein levels were analyzed after ERG siRNA transfection in VCaP cells. A decrease in IGF-1R was evident 96 h and 120 h after silencing. Conversely, IGF-1R protein expression was increased both in the non malignant RWPE-1 and malignant PC-3 cells stably transfected for tERG overexpression (RWPE-1_tERG and PC-3_tERG, respectively; Figure 2a), confirming the correlation between IGF-1R and the fusion gene. Moreover, an anti-ERG chromatin immunoprecipitation (ChIP) assay was performed in VCaP and parental PC-3 cells, which express ERG at high or low levels, respectively, as well as in RWPE-1_tERG cells. ChIP analysis indicated that ERG binds the *IGF-1R* gene promoter, and the amount of binding was higher in cells with tERG expression (Figure 2b). No consensus sequences were present in the promoter of *IR* (data not shown). Because the T2E fusion gene is regulated by androgens, the naturally expressing T2E VCaP cells were treated with abiraterone acetate, and IGF-1R protein levels were investigated upon stimulation. Abiraterone acetate is a second-generation anti-androgen drug that blocks the synthesis of androgens through the inhibition of 17 α-hydroxylase/C17, 20 lyase (CYP17A1). VCaP cells were treated for 72, 96 and 120 h with two concentrations of abiraterone acetate, and western blotting analysis showed that together with a strong ERG down-regulation, IGF-1R levels decreased upon 10 μM treatment in VCaP cells (Figure 2c).

tERG overexpression increases sensitivity to anti-IGF-1R agents

PCa cell lines were exposed to increasing concentrations of CP-751,871 or AVE1642, two anti-IGF-1R-HAbs, as well as NVP-AEW541, a selective IGF-1R tyrosine kinase inhibitor (TKI) [23–26]. As shown in Figure 3a, only VCaP cells showed remarkably high sensitivity to all anti-IGF-1R agents compared with the other PCa cell lines. Accordingly, PC-3_tERG cells showed increased sensitivity to CP-751,871 treatment compared to empty vector transfected cells. To address the role of the T2E/IGF-1R axis in influencing sensitivity to

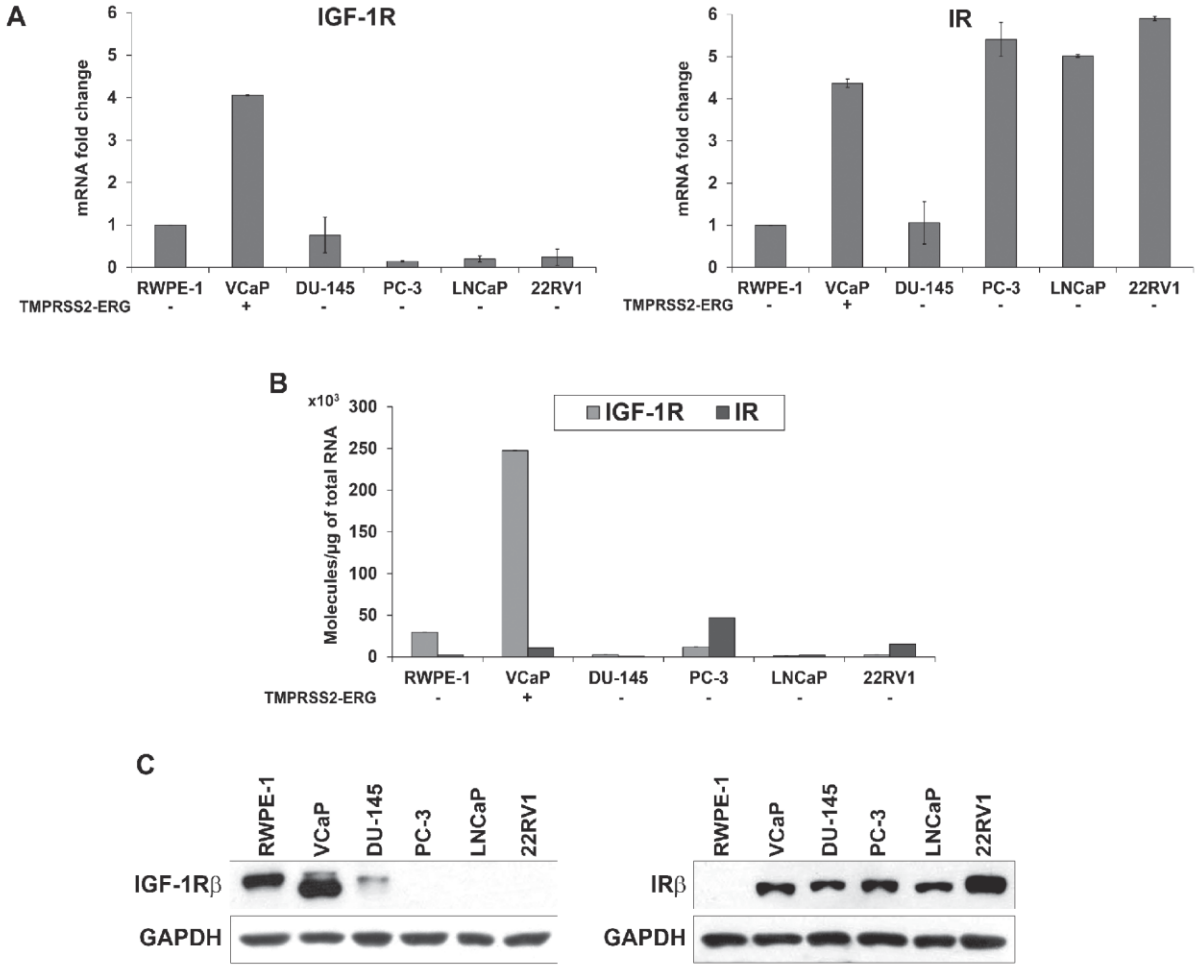

Figure 1: Evaluation of IGF-1R and IR basal expression in prostate cell lines. **(A)** Relative mRNA expression levels of *IGF-1R* and *IR* in prostate cancer cell lines. The RWPE-1 cell line was used as a calibrator ($2^{-\Delta\Delta Ct} = 1$). The columns represent the mean values of two independent experiments, and the bars represent the SE. **(B)** Absolute *IGF-1R* and *IR* mRNA quantification was assessed in the panel of cells. **(C)** Protein expression levels of receptors in prostate cells. The blots are representative of two independent experiments.

IGF-1R inhibitors, VCaP cells as well as PC-3_tERG cell line were deprived of ERG. The level of ERG expression significantly influenced the efficacy of anti-IGF-1R agents because its silencing cells reverted cell sensitivity toward CP-751,871 or NVP-AEW541 (Figure 3b). Notably, in prostate cancer, several clinical trials have investigated the effects of IGF-1R inhibitors in combination with other drugs, such as mitoxantrone (NCT00683475) or docetaxel [27]. Thus, because *TMPRSS2-ERG* expression is driven by androgens, we first investigated the response to abiraterone acetate in VCaP cells upon ERG silencing and observed that these genetically modified cells showed a significant decrease in sensitivity to abiraterone stimulation (Figure 4a). Interestingly, the simultaneous administration of anti-IGF-1R CP-751,871 HAbs and abiraterone but not cabazitaxel, a microtubule inhibitor recently introduced in PCa treatment, induced synergistic antiproliferative effects in VCaP cells (Figure 4b). Conversely, combined treatment of CP-751,871 and abiraterone gave subadditive effects in T2E-null DU-145 and LNCaP cell lines (CI = 2.88 ± 1.17 and CI > 100, respectively).

IGF-1R levels are associated with T2E expression in clinical samples

To confirm the clinical relevance of our experimental observations, we examined the gene expression levels of *IGF-1R* by qRT-PCR in a retrospective cohort of 270 primary prostate tumors (Figure 5a). Fisher's test revealed an association between *IGF-1R* and T2E expression in clinical samples ($p = 0.008$). In particular, patients harboring the fusion gene showed higher *IGF-1R* mRNA levels, in keeping with the increased binding of ERG to the *IGF-1R* promoter. This association was confirmed at the protein level. We analyzed the protein expression of ERG and IGF-1R in the same series of patients (Table 1). IGF-1R and ERG expression at the mRNA and protein levels (evaluation scores are reported in the Materials and Methods) were

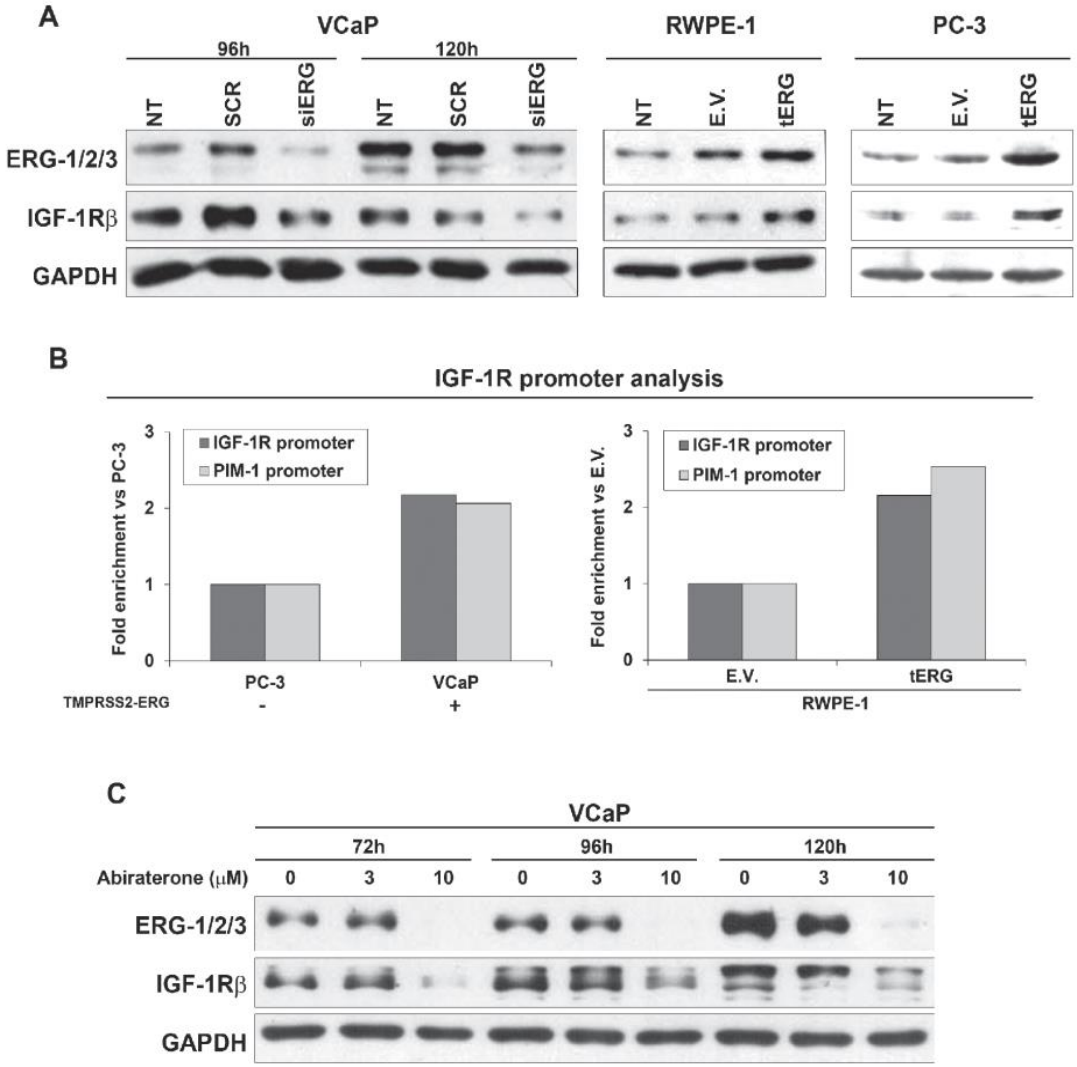

Figure 2: tERG-dependent IGF-1R induction in prostate cancer cells. (A) siRNA knockdown of ERG (siERG) in VCaP induces a decrease in IGF-1R levels compared with non-treated control (NT) or non-targeting siRNA (SCR) controls, whereas IGF-1R is over-expressed in RWPE-1 and PC-3 cells transfected with tERG compared with empty vector-transfected cells. The blots are representative of two independent experiments. GAPDH is shown as a loading control. **(B)** A ChIP assay was performed on VCaP and PC-3 prostate cancer cells, as well as on tERG- or empty vector-transfected RWPE-1 cells. ERG was precipitated with an anti-ERG-1/2/3 antibody. The results were obtained by quantitative RT-PCR. The data represent the recovery of each DNA fragment relative to the total input DNA. **(C)** Abiraterone acetate treatment induces down-regulation of ERG in VCaP cells and, consequently, down-regulation of IGF-1R. Cells were treated with abiraterone (3 and 10 μM) for the indicated time points. Representative blots are shown. GAPDH was used for normalization.

significantly correlated ($p = 0.047$ and $p < 0.0001$; Fisher's test, respectively). As observed at the mRNA level, IGF-1R protein expression was also found to be significantly associated with ERG expression ($p < 0.0001$; Fisher's test), further verifying the association between IGF-1R and T2E (Figure 5b).

DISCUSSION

The T2E fusion gene constitutes a critical event in development of PCa [2, 28], but other genetic alterations, such as loss of PTEN and PI3K pathway activation, are also required to induce malignant transformation [8]. Here, we provide evidence that IGF-1R is a target of tERG from T2E translocation and that this interaction has important implications in the field of personalized treatment through biomarker-driven patient selection. ChIP analysis showed ERG binding to the *IGF-1R* gene promoter, suggesting a direct transcriptional regulation of *IGF-1R* by ERG. Furthermore, we found greater ERG recruitment to the *IGF-1R* promoter in VCaP cells compared with PC-3 cells expressing low ERG levels, as well as in RWPE-1

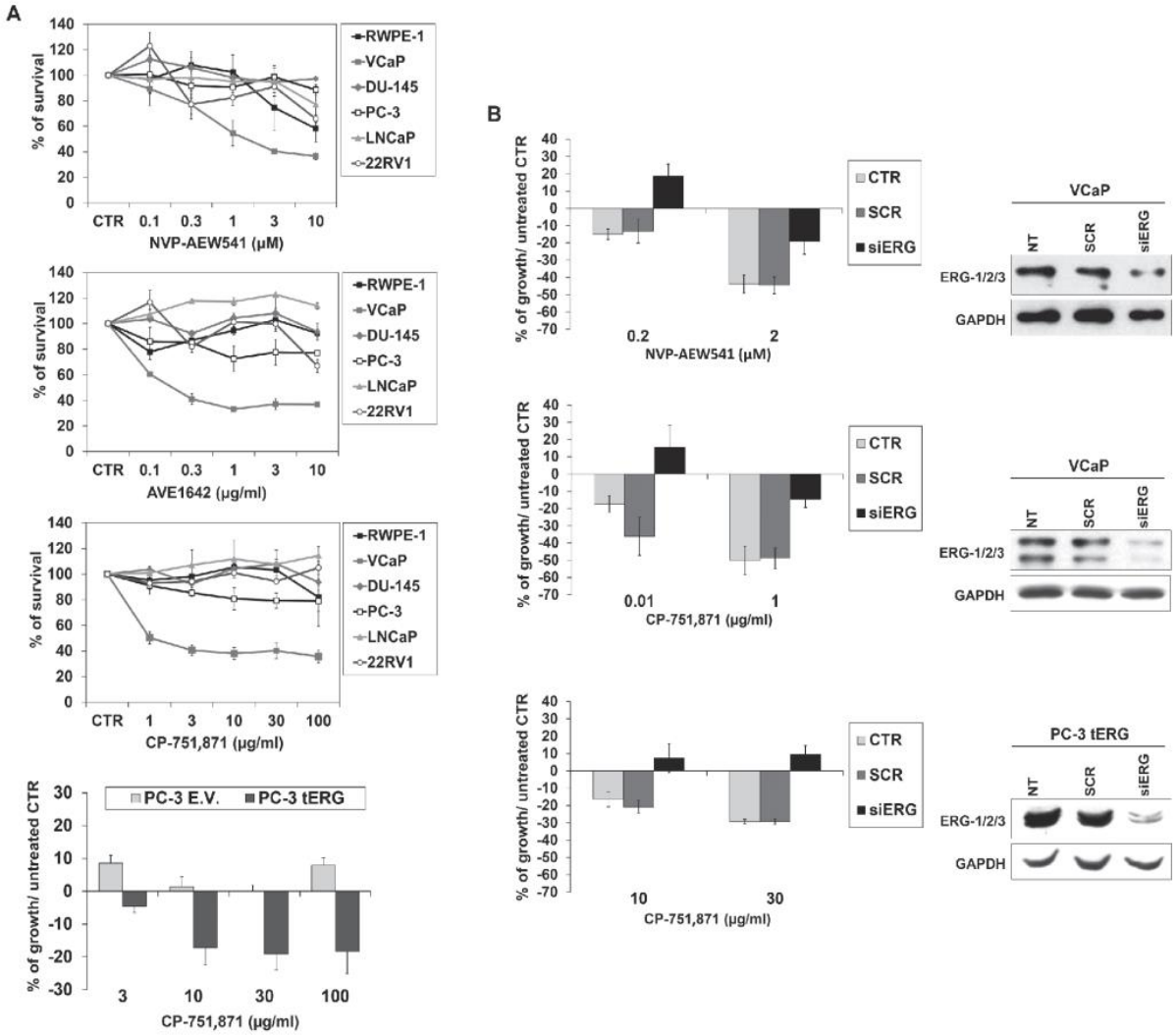

Figure 3: Efficacy of anti-IGF-1R agents in prostate cancer cells. **(A)** Cell growth was assessed using an MTT assay after a 72-h exposure to CP-751,871 or AVE1642, two anti-IGF-1R-HAbs, and NVP-AEW541, an anti-IGF-1R tyrosine kinase inhibitor (TKI) in prostate cell lines. PC-3 cells transfected with tERG or PC-3 empty vector-transfected cells were treated with indicated doses of CP-751,871 for 72 h. The results are displayed as the percentage of survival relative to controls. Points, mean of two independent experiments; bars, SE. **(B)** Reversion of sensitivity to anti-IGF-1R therapies by ERG knockdown. ERG silencing was achieved in VCaP or PC-3_tERG cells after a 48 h transfection of siERG (100 nM) or scrambled control siRNA (100 nM); GAPDH was used as a loading control. The transfected cells were treated as described in the Materials and Methods. Cell survival is shown as the percentage of growth respect to untreated control. The data represent the mean values of two independent experiments, and the bars represent the SE.

cells that over-expressed tERG compared with the empty vector. As a proof of concept, androgen deprivation induced by abiraterone acetate treatment in the androgen-responsive VCaP cells caused a significant decrease in ERG expression, as previously reported [29], but also a consequent inhibition of IGF-1R, confirming the presence of a T2E/IGF-1R androgen-regulated axis. Considering that RWPE-1 represents a model of non-tumorigenic immortalized cells but that VCaP cells are representative of advanced disease, the data indicate that the T2E/IGF-1R axis may represent a constant mechanism along different stages of pathology with putatively different consequences depending on pathological stage. The relationship between T2E and IGF-1R was also confirmed in radical prostatectomy specimens; patients expressing the fusion gene exhibited higher IGF-1R expression.

The *IGF-1R* gene has been identified as a molecular target for a number of stimulatory transcription factors and inhibitory proteins with important implications in cancer [30]. Aberrant fusion products, such as EWS-WT1 or EWS-FLI, the genetic hallmarks of desmoplastic small round cell tumor or Ewing sarcoma, were found to act as transactivators for the IGF-1R system, providing a selective growth advantage to tumor cells [31, 32].

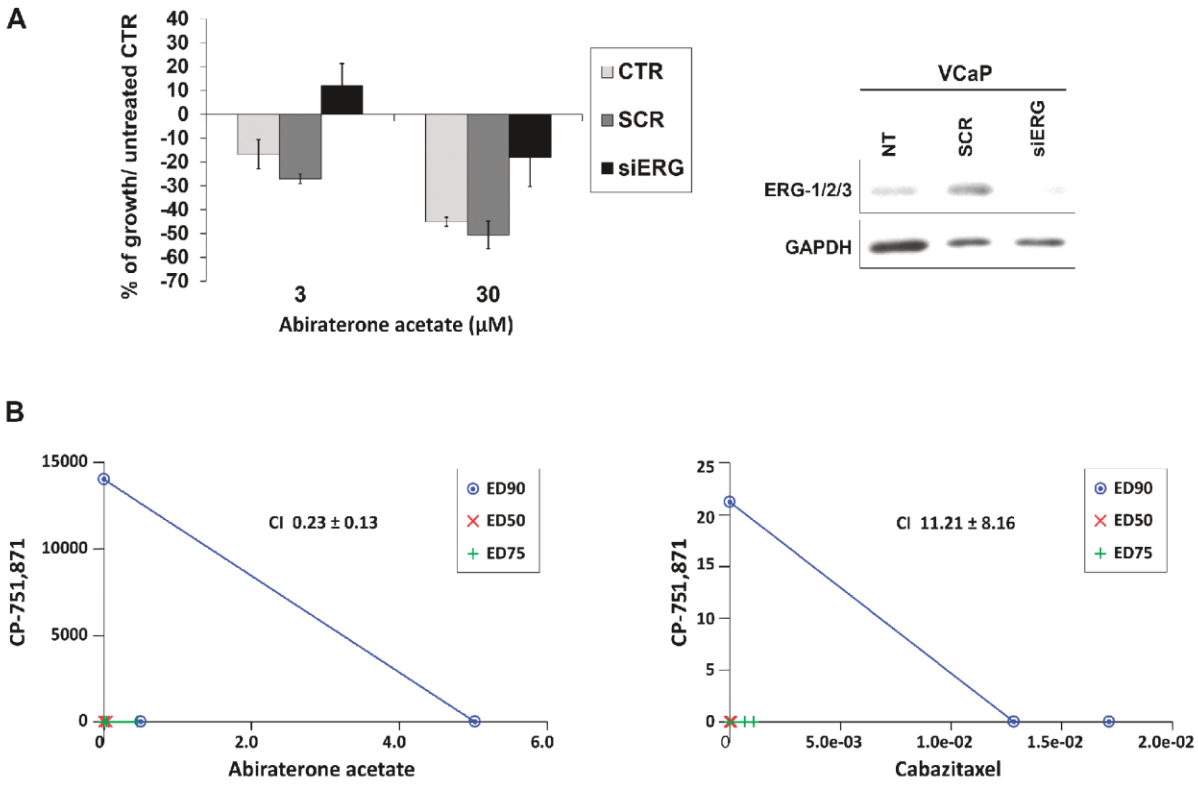

Figure 4: The combination of an IGF-1R inhibitor with an anti-androgen drug results in synergistic effects in TMPRSS2-ERG-positive cells. (A) ERG was silenced in VCaP cells with siERG (100 nM) or scrambled control siRNA (100 nM); GAPDH was used as a loading control. Cells were treated with abiraterone acetate for 72 h at the indicated doses, and the survival percentage with respect to untreated control is shown. The data represent the mean values of two independent experiments, and the bars represent SE. **(B)** The effects of simultaneous combined treatments of CP-751,871 in association with abiraterone acetate or cabazitaxel. Individual doses of CP-751,871, abiraterone acetate or cabazitaxel to achieve 90% growth inhibition (blue line; ED90), 75% growth inhibition (green line; ED75) and 50% (red line; ED50) growth inhibition are plotted on the x- and y- axes. CI values are represented by the points above on (indicating synergy), or below (indicating antagonism) the lines. The CI values representing ED90 are reported.

From a biological standpoint, the T2E/IGF-1R axis may be assumed to participate in establishing a biologically distinguished cellular context and promote a malignant cellular phenotype compared with cells that do not express T2E. From the clinical standpoint, this mechanism provides the rationale for the selective use of anti-IGF-1R agents for patients expressing the fusion gene. The contribution of IGF-1R to prostate carcinogenesis and progression remains controversial, but epidemiological, preclinical and clinical results indicate that IGF-1R overexpression plays an important role in the pathogenesis of CRPC [33]. This evidence in particular led to the enrollment of CRPC patients in several clinical trials investigating the effects of IGF-1R inhibitors. However, these clinical trials verified only very modest clinical benefits from IGF-1R inhibition [22, 27] and resulted in discontinuing the development of most of anti-IGF-1R agents. Here, we demonstrated that only PCa cells that express the translocation and therefore have higher IGF-1R expression displayed potentially interesting sensitivity to anti-IGF-1R agents. Accordingly, ERG silencing caused a decrease in treatment sensitivity, thus supporting the idea that only patients with PCa presenting with T2E may benefit from anti-IGF-1R therapy. This idea is in line with previous evidence demonstrating how PARP1 inhibitors blocked ETS-positive but not ETS-negative prostate cancer xenograft growth [34]. In addition, consistent with the observation that T2E-positive CRPC tumors display a better response to anti-androgen treatment compared with T2E-negative tumors [35], we found that sensitivity to abiraterone acetate significantly decreased upon ERG silencing. Abiraterone acetate is a selective small molecule inhibitor of CYP17, an enzyme that catalyzes generation of androgens and estrogens. In the clinic, the onset of androgen receptor-linked resistance mechanisms in CRPC patients treated with abiraterone is an important limitation, and the identification of a druggable target involved in the androgen receptor pathway may be an interesting opportunity to overcome resistance [36]. Combined therapies with abiraterone and targeted agents, such as Src inhibitors [37] or PI3K pathway inhibitors [38], have been proposed. Our results provide evidence for the first time

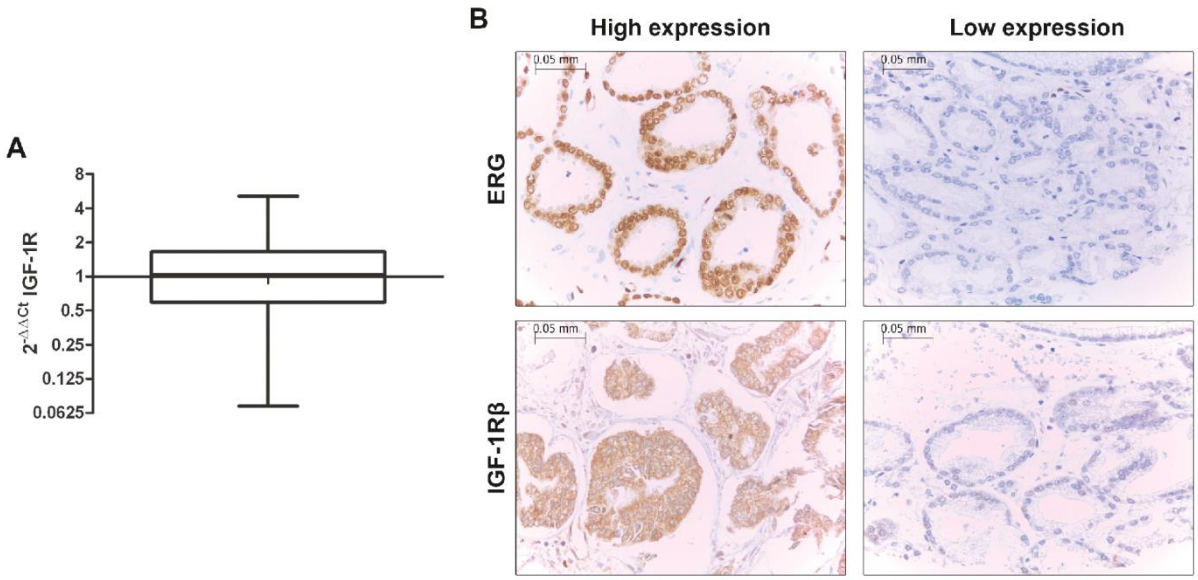

Figure 5: The fusion gene is directly correlated with IGF-1R in prostate cancer patients. (A) *IGF-1R* expression profile in 270 FFPE primary prostate cancer samples analyzed by qRT-PCR. Normal tissues were used as calibrator. *IGF-1R* was not differentially expressed with respect to normal tissue (median = 1.04; range = 0.07–5.12). **(B)** Representative expression of ERG (top) and IGF-1R (bottom) in prostate cancer tissue array samples by immunohistochemistry (magnification, x40). The cases were classified as 'high-expressors' when medium or high positivity was present and 'low-expressors' when no staining or low positivity was observed.

of a beneficial combination of abiraterone acetate and anti-IGF-1R agents. In VCaP cells, the association of anti-IGF-1R CP-751,871 HAb with abiraterone acetate produced synergistic effects, supporting the idea that the concurrent use of the two targeted agents deprive tERG-expressing cells of fundamental signaling pathways that operate in concert to sustain cell proliferation.

Overall, we suggest the application of T2E as a biomarker for patient selection in the field of personalized medicine. We demonstrated that IGF-1R is an important target of tERG and that this interaction leads to a higher IGF-1R expression in cell lines and patients. Thus, we observed a good response to IGF-1R inhibition in T2E-positive cells compared with T2E-negative cells. Considering that such a mechanism is driven by androgens, we provide the rationale for combining anti-IGF-1R agents to anti-androgen therapy in the subpopulation of patients expressing T2E.

MATERIALS AND METHODS

Cell lines

Prostate cancer cell lines PC-3, LNCaP, DU-145, VCaP were obtained from the American Type Culture Collection (ATCC). 22RV1 prostate cancer cell line was purchased from Sigma Aldrich. Immortalized non-malignant prostate cell line RWPE-1 and stable trasfectants RWPE-1_tERG or RWPE-1_empty vector were kindly provided by Dr. Gambacorti-Passerini, University of Milano-Bicocca [39]. PC-3, LNCaP and DU-145 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Lonza). RWPE-1 and transfectant cells were maintained in keratinocyte-serum free medium supplemented with epidermal growth factor and bovine pituitary extract (Life Technologies Inc.). 22RV1 cells were maintained in RPMI 1640 (Gibco) while VCaP cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) implemented with L-glucose and bicarbonate. IMDM, RPMI and DMEM media were supplemented with 10% inactivated Fetal Bovine Serum (FBS) (Lonza) and 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. All cell lines were tested for mycoplasma contamination every 3 months by MycoAlert mycoplasma detection kit (Lonza) and were recently authenticated by STR PCR analysis using genRESVR MPX-2 and genRESVR MPX-3 kits (Serac). The following locus were verified: D3S1358, D19S433, D2S1338, D22S1045, D16S539, D18S51, D1S1656, D10S1248, D2S441, TH01, VWA, D21S11, D8S1179, FGA, SE33.

Clinical prostate specimens

Formalin fixed and paraffin-embedded (FFPE) blocks corresponding to PCa patients were retrieved from the archives of the Biobank of the *Fundación Instituto Valenciano de Oncología* according to the following criteria: specimens obtained from radical retropubic prostatectomies from 1996 to 2002 and no history of

Table 1: Clinicopathologic features of the analyzed series

		qRT-PCR (n = 270)	IHC (n = 243)	
Parameter	**No. Pts**	**%**	**No. Pts**	**%**
Age				
≤ 55	15	5, 6	12	5
56–65	81	30	74	31
66–75	138	51, 1	124	52, 1
> 75	36	13, 3	28	11, 7
Gleason-sp:				
2–6	109	40, 4	87	36, 4
7	129	47, 8	123	51, 4
Greater than 7	32	11, 9	29	12, 1
PSA (ng/ml):				
10 or less	154	57	132	55, 6
10–20	74	27, 6	69	29, 1
Greater than 20	40	14, 9	36	15, 1
cT:				
cT2b or less	248	92, 2	219	92
cT3a or greater	21	7, 8	19	7, 9
pT:				
pT2 or less	135	50	115	48, 1
pT3 or greater	135	50	124	51, 8
pN*:				
pN0	236	95, 2	209	95, 4
pN1 or greater	12	4, 8	10	4, 5
Margins:				
Negative	137	50, 7	116	48, 5
Positive	133	49, 3	123	51, 4
TMPRSS2-ERG**				
Negative	92	34, 1	102	46, 5
Positive	178	65, 9	117	53, 4
IGF-1R***				
Low expressors	82	30, 4	55	24, 7
High expressors	188	69, 6	167	75, 2

SP, specimen; cT, clinical stage; PSA, prostatic specific antigen; pN, lymphnode pathological stage
*Lymphadenectomy was limited to the obturator fossa in most of the cases at the inclusion period
**IHC ERG expression was not detectable in 24/243 and negative in 85/219 cases (39%)
***IHC IGF-1R expression was not detectable in 21/243 cases and negative in 12/222 of the samples (5%).

previous treatment for PCa (including androgen deprivation therapy or chemotherapy prior to surgery). We identified 270 cases that met these criteria. All patients gave written informed consent for tissue donation for research purposes before tissue collection, and the study was approved by FIVO's Institutional Ethical Committee (ref. number. 2010–19). Clinical data were reviewed from clinical records and stored in a PCa-specific database. Patient characteristics, including the T2E fusion gene status, and demographics are shown in Table 1. Combined Gleason score was uniformly regarded by the same uro-pathologist (AC). For comparative and calibration purposes, we also analyzed 10 samples of normal prostate tissue obtained from patients operated of radical cystectomies without pathological evidence of prostatic disease. T2E gene fusion status was determined by RT-PCR and fluorescent *in situ* hybridization (FISH) as already described [40] and quantitative RT-PCR.

Gene expression analysis

Cell lines total RNA (2 mg) was extracted with TRIzol (Invitrogen) and purified by precipitation with isopropanol. Oligo dT primers (Applied Biosystems) were used to reverse transcribe RNA. Isolation of RNA from paraffin-embedded tissue was performed using RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion) following providers' specifications and reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's indications. For cell line analysis, Quantitative Real-Time PCR was performed on ABI Prism 7900 (Applied Biosystems) using TaqMan (*IGF-1R*) or SYBR Green assays (*IR*) (Applied Biosystems) as previously reported [26]. Primer Express software (Applied Biosystems) was used to design appropriate primer pairs for reference gene (glyceraldehyde-3-phosphate dehydrogenase) [26]. Clinical samples were analyzed using ABI 7500-Fast Thermocycler Sequence Detection System (Applied Biosystems), according to manufacturer's instructions. Predesigned TaqMan probes for target genes *IGF-1R* (Hs00181385_m1), T2E (Hs03063375_ft) as well as for endogenous control *β-2-microglobulin* (Hs99999907_m1) were used (Applied Biosystem). Two replicates per gene were considered. Relative quantification analysis was performed on ΔΔCt method [41]. cDNA from normal human prostate samples was used as calibrator for comparative analysis of PCa cases. Absolute quantification assay was performed for the measurement of total *IR* and *IGF-1R* [42].

Western blotting

Cell lysates were prepared and processed as previously described [43]. Membranes were incubated overnight with the following primary antibodies: anti-IGF-1Rβ, anti-IRβ, anti-GAPDH, anti-LAMIN B, anti-ERG-1/2/3 (Santa Cruz Biotechnology); anti-AR (Cell Signaling Technology); anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (GE Healthcare) were used as secondary antibodies.

Drugs

Anti-IGF-1R drugs were kindly provided by: ImmunoGen Inc. (AVE1642, a humanized version of anti-IGF-1R EM164 antibody), Pfizer (CP-751,871/ Figitumumab), and Novartis (NVP-AEW541). Abiraterone acetate (S1123) and Cabazitaxel (S3022) were purchased by Selleckchem.

In vitro assays

To assess drug sensitivity, MTT assay (Roche) was used according to manufacturer's instructions. Cells were plated into 96 well-plates (10, 000 cells/well). After 24 hours, various concentrations of AVE1642 (0.01–50 μg/ml), NVP-AEW541(0.03–5 μM), Figitumumab (0.5–500 μg/ml) were added and cells exposed to these drugs for up to 72 hours. phCMV2_HA_tERG plasmid containing the cDNA of the translated sequence of *TMPRSS2-ERG* (isoform 9) and phCMV2 empty vector were kindly provided by Dr. Gambacorti-Passerini, University of Milano-Bicocca [39]. PC-3 cell line was stably transfected with Calcium Phosphate Transfection Kit (Invitrogen) accordingly to manufacturer's instruction and selected for geneticin (Sigma) resistance at 0.75 mg/ml. PC-3_tERG and PC-3 empty vector transfected cells were treated with CP-751,871 (3, 10, 30, 100 μg/ml) for up to 72 hours and sensitivity was assessed with Trypan Blue cell count. Short interfering RNA knockdown of ERG was performed with siRNA from Thermo Scientific Dharmacon: siGENOME_ siRNA (D-003886–01) as reported in Tomlins et al. [5] and Magistroni et al. [39]. siGENOME_non targeting_ siRNA was used as control (D-001210–01-05). siRNA was transfected in VCaP or PC-3_tERG cells using siport NeoFX transfection agent (Life Technologies Inc.) according to manufacturer's instructions. Silencing was assessed after 48, 72, 96 and 120 hours from transfection. VCaP cells were pre-treated with ERG siRNA (100 nM) for 48 hours and then exposed to CP-751,871 (0.01–1 μg/ml), NVP-AEW541 (0.2–2 μM) or Abiraterone (3–30 μM) for 72 hours. PC-3_tERG cells were pre-treated with ERG siRNA (100 nM) for 48 hours and then exposed to CP-751,871 (10–30 μg/ml). ERG and IGF-1R protein expression was investigated upon 72, 96 and 120 hours of Abiraterone treatment (3–10 μM). For combined treatments, LNCaP, DU-145 and VCaP cells were treated for 72 hours with varying concentrations of CP-751,871 (1–100 μM) and Abiraterone (1–100 μM) or Cabazitaxel (0.003–0.3 μM).

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as previously described [43, 44] using anti-ERG-1/2/3 antibody (C-17, Santa Cruz Biotechnology). *IGF-1R* promoter was evaluated by

Real-Time PCR using the following custom TaqMan assay: forward 5′-AGGAGGAGGAGGAGG AGGAG-3′, reverse 5′-GCAGTTCGCAAGATCGCC-3′ and probe 5′-TTGACTCCGCGTTTCTGCCCCTCG-3′. For the TaqMan assay design TFSEARCH - Searching Transcription Factor Binding Sites, version 1.3 free website was used for the prediction of ETS binding sites in the promoter of *IGF-1R* gene and the sequence spanning from 1041bp to 1051bp was identified as the best. Beacon Designer 4 software was used for the design of the assay spanning from 1005bp to 1114bp. PIM-1 promoter fragment containing ETS consensus sequence was used as immunoprecipitation positive control [39] by Real-Time PCR using the following SYBR Green assay: forward 5′-GTGCTAGGCGAGTGGGAACAACTG-3′ and reverse 5′-AATGACCCAAATTCACCTCCTGAG-3′. Quantification analysis was calculated with the following formula: % of recruitment = $2^{\Delta Ct}$ x input chromatin percentage where ΔCt = Ct (INPUT) - Ct (IP:ERG) [45].

Immunohistochemistry

PCa specimens were incorporated in 11 tissue microarrays (TMA). Two or three representative areas (1 mm in diameter) of each tumor were selected for TMA production by first examining hematoxylin and eosin-stained prostatectomy tumor slides and then sampling tissue from the corresponding paraffin blocks. A tissue microarray instrument (Beecher Instruments) was used for TMA assembly. From TMA blocks, 3-μm-thick sections were immunostained using rabbit anti-human IGF-1Rβ (Santa Cruz Biotechnology) or anti-human ERG clone EP111 polyclonal-Ab (Dako). Percentage of IGF-1R-positive cells and cytoplasmic staining intensity were scored semiquantitatively, forming four groups (from 0 to 3). Cases were scored as low expression when staining intensity was between 0 and 1, and high expression when intensity was 2 and 3.

Statistical analysis

Differences among means where analyzed using two-sided Student's *t* test. To define drug-drug interactions combination index (CI) was calculated with the isobologram equation [46] using CalcuSyn software (Biosoft). Correlations analysis was performed using Fisher's exact test.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed by the authors.

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Summary of results and discussion

In this section the summary of the results will be exposed according to the established objectives highlighting those aspects with a translational potential into the clinical setting.

Objective 1: To identify miRNAs that could be used as potential biomarkers for PCa diagnosis and prognosis using a discovery approach based on miRNA microarray analysis (Studies I and II).

The current tools for PCa diagnosis (PSA and DRE) are limited by a low predictive value and a low rate of specificity leading to disease overdiagnosis and a high probability of mistaken results. In this context, the identification of new biomarkers for PCa diagnosis is a constant need. Besides of improving diagnosis accuracy it is also imperative to have biomarkers able to distinguish between indolent and aggressive disease. In this line, miRNAs have emerged as a new source of biomarkers since they have been found to be de-regulated in different types of tumors including PCa [77]. Several studies have already described that the aberrant expression of miRNAs is related with the development of PCa and they have also been found to be correlated with disease stage and prognosis (*Table 1. Study I*). For instance, miR-125b plays an important role in CRPC since its expression is directly regulated by androgen signaling (*Figure 2. Study I*), which consequently will modulate the expression of its targets implicated in the apoptotic pathway. Other miRNAs have been described to be associated with advanced tumor stages. For instance, up-regulation of miR-141 and miR-375 seems to be a common event in the progression of PCa to metastatic disease [76, 87]. Despite the increasing number of miRNA profiling studies there is still no agreement in which will be the miRNA specific signature for PCa. Hence, considering the important role of miRNA in the biology and progression of PCa we designed a strategy to identify miRNAs that could distinguish between cancer and healthy patients and could be also useful in both diagnostic and prognostic settings.

With this aim we performed a miRNA microarray approach to identify miRNAs differentially expressed between normal and PCa tissue (GEO (Gene Expression Omnibus) database Accession No. GSE45604. http://www.ncbi.nlm.nih.gov/geo/). A total of 11 smallRNAs (sRNAs) were found to be differentially expressed (Bonferroni test $p<0.05$) between PCa and normal tissue. From these sRNAs 5 miRNAs were

significantly down-regulated in PCa (miR-187, miR-224, miR-34a*, miR-221 and miR-34c) while there was just one miRNA found over-expressed (miR-182) (*Figure 1. Study II*). Our results were also consistent with previous works where miR-187, miR-224, miR-34 and miR-221 under expression was already described [139]. According to our data the up-regulation of miR-182 was also previously reported in PCa and other tumors [140, 141]. To confirm our findings miR-182 and miR-187, that were the most significantly regulated miRNAs in our series with a FC of +4.7 and -12 respectively, were selected for further validation. The differential expression of both miRNAs was further demonstrated by RT-qPCR in a training set of samples comprising 50 prostate tumors and 10 normal fresh tissues as well as in a retrospective cohort of 273 primary tumors with more than 5 years of follow-up. We also assessed the relationship between the expression of these 2 miRNAs and different clinico-pathological parameters such as pT, cT, Gleason score and *T2E* status as well as patient outcome (BPFS and PFS). Interestingly, we found for the first time an inverse association between miR-187 expression, pT (p=0.0002) and Gleason score (p=0.003) (*Figure 4S. Study II*). Moreover, to date the only association between *T2E* status and miRNAs was found by Gornadpour et al. who found how miR-221 loss was associated with the presence of the translocation [88]. However, we were also able to find a significant inverse correlation between miR-187 and *T2E* (p=0.003) (*Figure 4S. Study II*). Despite previous studies have found significant association between miR-182 and different clinico-pathological parameters [142] we could just found a significant association between miR-182 and Gleason score in our microarray data but not in the validation cohort of samples. However a robust and independent correlation between miR-182 expression and PCa prognosis for both BPFS (p=0.02) and PFS (p=0.04) was found (*Table 2. Study II*). The higher expression of miR-182 was significantly associated with a higher risk of biochemical recurrence and distant progression. This association of miR-182 with prognosis was also described in other tumors such as glioma and colorectal cancer [140, 141]. Considering that miR-182 was associated with Gleason score in our microarray data we proposed a new variable combining miR-182 expression (taking median values as cut-off) and Gleason score and we found that this combined variable was able to discriminate between groups at different risk of progression inside each

Gleason score category (p<0.0001) (**Figure 17**). Hence, in the group of patients with a Gleason score of 7 we could distinguish 2 populations with different risk to progress depending on miR-182 expression levels and could suggest the definition of a new risk category to better distinguish patients who are suitable candidates for active surveillance from those who will need a more radical treatment.

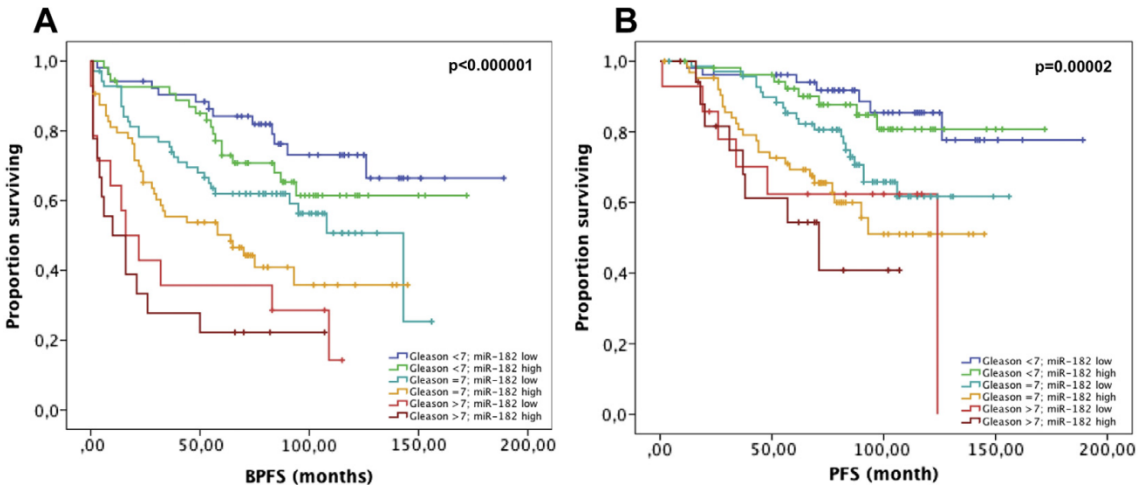

Figure 17. miR-182 expression classifies patients in groups at different risk of progression within each Gleason score.

Since miRNAs have been described to be also stable in different body fluids such as plasma, serum or urine [92], we aimed to study the potential role of the selected miRNAs (miR-182 and miR-187) as biomarkers in an easy and non-invasive diagnostic context. Therefore, we further analyze a cohort of 92 urine samples to assess the expression of miR-182 and miR-187 together with other already known biomarkers like *PCA3*, *T2E*, *GOLPH2* and *SPINK1*. As Laxman et al. showed in their study we also reported the utility of generating a multiplexed urine based diagnostic test combining several biomarkers [143]. In our case we established a prediction model including serum PSA, urine *PCA3* and miR-187 and we found that it could predict a positive prostate biopsy with a higher probability than PSA alone. Our model achieved 88.6% sensitivity and 50% specificity with 69.3% diagnostic precision (**Figure 18**). Therefore, miR-187 appears to be a promising biomarker for early diagnosis in PCa.

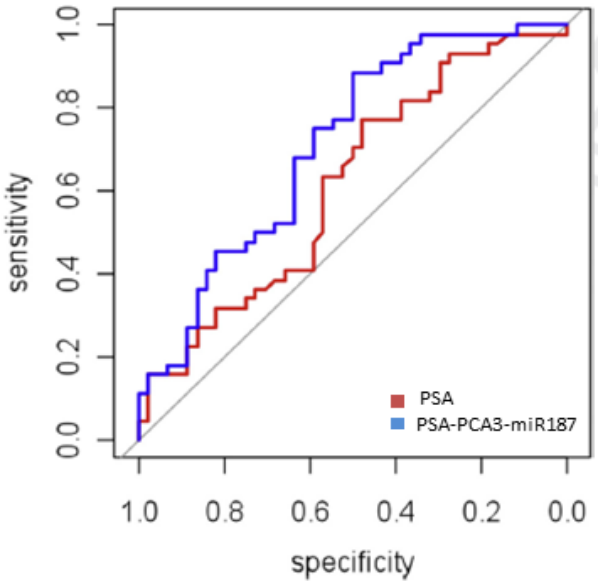

Figure 18. A multivariate combined model incorporating the expression of miR-187 improves the sensitivity and specificity of PSA alone to predict the result of a prostate biopsy in post-DRE urines of patients.

Milestones:

Our results suggest that both miR-182 and miR-187 play a key role in the pathogenesis and development of PCa, and that especially miR-182 constitute a promising biomarker for PCa prognosis and miR-187 could be a useful biomarker for the PCa diagnostic setting.

Translational impact derived from Objective 1:

In the prognostic context, we are currently developing in collaboration with Panomics (Affymetrix®) an in-situ hybridization assay to discriminate in needle biopsy PCa specimens, according to miR-182 expression, patients with a different clinical outcome. The application of this assay into the clinical practice would distinguish patients with indolent disease from those with tumors with a more aggressive behavior.

Objective 2: To perform a proteomic approach based on 2D-DIGE and MS analysis to identify new mRNA targets of miR-187 and to evaluate the potential role of these targets as novel biomarkers for PCa (Study III).

Despite we could demonstrate the utility of miR-187 in the diagnostic setting the fact that we found this miRNA down-regulated in PCa complicated its translation into a clinical applicable test. Nevertheless, miRNAs are known to regulate gene expression through translational repression and mRNA cleavage of more than 60% of protein coding genes. Therefore we hypothesized that if we would be able to identify miR-187 targets in PCa, that were not experimentally confirmed to date, we could find a potential biomarker whose expression will be up-regulated upon loss of miR-187 expression. To identify potential targets of miR-187 in PCa a proteomic approach based on 2D-DIGE followed by matrix-assisted laser desorption-ionization tie-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed. The use of this methodology allows us to identify physiological miRNA-mRNA relationships that cannot be predicted using *in silico* algorithms. In fact, results from previous works confirmed than less of 10% of proteins identified by a proteomic approach could have been predicted by commonly used algorithms such as Pictar, Targetscan and miRanda [122, 130]. Therefore to assess which proteins were regulated by miR-187 we performed a DIGE and LC-MS/MS analysis in an in vitro PCa model (PC-3 cell line) where we have synthetically recovered the expression of miR-187 by transfecting a miRNA mimic. After separating the protein extracts from PC-3 transfected with miR-187 mimic and PC-3 transfected with the negative control and fluorescence scanning, 9 differentially spots were detected. From these 9 spots detected, 7 of them showed a down-regulation upon miR-187 recovery (PC-3 miR-187 mimic transfected cells), which was consistent with the expected inhibitory effect of the miRNA through its targets (**Figure 19**).

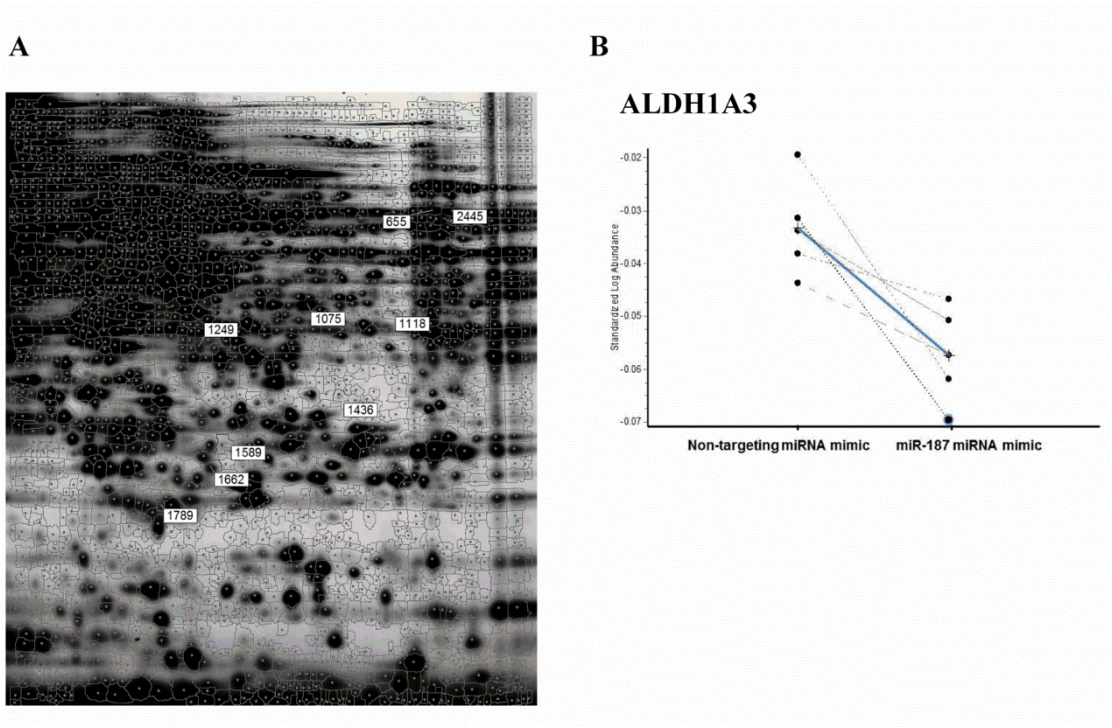

Figure 19. A proteomic approach based on 2D-DIGE followed by MS analysis lead to the identification of ALDH1A3 as a potential target of miR-187.

Among the 7 putative targets identified we selected ALDH1A3 for further validation. ALDH1A3 is an aldehyde dehydrogenase that was already described to be important for normal prostate development [134]. Aldehyde dehydrogenase family catalyzes the oxidation of retinal to retinoic acid and has been linked with cell proliferation, differentiation and survival. Furthermore the expression of ALDH1A3 has been described to be directly regulated by androgens [132]. Therefore, since ALDH1A3 was found to be correlated with both important parameters of cancer such as proliferation, survival and pluripotency [135, 136] together with essential signaling pathways for the prostate such as the androgens we decided to first study its role as a potential target for miR-187 in PCa. To demonstrate the role of *ALDH1A3* as a potential miR-187 target we first confirmed the presence of putative miR-187 binding sites in *ALDH1A3* mRNA sequence using RNA22 mRNA-miRNA heteroduplex prediction software (*Figure 3A. Study III*). Furthermore, three different cell line models: PC-3, LNCaP and DU-145 were transfected with miR-187 mimic to confirm the effect of the miRNA on ALDH1A3 expression. Western blot analysis confirmed a reduction in

ALDH1A3 expression upon re-introduction of miR-187 in all the studied models (*Figure 3B. Study III*). This inhibitory effect of miR-187 through *ALDH1A3* was further confirmed in a luciferase reporter assay, where those cells with a recovered expression of miR-187 (PC-3 miR-187 mimic) experienced a decrease in luciferase signal when a firefly luciferase reporter plasmid under the control of *ALDH1A3* 3'UTR region was co-transfected into the cells (*Figure 3C. Study III*). In addition, the RT-qPCR analysis of the expression of *ALDH1A3* demonstrated the up-regulation of this mRNA in a cohort of 96 FFPE and 10 fresh tissue PCa patients who also shared a strongly down-regulation of miR-187 (*Figure 3D and Figure 1S. Study III*). However, no correlation between clinico-pathological parameters or prognosis and *ALDH1A3* mRNA expression was found. ALDH1A3 protein expression was also assessed by immunohistochemistry (IHC) in a cohort of 195 PCa patients where it was found to be significantly over-expressed ($p<0.0001$) and directly correlated with Gleason score ($p=0.05$) (**Figure 20**).

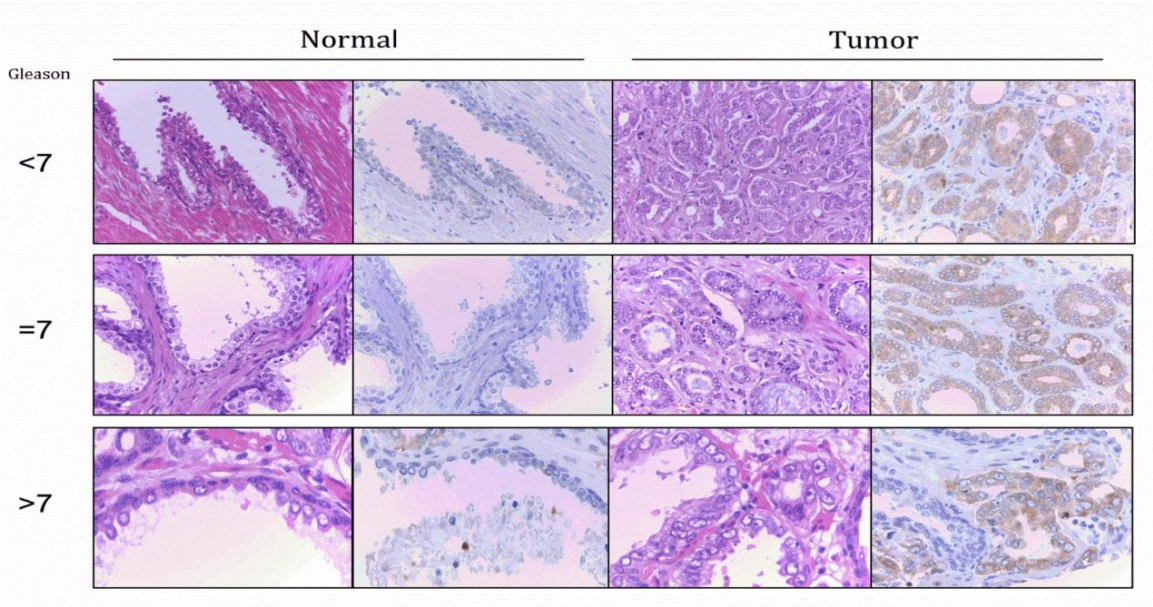

Figure 20. ALDH1A3 evaluation by IHC in prostatectomy pieces of PCa and normal prostate showed a differential expression, indicating its potential role as new biomarker in PCa.

Since we have previously postulated a potential role of miR-187 in the diagnostic setting we further analyze the role of its target ALDH1A3 as a potential biomarker for PCa diagnosis. For this purpose, ALDH1A3 expression was evaluated using an ELISA immunoassay in urine samples from patients with suspicious of PCa. These results were translated into a univariate logistic regression model where the predictive capability of this biomarker, together with PSA, for the presence of PCa in diagnostic biopsies was evaluated. Interestingly, in the same way than miR-187 expression in urine was cooperating with other biomarkers to predict appositive prostate biopsy, we found that ALDH1A3 was also cooperating with PSA and at a significance level of 10% both of them were significantly associated with a positive biopsy of PCa (*Figure 5. Study III*). Therefore, our results are in agreement with previous reports [22, 143] that postulate that a combination of multiple biomarkers may increase sensitivity and specificity over use of individual markers. Moreover, in the context of miRNAs it is also important to remember that a single miRNA can modulate several genes [40, 121, 144] and probably the effects of miR-187 expression is broader than the observed in a single target gene.

Milestones:

Our data illustrate for the first time the role of ALDH1A3 as a miR-187 target in PCa and provide insights in the utility of using this protein as a new biomarker for PCa.

Translational impact derived from Objective 2:

In order to translate our results into the clinical practice we aim to evaluate the utility of ALDH1A3 as a new biomarker for PCa in both body fluids and tumor samples (prostate biopsies) to apply it into a diagnostic context.

Moreover we would also like to explore the role in PCa diagnostic and prognostic settings of other proteins identified in our proteomic approach as potential targets of miR-187

Objective 3: To evaluate the molecular alterations of *SPOP* gene in PCa by NGS technology to determine its role as a new prognostic and therapeutic biomarker and implications as new PCa biotype (Study IV).

In our aim to translate the molecular heterogeneity found in PCa into a collection of homogeneous molecular subtypes ("biotypes"), we also studied the molecular alterations of the recently discovered *SPOP* gene in our cohort of PCa patients. Since *SPOP* gene was described to be the most common non-synonymous mutation in PCa we decided to assess both the expression profile and mutational status of *SPOP* gene together with the relationship with clinico-pathological parameters in a retrospective cohort of 265 primary PCa patients with more than 5 years of follow-up.

SPOP expression was evaluated by RT-qPCR and relative expression was determined using normal prostate as control calibrator. Interestingly we found that *SPOP* gene was down-regulated in 93.5% of the studied samples when compared with normal tissue (*Figure 1. Study IV*). Moreover *SPOP* down-regulation was inversely correlated with Gleason score (p=0.045) and log-rank analysis for both BPFS and PFS showed a significant association between prognosis and *SPOP* expression. The lower expression of *SPOP* gene was found to associate with a higher risk of biochemical recurrence (p=0.003) and distant progression (p=0.023) after prostatectomy and lower levels of *SPOP* were even able to independently predict a worse prognosis in the Cox proportional hazard multivariable analysis (BPFS HR: 0.5; CI 95% [0.4-0.8], p=0.005. PFS HR: 0.6; CI 95% [0.4-1], p=0.045). This association with prognosis was even stronger in the subgroup of tumors negative for the translocation *T2E* (**Figure 21**). It is already well known that *T2E* defines a subgroup of PCa patients with different clinico-pathological parameters and it has also been found to be mutually exclusive with the presence of certain lesions [46]. For instance, *SPOP* mutants have only been found in those patients that do not harbor the *T2E* translocation [63, 145]. Although previous studies performed in smaller cohorts of patients have already showed a down-regulation of *SPOP* gene and protein in PCa [63, 145, 146] none of them found any association with neither clinico-pathological parameters nor prognosis. Therefore we

have described for the first time how the loss of expression of *SPOP* is related with a worse patient outcome confirming its role as tumor suppressor in PCa.

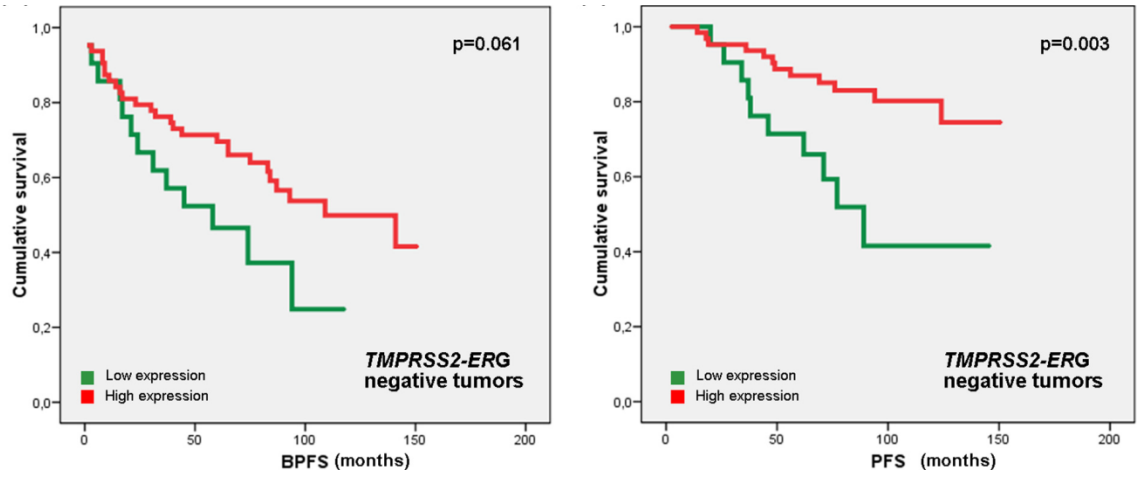

Figure 21. Loss of *SPOP* expression is associated with a higher risk of biochemical progression in PCa and this result is more evident in the subgroup of patients not expressing the *T2E* fusion gene.

Besides the expression analysis we also performed a mutational profile of *SPOP* gene in *T2E* negative tumors. Since it has already been reported that *SPOP* mutations were mutually exclusive with the presence of the *T2E* fusion gene, we only evaluated the presence of mutations in 90 cases already assessed as negative for *T2E* by RT-PCR, FISH and qPCR. Mutations were identified using the 454 GS-Junior NGS platform and confirmed by Sanger sequencing. *SPOP* gene was found to be mutated in 10% of the specimens and mutations were located in exons 5 and 6, which is in line with previous studies [63, 145]. Despite some of the mutations found were already described in previous works we were able to report for the first time mutations p.F104V, p.D153N and p.Q120Stop. When analyzing the prognostic role of *SPOP* mutations in PCa we found a significant direct correlation between *SPOP* mutants and a worse BPFS (p=0.009) (**Figure 22**). Moreover, *SPOP* mutations also constituted an independent variable of poor prognosis after Cox proportional hazard multivariable analysis (HR: 3.4; IC 95% [1.5-7.6], p=0.004). In a previous work performed by Blattner et al. they also studied the association between *SPOP* mutations and prognosis however they did not

find any statistically significant correlation [145]. The discrepancy with our results could be explained because of the differences in the patient cohorts analyzed. The distribution of higher risk PCa patients is significantly different between the two studies since the proportion of these tumors is very low in the specimens collected in our institution.

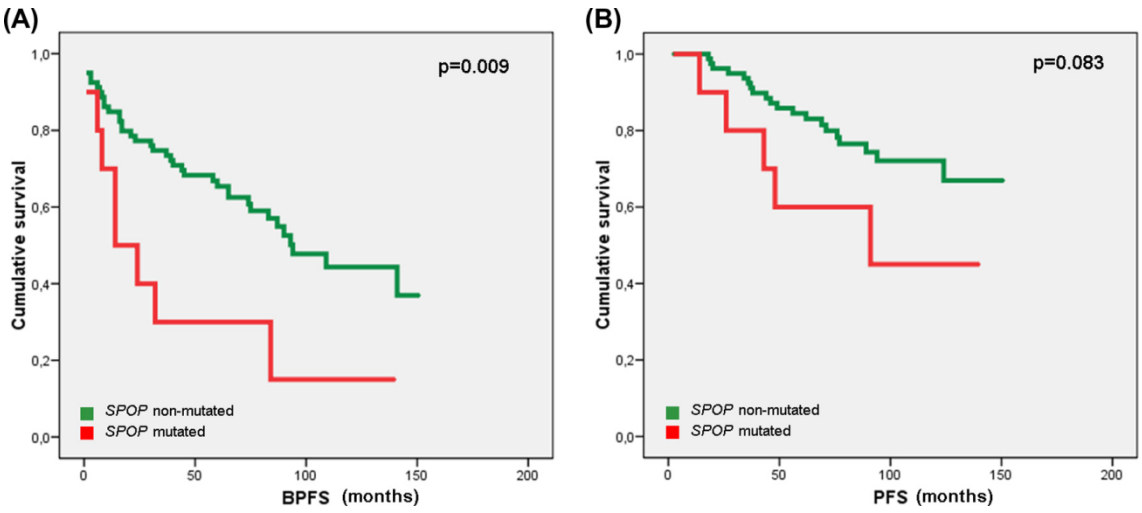

Figure 22. The presence of mutations in *SPOP* gene confers a higher risk of biochemical progression.

Additionally we also studied the association between *SPOP* mutations and loss of expression but no significant correlation was found. However, we interestingly found that all the mutated cases showed a down-regulation of *SPOP* gene. This result indicated us that besides the presence of mutations there should be other mechanisms leading to the loss of expression of this gene. Genomic loss of *SPOP* gene locus has already been described and in fact the 17q21 chromosome, where *SPOP* gene is located, has been reported to be a region with a high allelic imbalance in different tumors including PCa [65]. Moreover, miR-145 has recently been described to regulate *SPOP* expression what could also explain part of this loss of expression [147].

Our findings give more strength to the fact that *SPOP* gene is defining a new biotype in PCa that could be also associated with different risk of progression and could be subjected to different therapeutic options.

Milestones:

Loss of expression and alterations in *SPOP* gene demonstrate that it acts as a tumor suppressor gene in PCa and defines a new subtype of PCa tumors associated with a worse prognosis.

Translational impact derived from Objective 3:

One of our future objectives consists on the assessment of *SPOP* expression by IHC in prostate biopsies in order to classify patients at different risk of progression.

Furthermore, upon the characterization of mutations found in *SPOP* gene, a high sensitive genetic test could be designed (RT-qPCR, digital PCR) in order to detect these alterations in body fluids such as urine or plasma that can be useful for monitoring patient outcome.

Objective 4: To determine the relationship between *T2E* and IGF-IR in PCa and evaluate the potential implications of this cross-talk for the design of new therapeutic strategies (Study V).

T2E represents the most common event in PCa being expressed in 40-70% of tumors. Moreover several studies have defined distinct gene expression profiles in ETS fusion-positive and ETS fusion-negative PCas [45, 49, 148]. Therefore, the attempts to molecularly characterize PCa into distinct biotypes often begin with division into subgroups according to *T2E* status.

T2E has been described to represent an early event in PCa development and has been reported to be mutually exclusive with certain molecular lesions (i. e. *PTEN*, *SPOP*) or cooperate with others (i.e AR signaling). Furthermore, due to the high prevalence of this translocation in PCa there is also an increasing interest in potential therapeutic targeting of this subgroup of patients. In this context we aimed to study the relationship between *T2E* and IGF system.

IGF system has already been extensively studied in PCa although most of the works have produced controversial evidences [149]. Nevertheless, there is a broad consensus in the critical role played by IGF-IR in normal prostate development as well as in cancer initiation and progression [150, 151]. In fact, several phase II studies have been developed in PCa using IGF-IR inhibitors, although none of them reported any promising results [152, 153].

In our work we have assessed the expression of the two main receptors of IGF system (IGF-IR and insulin receptor [IR]) at mRNA and protein level in a panel of PCa cell lines comprising 5 tumor cell lines (VCaP, DU-145, PC-3, LNCaP and 22RV1) and a non-malignant prostate cell line model (RWPE-1) (*Figure 1. Study V*). We interestingly found that IGF-IR expression was predominantly low in most of the cell lines analyzed but VCaP model, which is the only cell line that harbors the translocation *T2E*. Therefore, to further investigate the relationship between the presence of the fusion gene and the expression of IGF-IR we developed a model where we modulated the expression of *ERG* by siRNA transfection in VCaP cells. We analyzed IGF-IR

expression upon *ERG* silencing by Western blot and a decrease of receptor expression was found at 96 and 120 h. Moreover we also confirmed that the modulation of IGF-IR expression was dependent of *T2E* status in other two models where tERG (the protein product of *T2E* translocation) expression was re-introduced by stably transfection (RWPE-1 tERG and PC-3 tERG) (**Figure 23**). To confirm the observed effect of *T2E* on IGF-IR levels an anti-ERG chromatin immunoprecipitation (ChIP) assay was performed. This assay confirmed the binding of *T2E* into *IGF-IR* promoter leading to the modulation of its expression (*Figure 2. Study V*).

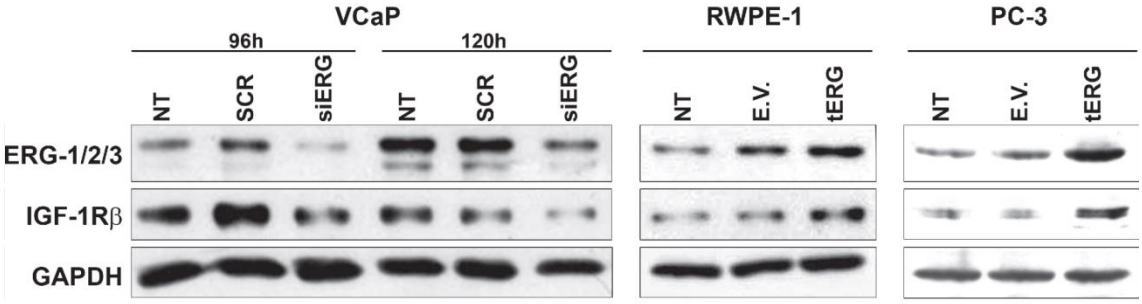

Figure 23. The expression of IGF-IR is dependent on the levels of ERG since its modulation notably affects the levels of IGF-IR.

To confirm the clinical relevance of the observed cross-talk between *ERG* and *IGF-IR* we also assessed *IGF-IR* expression profile by RT-qPCR in our retrospective cohort of 270 primary PCa. Interestingly we found a direct correlation between *IGF-IR* expression and the presence of *T2E* (p=0.008) implicating that those tumors harboring the fusion gene showed increased levels of *IGF-IR*. This association was further confirmed at protein level since we also analyzed ERG and IGF-IR by IHC in the same cohort of patients (*Figure 5. Study V*)

There are several studies evidencing the interaction between *T2E* and androgen signaling axis. In fact, *TMPRSS2* promoter is known to be directly regulated by androgens. Therefore we decided to study the effect of ERG and IGF-IR expression upon Abiraterone treatment. An strong ERG down-regulation together with a decrease

of IGF-IR expression was showed after treatment at different time points (72, 96 and 120 h) with the higher used concentration of Abiraterone (10 μM) (*Figure 2. Study V*). Although the decrease of ERG expression upon Abiraterone treatment was already described [154] any other work has ever showed the consequent inhibition of IGF-IR which confirms the presence of a *T2E*/IGF-IR androgen regulated axis.

In parallel we also assessed the effect of IGF-IR inhibition in PCa cell lines. Hence when exposing the panel of PCa cell lines to different concentrations of the IGF-IR human monoclonal antibodies (HAbs) CP-751,871 and AVE1642 or the IGF-IR TKI NVP-AEW541 only VCaP showed a remarkably high sensitivity to the treatment. Moreover, when silencing *ERG* in VCaP cells this effect upon IGF-IR inhibition was lost. Moreover, PC-3 cells transfected with tERG also showed a higher sensitivity to the treatment with CP-751,871 (*Figure 3. Study V*). Our data confirmed for the first time that the level of ERG expression significantly influenced the efficacy of anti-IGF-IR agents (**Figure 24**).

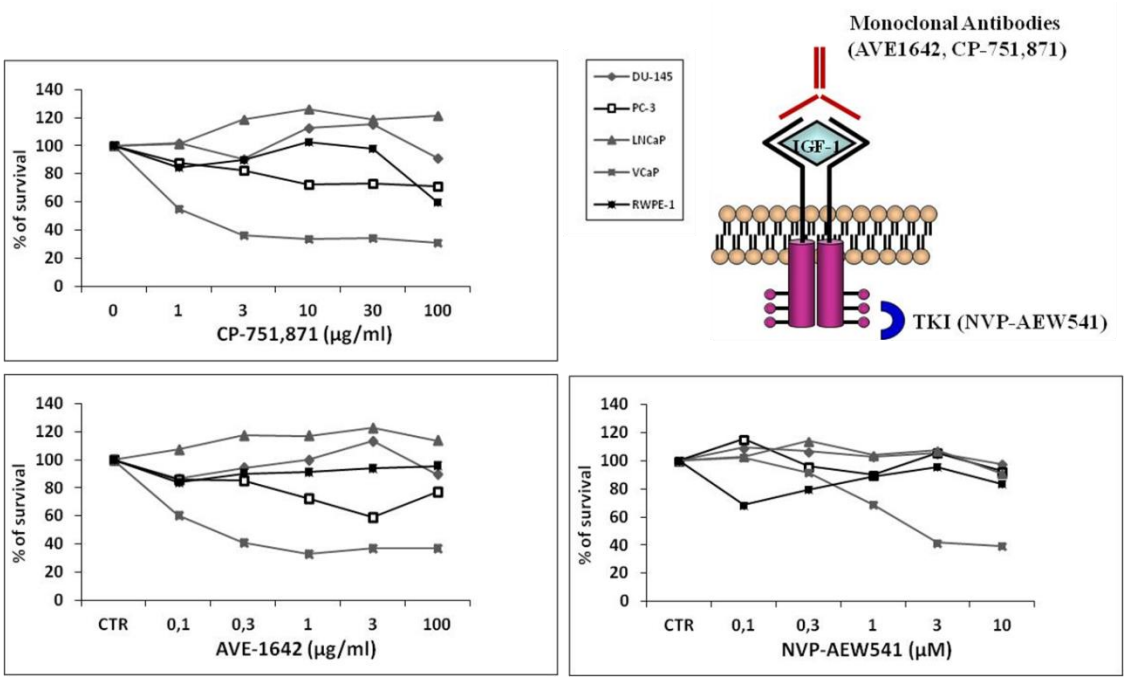

Figure 24. The treatment with different HAbs anti-IGF-IR or TKIs only showed efficacy in the cell line model harboring the translocation *T2E* (VCaP).

Taken together our results confirming the presence of a *T2E*-IGF-IR–AR axis we also proposed a combination therapy administrating simultaneously anti-IGF-IR CP-751,871 HAbs and Abiraterone or Cabazitaxel in PCa cell lines. We found that IGF-IR inhibition together with Abiraterone treatment induced synergistic antiproliferative effects in VCaP cells while no beneficial effect was observed in other cell line models negative for *T2E* translocation such as DU-145 or LNCaP (CI= 2.88±1.17 vs CI > 100). From a clinical perspective, this mechanism provides the rationale for the selective use of anti-IGF-IR agents for patients expressing *T2E*. In fact, several clinical trials have been developed for IGF-IR inhibitors, in CRPC patients, such as Cixutumumab (Phase II, NCT00520481 and NCT00683475) and Figitumumab (Phase I, NCT00313781) as a single agent or in combination with other drugs. However, patients only experienced a partial response to therapy and a lot of side toxicities (neutropenia, diarrhea, hyperglycemia,etc.) were found [155]. Probably, the clinical efficiency of these compounds could be notably improved if we would be able to determine those respondent tumors with specific alterations or biotypes such as *T2E* status. Moreover, due to the molecular complexity observed in human tumors, multiple drugs in combination are often administered simultaneously to hit different pharmacological targets and thus improve efficacy and decrease resistance. This is the rationale behind the so called polymer-based combination therapy that becomes an excellent tool for developing this interesting concept [156].

Milestones:

T2E expression directly modulates IGF-IR levels in PCa. As a consequence, the subgroup of patients harboring the *T2E* gene fusion is more sensitive to IGF-IR inhibition, and its cytotoxic effect becomes enhanced if combined with androgen ablation with Abiraterone.

Translational impact derived from Objective 4:

In view of our results we are currently developing a polymer-based combined therapy using two targetable drugs, Abiraterone that blocks the AR pathway; and a humanized monoclonal antibody anti-IGF-IR that affects the PI3K pathway among others in the subtype of CRPC tumors that harbor the fusion gene *T2E*.

Conclusions

Our work demonstrates and gives support to the highly molecular heterogeneity found in PCa. The possibility of identifying new biotypes in PCa, able to stratify patients in different subgroups according to their molecular profile, represents a big impact for diagnosis, prognosis and therapeutic intervention, making the concept of *precision medicine* a reality.

1. Many miRNAs are de-regulated in PCa and play a role in tumor progression, highlighting its role as potential biomarkers in PCa.

2. miR-182 plays a role as prognostic biomarker for both biochemical and clinical progression. The categorization of Gleason score according to the expression level of miR-182 identifies patient groups with different risk of progression. Our findings suggest that the miR-182 overexpression could potentially be incorporated into the clinical decision making algorithms once its role as biomarker has been validated on prospective series of patients.

3. miR-187 has been shown to be a potential diagnostic biomarker in urine samples by improving the PCa detection rate compared with PSA alone.

4. Restoration of miRNA expression in cell models followed by a proteomic approximation constitutes a useful tool for the functional identification of miRNA targets that can be tested as potential biomarkers.

5. For the first time, we have identified ALDH1A3 as a miR-187 target in PCa and described its role as potential new biomarker in PCa.

6. Approximately 10% of *T2E*-negative PCa harbors *SPOP* mutations defining a group of patients with special worse prognosis.

7. We have been the first group in describing a prognostic role for *SPOP* alterations in PCa progression suggesting its translation into the clinical context by the identification of patients with poor prognosis that could benefit from more radical treatments.

8. Herein it has been evidenced for the first time that IGF-IR is directly regulated by *T2E* and how the impact of this association affects to the response to IGF-IR

inhibition. Hence, we suggest that *T2E* PCa patients are sensitive to IGF-IR inhibition and become potential candidates to IGF-IR directed therapies.

9. The cross-talk found in the *T2E*-IGF-IR–AR axis leads us to design a combination therapy able to target both IGF-IR and AR in a subgroup of PCa patients ('biotype') with synergistic effects.

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Annex I. Other publications derived from the doctoral thesis

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DIAGNÓSTICO NO INVASIVO DEL CÁNCER DE PRÓSTATA; MARCADORES SÉRICOS Y EN ORINA

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Resumen.- *El gran número de biomarcadores que la investigación básica plantea en distintos escenarios clínicos de cáncer de próstata (CaP) exige de la comunidad científica un rigor en su desarrollo molecular y clínico para la selección de aquellos que puedan aportar información diagnóstica o pronóstica a los nomogramas de factores clínico-patológicos establecidos. El CaP necesita por su prevalencia y heterogenicidad un diagnóstico más dirigido, la caracterización de su potencial maligno y la monitorización de sus múltiples tratamientos. En este artículo de revisión pretendemos repasar la reciente incorporación de nuevos biomarcadores séricos y en orina en el manejo clínico de este tumor, haciendo hincapié en aquellos con mayor desarrollo clínico.*

Palabras clave: *Cáncer de próstata. Biomarcadores. Diagnóstico. Pronóstico. Biología molecular.*

Summary.- *The great number of biomarkers basic research is presenting in different clinical scenarios of prostate cancer demands the scientific community rigor in their molecular and clinical development for the selection of those which could supply diagnostic and prognostic information for the established nomograms of clinical-pathological factors. Prostate cancer, due to its prevalence and heterogeneity, needs a more directed diagnosis, characterization of malignant potential and monitoring of its multiple therapies. In this review article we try to go over the recent incorporation of new serum and urine markers in the clinical management of this tumor, emphasizing those with greater clinical development.*

Keywords: *Prostate cancer. Biomarkers. Diagnosis. Prognosis. Molecular biology.*

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INTRODUCCIÓN

La necesidad de nuevos biomarcadores en cáncer de próstata (CaP) nace de los datos del programa de screening de CaP (1), donde se demuestra que el uso masivo del PSA disminuye la mortalidad

por este tumor pero con un coste en sobrediagnóstico de enfermedad indolente con su consabido sobretratamiento. La investigación básica en biomarcadores debe mejorar el diagnóstico y perfilar la indicación del screening a determinada población de riesgo de CaP. Además a los nuevos biomarcadores se les debe exigir información pronóstica, hecho vital en un cáncer del que 18% de los hombres podrían ser diagnosticados a lo largo de sus vidas pero que solo matará a 3 de ellos (REF). La elección o rechazo de determinadas alternativas terapéuticas frente a un CaP es un campo abierto a la caracterización por biomarcadores, así como la monitorización de las respuestas a distintas terapéuticas.

Si en un buscador cruzamos las palabras "biomarker & prostate cancer", obtenemos 21845 referencias; de 10 en 10 años en 1982 obteníamos 7 referencias, 282 en 1992, 1027 en 2002 y finalmente 1488 en 2011; esta ingente avalancha de nuevos biomarcadores ha obligado a estandarizar científicamente tanto su desarrollo experimental y clínico y a exigir a los nuevos biomarcadores que demuestren una suma de valor diagnóstico y pronóstico a lo ya existente, buscando una estrategia similar al desarrollo de un nuevo fármaco, pero este importante aspecto ya es tratado en otro capítulo de esta monografía.

El marcador tumoral ideal sería aquél que cumpliera las siguientes características; no ser invasivo, ser fácilmente realizable, reproducible y barato y que sus estadísticos, sensibilidad (S), especificidad (Sp), valor predictivo negativo (VPN) y positivo (VPP), permitieran en conjunción con los parámetros clínico-patológicos una mejoría en la decisión clínica planteada. No es objeto de esta revisión describir el desarrollo molecular y tecnológico de cada biomarcador que el lector puede encontrar también en distintas partes de esta monografía, sino revisar los nuevos biomarcadores desde las distintas controversias clínicas que en el manejo de CaP se podrían beneficiar de ellos, centrándonos en los de mayor desarrollo clínico. En la Tabla I se muestran parte de otros biomarcadores descritos en la literatura con menor desarrollo clínico.

BIOMARCADORES RELACIONADOS CON UN MAYOR RIESGO DE PADECER CaP;

Single Nucleotide Polymorphisms (SNPs o polimorfismos de un solo nucleótido)

Múltiples estudios caso-control encuentran distintos SNPs descritos en la literatura como factores predisponentes de CaP (2). Recientemente, variantes de SNPs del gen HOXB13 se han asociado a predisposición a CaP familiar, aunque aparecen en <1% de los pacientes con CaP (3).

Sin embargo, en una población de 2829 hombres no sujetos a diagnóstico precoz de CaP estudiados de forma prospectiva comparándolos a 943 hombres con CaP pareados, el panel de SNPs descritos en la literatura no aumenta la capacidad predictiva del PSA para detectar CaP (4). Estos estudios genómicos siempre estarán sujetos a la duda del infradiagnóstico de CaP en biopsia frente a las cifras de CaP en estudios de autopsia y a las variaciones interétnicas. Además, los SNPs asociados a un mayor riesgo de padecer CaP probablemente sean una pequeña muestra de todos los SNPs posibles aún por demostrar. Por todo ello, este es un campo en plena investigación y se espera que en un futuro, el perfil génico pueda ayudar a seleccionar qué hombres se beneficiarían de un screening precoz y más intensivo.

Cambios epigenéticos

Se han descrito diferencias en la metilación de los genes GSTP1 y CD44 entre afro-americanos, asiáticos y caucásicos (5).

BIOMARCADORES QUE PUEDAN MEJORAR EL SCREENING DE CaP

PCA3 (Gene Probe, San Diego, CA, USA);

El desarrollo de este biomarcador se ha realizado en poblaciones de riesgo de CaP o en biopsias de repetición; su punto de corte de 35 no ha sido valorado prospectivamente en un contexto de detección oportunista de CaP. Solo la rama holandesa del ERSCP lo testó con un punto de corte de 10 en un contexto de test de primera línea de screening. El PCA3 tuvo una S y un VPP ligeramente mejores que el PSA para detectar CaP, apuntando la posibilidad de mejorar la detección de CaP agresivo por debajo de 3ng/dl de PSA (6). Su precio y su pérdida de Sp a ese punto de corte hacen difícil su aplicación en primera línea.

La independecia del PCA3 del volumen glandular a puntos de corte de 20 o 35 (7-9), del PSA sérico (9, 10) y de los fenómenos inflamatorios de la próstata (11) y su independencia del uso de inhibidores de la 5α-reductasa tipo I y II (8) son características que lo harían atractivo en un escenario de screening. Por todo ello iniciamos en nuestro Centro hace 2 años un estudio prospectivo en un escenario de screening dual donde si el PSA es ≥ 3ng/ml y/o el tacto rectal es sospechoso se realiza PCA3; si éste

Tabla I.

	Función	Utilidad clínica potencial	Refs (1er autor/ rev/ año)
Annexin A3	Proteina ligadora de calcio	Mejora AUC del PSA para Dx, con valores menores en CaP frente a HBP	Schostak, Urology, 2009
MMP9	Proteinasa de matriz extracelular	Mejora Dx, S 74% y Sp 82%	Roy, Clin Cancer Res, 2008
Sarcosina	Metabolito de grupo N-methil	Relacionado con progresión y M+	Sreekumar, Nature, 2009
Metilación GSTP1 / APC (+ paneles múltiples de hipermetilación)	Metilación epigenética	Capacidad de mejorar el diagnóstico de CaP. Resultados pronósticos controvertidos	Payne, Prostate, 2009, Roupret M, Clin Cancer Res 2007 Trock, BJU Int 2011
TGF-β1	Factor de crecimiento relacionado con proliferación celular	Resultados controvertidos en cuanto a relación con progresión del CaP	Shariat, Clin Cancer Res, 2004 y Kattan, JCO, 2003
IL-6	Citokina relacionada con crecimiento y diferenciación celular	Resultados controvertidos en cuanto a relación con progresión y supervivencia	Kattan, JCO, 2003
AMARC	Enzima relacionada con el metabolismo graso	Potencial diagnóstico y pronóstico en tejido	Rubin, JAMA 2002, Rubin Cancer Epidemiol. Biomarkers Prev 2005
CCP score	Panel de 31 genes relacionados con el ciclo celular	Caracterización pronóstica de CaP tras prostatectomía radical y en Bx	Cuzick, Lancet Oncol, 2011, Br J Cancer 2012
IGF & IGFBP	Metabolismo glúcido	Relación con iniciación & progresión del CaP	Rowlands, Cancer Res, 2012
hK2	Serinproteasa similar al PSA	Potencial diagnóstico en suero	Vickers BMC Med, 2008
uPA / uPAR	Degradación de matriz intracelular	Posible valor pronóstico	Gupta,Eur Urol, 2009 Shariat, JCO, 2007
EPCA	Proteína de matriz nuclear	Potencial valor diagnóstico y pronóstico en tejido y suero	Leman, Urology 2007 Urology, 69: 714, 2007
Metabolómica	Análisis de los metabolitos en orina	Resultados controvertidos en diagnóstico y pronóstico	
BPSA	Marcaje de la HBP	Caracterización HBP	Canto, Urology, 2004
CD105 Endoglina	Relación con angiogénesis	Posible valor pronóstico	Svatekl, Clin Cancer Res, 2008

APC; Adenomatous Polyposis Coli, AUC; área bajo la curva, BPSA; PSA relacionado con la hipertrofia benigna de prostate, CCP score: panel de 31 genes relacionados con el ciclo celular normatizados en expresión de ARN a 15 genes estructurales, Dx: diagnóstico, EPCA; antígeno precoz de CaP, GSTP1; glutation S transferasa P, HBP; hipertrofia benigna prostática, IGF; insulin growth factor, IGFBP; IGF binding proteins, IL; interleukina, MMP; metaloproteinasa de matriz 9, M+; metastasis, , p2PSA; isoforma del PSA libre [_2]proPSA, , S; sensibilidad, Sp; especificidad, TGF; transforming growth factor, uPA: urokinasa plaminogeno y receptor

es ≥35 todos se biopsian (12 cilindros). Si es <35 se randomizan 1:1 a realizar biopsia u observación. El objetivo primario del estudio es calcular el posible ahorro de biopsias en primera ronda y en el seguimiento, y los objetivos secundarios calcular los falsos negativos del PCA3 y las diferencias anatomopatológicas entre los CaP encontrados en cada rama. A 18 meses de reclutamiento, 1847 pacientes han sido sometidos a screening oportunista y solo 185 han requerido realizarse un PCA3 (morbilidad de 10.01%), habiendo obtenido una tasa de 6.76% de falsos negativos en la rama de PCA3 negativo. La potencial aplicación de este protocolo dual ahorraría un 71.9% de biopsias en 1ª ronda y un 60.5% con un seguimiento medio de 7 meses.

Isoforma del PSA [_2]proPSA (p2PSA) y derivados; p2PSA / PSA libre (%p2PSA; [(p2PSA pg/ml) / (free PSA ng/ml _ 1000)] _ 100) y Prostate Health Index (PHI; [p2PSA / free PSA] _HtPSA) (Beckman Coulter; Brea, CA, USA)

Se han descrito distintas formas moleculares del PSAl, precursores inactivos que se expresan diferencialmente en HBP como el PSA-benigno y el PSA-intacto, o el proPSA, que se expresa más en CaP y que es enzimáticamente inactivo. Una de las isoformas del proPSA, la -2proPSA se ha erigido en un potencial biomarcador al comprobarse su mayor concentración en tejido canceroso frente a otras isoformas (12). El desarrollo clínico de este nuevo biomarcador ha permido integrarlo en un modelo matemático junto al PSAt y al PSAl para generar el Beckman Coulter prostate health index, más conocido como índice PHI. Es importante recordar que el p2PSA tiene mínimo valor como marcador único y sin embargo, al igual que con el PSA libre, su ratio frente a éste y su conjugación en la fórmula del índice PHI son las que ofrecen resultados diferenciales entre CaP y próstata benigna y en su relación con el índice Gleason.

En su manejo clínico, hay que tener en cuenta posibles factores que afectan la determinación de este biomarcador: hormonoterapia, alteraciones proteinograma, hemofilia y politransfusiones entre otros. Sin embargo, la raza o la edad no parecen marcar diferencias en sus valores (13). Su tasa de detección hasta 3pg/ml permite usar la determinación del proP SA en muestras con PSAt > 0.5 ng/ml.

En un escenario de screening, un estudio en 2034 hombres, el %p2PSA y el índice PHI mostraron las AUC más elevadas (0.76 y 0.77 respectivamente) y en el caso del índice PHI triplicando y duplicando la especificidad frente al PSAt y al PSAl respectivamente a una sensibilidad del 88.5% (14).

Hipermetilación epigenética

El uso de paneles de detección de múltiples genes, y no solo el GSTP1, ha demostrado que manteniendo la Sp de la detección de éste en orina (86-100%), mejora mucho su S hasta cifras de 86% (15). Estos paneles también pueden detectarse en suero y sobre tejido prostático, pero su caracterización no es aún definitiva pues la selección de los genes a estudiar puede alterar las cifras de especificidad del test múltiple.

BIOMARCADORES IMPLICADOS EN MEJORAR EL DIAGNÓSTICO EN 1ª BIOPSIA

PCA3 score

El desarrollo clínico inicial de este marcador fue en un escenario de biopsia de repetición, pero pronto su aplicación se testó en indicación de primera biopsia con resultados equiparables (16) aunque en nuestra experiencia donde mejor rentabilidad tiene es precisamente en indicación de primera biopsia (7). Un meta-análisis de nuestro país que analizó una selección de 14 artículos de entre 403 citas bibliográficas a cerca del PCA3 ofrece un rango de S de 46.9-82.3%, de Sp entre un 56.3-89%, de VPP entre 59.4-97.4% y de VPN entre 87.8-98%, reconociendo unos aceptables índices de validez diagnóstica para el uso del PCA3 en el diagnóstico de CaP (17) (Tabla II).

No está claro aún el punto de corte que ofrece más rentabilidad al test; si el objetivo es detectar más tumores, nuestra experiencia nos muestra que un punto de corte de 20 incrementa la sensibilidad del test 10 puntos, a costa de una pérdida de 13 puntos en especificidad (7). Dado que el objetivo es detectar más tumores clínicamente significativos, y éstos son raros por debajo de 35, la mayor rentabilidad diagnóstica de este punto junto al escaso porcentaje de tumores clínicamente significativos que se escapan al mismo, ha hecho que se acepte 35 como punto de corte de PCA3, sin olvidar (8, 18) que lo más informativo es entender el PCA3 como variable continua.

En un escenario de primera biopsia, un estudio multicéntrico sobre 516 pacientes con PSA 2.5-10 ng/ml, con punto de corte de PCA3=35 demostró que se hubieran podido ahorrar 60% de biopsias dejando de detectar 11% de CaP Gleason ≥7 (cifra similar a la obtenida en nuestro programa de screening dual); si el punto de corte de PCA3 hubiera sido 20 dichos porcentajes serían 40% y 2% (19).

Tabla II. Estadísticos del PCA3 en series con más de 400 pacientes analizados.

Autor/año	N	%CaP	AUC	S (%)	E (%)	VPP (%)	VPN (%)
Van Gils/2007	534	33	0.66	65	66	48	80
Ankerst/2008	443	27.8	0.67	63	60	38	81
Deras/2008	570	36.1	0.69	54	74	58	74
Haese/2008	463	27.6	0.66	47	72	39	78
Auprich/2010	621	41.1	-	88	45	53	84
Roobol/2010	721	16.9	0.64	68	56	24	90
Chun/2010	809	39.1	0.68	81	45	49	79
Rubio-Briones/2011	474	34	0.67	85	33	39	81

El desarrollo clínico del marcador ha hecho que llegue a incorporarse como factor pronóstico independiente en varios nomogramas dirigidos a cuantificar la posibilidad de una biopsia positiva, mejorando la capacidad predictiva de los modelos previos sin su concurso (18, 20). La comparación bioestadística de los diferentes nomogramas que incluyen el PCA3 permite recomendar el de Chun y cols en el escenario de 1ª biopsia, que usa el punto de corte de PCA3=17, edad, PSA, TR, volumen prostático y si se han biopsiado o no previamente como variables (21).

Por lo tanto, el uso del PCA3 score proporciona una tasa de ahorro de biopsias entre el 40 y el 67% según la literatura (9, 19, 22), en concreto del 48.9% en nuestra propia experiencia, combinándolo con el PSA y el TR (7).

Sin embargo, el uso expandido del PCA3 ha mostrado claramente que no puede ser generalizado y ser utilizado como marcador único, dado que se detecta en HGPIN (23), en algún CaP por debajo de 35 y ocasionalmente en algún caso con PCA3>100 no se detecta CaP (6); es por tanto primordial reseñar que la capacidad predictiva del PCA3 se ve incrementada cuando se complementa con el valor del PSA y el TR (16) o cuando se añaden nuevos biomarcadores, como se explicará más adelante.

[_2]proPSA (p2PSA) y Prostate Health Index (PHI)

En varios estudios retrospectivos usando sueros archivados, el p2PSA y sus derivados demostraron una mejor fiabilidad que el PSAt y el cociente PSAl/t para detectar CaP en 1ª biopsia (13, 24, 25). Cuando se analizó el valor aditivo de %p2PSA en redes neuronales artificiales o en modelos de regresión, se objetivó como éste tenía el impacto más importante para ambos modelos (p<0.0001); su exclusión del modelo disminuía el AUC de 0.85 a 0.74 y su inclusión en la red neuronal artificial añadía su mayor eficacia diagnóstica en el rango de sensibilidad de entre 82-90%, donde añadía 12% de especificidad (26).

Más recientemente y de forma prospectiva, se han reproducido estos resultados en pacientes con PSAt entre 2.5-10 ng/ml usando un protocolo de 18-22 cilindros en biopsia de inicio; los autores encuentran que para una especificidad de 90%, la sensibilidad de PHI (42.9%) y %p2PSA (38.8%) mejoraban las de el %PSAl/t (20%) y la del PSAd (26.5%). La precisión del modelo basal para detección de CaP mejoraba en +11% con el PHI y en +10% con el %p2PSA (p<0.001) (27).

BIOMARCADORES IMPLICADOS EN MEJORAR EL DIAGNÓSTICO DE BIOPSIAS DE REPETICIÓN

PCA3 score

Como hemos indicado previamente, el PCA3 fue valorado inicialmente tras primera biopsia negativa estableciendo el cut-off de 35 en este contexto; entre 463 pacientes Haese et al encontraron 39% de biopsias de repetición positivas si el PCA3>35

frente a 22% con PCA3<35 (9). Dentro del estudio REDUCE se ha objetivado un 52% de ahorro de biopsias si a los 1024 hombres de la rama placebo (con una biopsia previa negativa, PSA 2.5-10 ng/dl y con biopsias de repetición por protocolo a los 2 y 4 años) se les hubiera tenido en cuenta el PCA3 (cut-off 20) que se hizo paralelamente de forma experimental. No se hubieran diagnosticado 49 casos (9.1%) de CaP Gleason < 7 y 7 casos (1.3%) de CaP Gleason ≥7 (8). En base a todos estos estudios, recientemente la FDA ha aprobado su uso en esta indicación con un punto de corte de 25, remarcando su alto VPN (90%; IC95% = 86.5-93.1), con una S de 77.5%, una Sp de 57.1% y un VPP de 33.6%. El cut off de 25 para el PCA3 score ya se ha visto refrendado en publicaciones recientes como el que mejor estadísticos tiene para la detección de CaP en biopsia de repetición (28).

También se ha valorado la rentabilidad del PCA3 en relación con el número previo de biopsias negativas; nuestra experiencia y la de otros objetiva que el PCA3 ofrece sus mejores estadísticos en biopsia inicial o en la 1ª biopsia de repetición, siendo superado por otros marcadores como el %PSAl/t a partir de la 2ª biopsia de repetición (7, 29). Sin embargo, otros autores han obtenido estadísticos similares para el PCA3 independientemente de si la biopsia de repetición era la 1ª o la 2ª (9).

Gen de fusión

Son varias ya las evidencias de la reproducibilidad de la detección del gen de fusión (GF) en orina por TMA a su presencia detectada por FISH o RT-PCR en pieza de prostatectomía; este hecho es importante, puesto que la presencia del GF en orina y la no detección de CaP en la biopsia empieza a plantearse como una indicación clara de rebiopsia temprana pues dada la alta especificidad de este biomarcador, el CaP "está pero no se ha detectado", lo que sabemos ocurre en más del 40% de las biopsias que realizamos siguiendo el esquema de 10-12 cilindros estándar. La heterogenicidad de la fusión TMPRSS2-ERG, en el contexto de su uso como biomarcador en orina, estaría detrás de un test positivo para la fusión y la detección de un CaP negativo para la fusión.

Hipermetilación del tejido de la 1ª bx

Aunque no se trate de un biomarcador sérico, en sujetos con 1ª bx negativa pero alta sospecha de CaP por parámetros clínicos o antecedentes de PIN o ASAP en la 1ª, la hipermetilación del gen APC (adenomatous polyposis coli) en el tejido de la 1ª bx negativa podría ahorrar un 30% de biopsias dado su alto VPN (96%) (30), pero faltan estudios que reproduzcan estos resultados.

Tabla III. Relación de la expresión de PCA3 con distintas variables patológicas pronósticas en pieza de prostatectomía radical, expresándola como relación estadísticamente significativa (SI) o no estadísticamente significativa (NO)

Autor/año	n	CaP insignificante	Volumen Tm	Gleason score ≥7	pT≥3
Whitman / 2008	72	SI	SI	NO	SI
Nakanishi / 2008	83	SI	SI	SI	NO
Hessels / 2010	70	NO	NO	NO	NO
Liss / 2011	100	NO	No valorado	NO	NO
Auprich / 2011	305/160*	SI	SI	SI	NO
Vlaeminck-Guillem / 2011	102	No valorado	SI	NO	NO
van Poppel / 2011	175	No valorado	No valorado	SI	SI
Ploussard / 2011	106	SI	SI	NO	NO
Serie IVO / 2012**	68	No valorado	SI	NO	NO
Durant / 2012	160	No valorado	SI	SI	SI

*Solo 160 tenían el volumen tumoral calculado por planimetría computarizada. ** Resultados no publicados*

BIOMARCADORES CON VALOR PRONÓSTICO EN CAP

En CaP, es sin duda en este campo donde necesitamos de forma más urgente desarrollar biomarcadores que sean validados clínicamente y se puedan incorporar a los distintos nomogramas de variables clínico-patológicas existentes para distintos escenarios.

PCA3 score

En la Tabla III resumimos las distintas variables patológicas clásicamente relacionadas con el pronóstico del CaP y la expresión del PCA3. Como se puede objetivar, existen resultados controvertidos y, de momento, el PCA3 no se puede considerar como un factor pronóstico del peso del Gleason score o del estadio patológico.

La adicción del PCA3 score puede mejorar los modelos predictivos de bajo volumen tumoral (+ 2,4-5,5%) y de CaP insignificante (+3-3,9%), mientras que no mejora la capacidad predictiva de enfermedad extracapsular o invasión de vesículas seminales (31).

El bajo número de pacientes analizados (tabla 2) puede explicar las controversias en el valor pronóstico del PCA3; sin duda el valor pronóstico de este biomarcador merece un análisis con un mayor número de pacientes y examinarlo además como variable pronóstica independiente en estudios multivariados frente a las variables ya conocidas.

[_2]proPSA (p2PSA) y Prostate Health Index (PHI)

La relación entre el p2PSA y sus derivados con el Gleason de la biopsia también ofrece resultados controvertidos en la literatura; existen grupos que apuntan una relación significativa (13, 24) frente a grupos que no observan (25). En un análisis de 384 PR por CaP cT1c se observa una relación significativa con el Gleason de la pieza y con el pT (26). Otro estudio de 311 PR demuestra en el análisis multivariable que la inclusión de %p2PSA o PHI mejoraba la precisión de un modelo basal estándar de 2.4% al 6% para la predicción de factores patológicos de mal pronóstico en el análisis de la pieza (32).

Gen de Fusión

Las distintas poblaciones de pacientes estudiadas y las diferentes técnicas utilizadas para su detección estuvieron de nuevo detrás de la controversia en cuanto al valor pronóstico de la fusión TMPRSS2: ERG. Por ejemplo, en un estudio de 521 PR se relacionó la presencia del gen de fusion con Gleason <7, pero no con el estadio ni con el pronóstico (33) y de forma similar otros autores con 214 casos seguidos más de 12 años no observaron que la fusión en sí marcara un pronóstico diferente (34). Nuestra experiencia en pacientes operados con PR sin hormonoterapia es que la fusión en sí no marca diferencias en SLPBq ni SLP, aunque si se dicotomizan los pacientes en función de la presencia o ausencia de la fusión, se obtienen paneles de factores clínico-patológicos independientes (35). Por el contrario, un estudio sueco de CaP sometido a observación, objetivó que la presencia de la fusión se asociaba a una mayor mortalidad por CaP (36). Mehra et al, en una serie de CPRC metastático demostró que todas las metástasis portadoras de la fusión se asociaban a la variable 2+Edel (37). Dado el potencial pronóstico de conocer el mecanismo de la fusión, y dado que esto se podría caracterizar por FISH en biopsia, ello podría añadir valor pronóstico a la misma.

Más recientemente, la validación del score del gen de fusión como marcador en orina se ha objetivado que sí que está relacionado estadísticamente con el Gleason tanto de la biopsia como de las PR ulteriores, así como con la presencia en pieza de CaP significativo, demostrando que la estratificación de los CaP detectados según el score combinado de PCA3+Gen de fusión ofrece claramente información pronóstica (38).

Existe controversia acerca del valor pronóstico de la expresión de ERG en CaP; una positividad alta a la proteína ERG se asoció Gleason bajo, mayor supervivencia global y un mayor tiempo en desarrollo de resistencia a la castración (39). Sin embargo, en una cohorte de 481 pacientes provenientes del ERSPC, la expresión de ERG (65% de los casos) no se relacionó con ninguna de las variables patológicas clásicas ni con la supervivencia libre de progresión bioquímica (40).

SNPs

El genotipado de SNPs en 156 genes en una población de 1309 pacientes de CaP encontró una expresión diferencial en 5 de ellos (LEPR, CRY1, RNASEL, IL4 y ARVCF) cuya presencia podría marcar diferencias en mortalidad cáncer específica (41). De nuevo, la falta de reproducibilidad de estos estudios y las diferencias entre poblaciones, hacen que estos datos deban ser tamizados y refrendados por otros grupos.

BIOMARCADORES PREDICTIVOS DE PROGRESION BIOQUIMICA POST PROSTATECTOMÍA RADICAL

Tras PR, existen nomogramas robustos y validados basados en variables clínico-patológicas (42-44); se han propuesto variables moleculares que puedan mejorar su capacidad de predicción de supervivencia libre de progresión bioquímica. La metilación de GSTP1 en suero se ha descrito como un factor predictivo independiente de la progresión BQ tras PR en CaP localizado (45).

Se ha descrito de forma preliminar un panel de miRNAs en tejido de PR que conformaban una expresión diferencial entre casos de recurrencia bioquímica precoz y sin recidiva o con recurrencia bioquímica tardía (46). La diferente expresión de SNPs también se ha relacionado con la posibilidad de desarrollar progresión bioquímica tras prostatectomía (47).

Varios grupos han estudiado la expresión de SNPs relacionado con este objetivo; la obtención de diferentes SNPs en los diferentes trabajos y su diferente capacidad predictiva hacen que debamos considerar tanto los paneles de diferentes miRNAs como de SNPs estudios muy preliminares (47-51).

BIOMARCADORES PREDICTIVOS DE RESPUESTA A RADIOTERAPIA

El fallo al tratamiento con RT probablemente atienda a una combinación de factores genéticos y epigenéticos muchas veces compartidos por los fallos a la cirugía o la hormonoterapia. Lo interesante de su caracterización inmunohistoquímica en tejido de biopsia es que pudieran desaconsejar un tratamiento radioteápico del CaP si nos aseguraran la radiorresistencia.

Distintos marcadores tisulares se han estudiado para predecir la posible radiorresistencia de las células tumorales del CaP. El bloqueo de la expresión de CD44 en distintas líneas celulares de CaP se correlacionó con una mejor radiosensibilidad de las mismas (52), por lo que la expresión de CD44 podría ser un ejemplo de molécula a investigar como posible biomarcador de respuesta a RT.

La RTOG ha testado varios marcadores tisulares en sus ensayos y las inmunohistoquímicas con Ki-67, p16, COX-2, MDM2 y PKA se encuentran entre las más usadas con ese fin, pero al ser marcadores tisulares remitimos al lector a excelentes artículos de revisión en este campo (53).

Las CTC también se empiezan a testar en la respuesta a radioterapia de rescate tras PR, observándose de forma preliminar un descenso en el contaje de las mismas cuando existe respuesta a la RT (54). También determinadas fusiones génicas en CaP puede alterar la radio y quimio sensibilidad de las células tumorales, lo que abre en el futuro una mejor caracterización molecular a determinados tratamientos (55).

BIOMARCADORES PREDICTIVOS DE RESPUESTA A HORMONOTERAPIA o QUIMIOTERAPIA

El abiraterone es un buen ejemplo de la investigación que últimamente se ha llevado a cabo buscando nuevos marcadores como las CTC en el CPRC. En esta línea, el GF se ha demostrado en 41% de CPRC mediante CTC y se ha observado que los tumores ERG+ tenían una respuesta a abiraterone en más del 80% de los casos (56). En otro estudio, se detectaron por RT-PRC en 7.5ml de sangre periférica un 37% de CTCs + para el GF; la respuesta al abiraterone fue ligeramente superior (47%) entre los pacientes con CTCs + que entre los pacientes CTCs – (38%) y un contaje de CTCs <5 tras el abiraterone se relacionó con una mejor supervivencia (57). En otro estudio, el contaje de CTC tras un tratamiento mejoraba de forma significativa la predicción de muerte por el CPRC frente al clásico parámetro de reducción del 30-50% del PSA (58).

La presencia del gen de fusión TMPRSS2-ERG en pacientes con CaP pN+ no supuso una respuesta diferente a la hormonoterapia que la los pacientes sin la fusión, no siendo por tanto útil como predictor de respuesta a dicho tratamiento (59). Por el contrario, otro estudio demuestra que los pacientes con CaP ERG+ tenían una respuesta mejor al la supresión androgénica adyuvante que los CaP ERG- (60).

BIOMARCADORES PREDICTIVOS EN PROTOCOLOS DE VIGILANCIA ACTIVA

La cada vez mayor implementación de protocolos de VA derivados del screening oportunista en CaP demanda la validación de biomarcadores que apoyen los criterios de inclusión, progresión y exclusión actualmente basados en criterios clínico-patológicos y donde la cinética del PSA tampoco ofrece resultados definitivos. El PCA3 inicial se ha testado en una serie de vigilancia activa, mostrando una media de 72 los pacientes que progresan en las biopsias de seguimiento frente a 50 los que no lo hacen ($p = 0.08$), no llegando a mostrar su indepen-

dencia como factor pronóstico para ello en el multivariado (p= 0.15) (61). Pese a las discrepancias mostradas en la Tabla II, distintos estudios parecen apuntar que los niveles de PCA3 podrían caracterizar a los tumores en su "perfil de buen pronóstico", es decir, aquellos con un bajo volumen tumoral y con características de CaP insignificante candidatos a VA (31, 62, 63).

De forma similar, los niveles iniciales de -2proPSA en pacientes incluidos en un estudio prospectivo de VA eran significativamente más altos (0.87 ± 0.44 versus 0.65 ± 0.36 pg/mL; P = 0.02) en aquellos pacientes que tuvieron criterios de progresión en las biopsias de seguimiento y se asociaron significativamente con la ocurrencia de una biopsia desfavorable mediante los análisis de Cox y Kaplan-Meier [hazard ratio, 2.53 (1.18-5.41); P = 0.02] (64).

En la actualidad existen gran número de protocolos de VA en marcha y en muchos de ellos se incluyen el análisis de distintos biomarcadores que esperemos próximamente mejoren la selección de pacientes y el seguimiento de los incluidos en esta alternativa terapéutica. Dado el perfil de pacientes de bajo o muy bajo riesgo que se incluyen en VA, podríamos pensar que será más difícil desarrollar biomarcadores que establezcan diferencias pronósticas de inicio; pensamos que el primer objetivo que tendrían que estos potenciales biomarcadores séricos o en orina sería predecir la progresión patológica en las biopsias de seguimiento para poder obviarlas.

PANELES DE MÚLTIPLES BIOMARCADORES

Como queda reflejado en esta revisión, existen múltiples situaciones clínicas donde se necesitan biomarcadores nuevos y más fiables que el PSA. Por otro lado, es lógico que inicialmente el nuevo biomarcador se desarrolle en un contexto de mejorar el diagnóstico; la alta prevalencia del CaP, su heterogenicidad y las distintas vías moleculares potencialmente implicadas en su patogénesis hacen que sea improbable que un único marcador molecular tenga una sensibilidad y especificidad suficientemente altas para considerarlo perfecto en este contexto.

Es lógico pensar que moléculas como el PCA3 y el GF se puedan combinar para mejorar su capacidad diagnóstica, aunando la sensibilidad de aquél y la especificidad de éste, como se demuestra en un estudio piloto combinado de ambos marcadores donde el PCA3 obtuvo una sensibilidad de 93% y la fusión TMPRSS2:ERG una especificidad del 87% (65). A nivel de expresión sobre tejido, ya se ha comprobado como la detección del GF permite disminuir el número de falsos negativos que se le pueden escapar al PCA3 mejorando la sensibilidad de un test combinado (66). En la Tabla IV se muestra los paneles múltiples de marcadores que, asociados al PCA3, aumentan el AUC de éste.

Algunos autores ya presentan aplicaciones prácticas de estas combinaciones de biomarcadores; Salami y cols, en un estudio piloto, proponen dicoto-

Tabla IV. Paneles de biomarcadores múltiples que, asociados al PCA3 score, aumentan el AUC de éste usado de forma aislada.

Autor/año	n	Además de PCA3;	AUC PCA3	AUC modelo
Laxman/2008	257	GOLPH2 / SPINK1 / Gen de fusión	0.662	0.758
Aubin/2008	105	Gen de fusión / PSA	0.650	0.801
Rigau/2010	154	PSMA / PSGR	0.60	0.74
Tomlins/2011	1312	PSA / Gen de fusión / PCPT risk calculator	-	0.75
Salami/2011	45	Gen de fusión / PSA	0.65	0.88
Jamaspishvili/2011	176	AMACR / MSMB / TRPM8	0.671	0.771
Cao/2011	131	Gen de fusión/ AnexineA3/Sarcosine/PSA	0.733	0.856
Leyten/2012	443	Gen de fusión	0.720	0.758

mizar los pacientes candidatos a biopsias según un PSA mayor o menor de 10 ng/ml, y en cada grupo indicar o no la biopsia según la presencia de PCA3 +/- GF detectados en orina, pudiéndose ahorrar hasta 67% de las biopsias innecesarias con solo un 15% de biopsias falsas negativas (65).

CONCLUSIONES

La necesidad de nuevos biomarcadores en distintas situaciones clínicas que plantea el CaP es una obviedad que obliga a estar muy atentos a la ingente investigación básica y exigirle a ésta el cumplimentar todos las fases exigidas al desarrollo de un biomarcador para su implementación en clínica y un buen y reciente ejemplo de ello ha sido el PCA3, que ha supuesto un renacer en el interés de la comunidad urológica por el refinamiento diagnóstico y la caracterización pronóstica del CaP.

El acceso a una determinación asequible y reproducible de nuevos biomarcadores como el GF y las isoformas del PSAl hará que próximamente debamos comparar bioestadísticamente unos biomarcadores con otros en distintos escenarios. En CPRC será importantísimo caracterizar biomarcadores que permitan caracterizar qué tratamientos aplicar en cada caso, dado el gran número de alternativas que se nos abren y el coste de las mismas; es posible que las CTCs encuentren su sitio en la caracterización y seguimiento de las terapéuticas para el CPRC.

Pensamos que sin duda el uso combinado de los distintos marcadores en orina y suero en paneles de biomarcadores múltiples será, probablemente junto a la caracterización radiológica del CaP, el escenario que marcará el diagnóstico y el perfil pronóstico del CaP en un futuro a medio y largo plazo, obviando biopsias innecesarias, disminuyendo la tasa de sobrediagnóstico y sobretratamiento, perfeccionando los protocolos de VA y seleccionando y valorando los distintos tratamientos en CPRC.

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ORIGINAL ARTICLE

Optimizing prostate cancer screening; prospective randomized controlled study of the role of PSA and PCA3 testing in a sequential manner in an opportunistic screening program☆

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KEYWORDS
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Abstract
Objectives: To reduce unnecessary biopsies (Bx) in an opportunistic screening programme of prostate cancer.
Material and methods: We performed a prospective evaluation of PCA3 as a second-line biomarker in an opportunistic screening for prostate cancer (PCa). From September 2010 until September 2012, 2,366 men, aged 40–74 years and with >10 years life expectancy, were initially screened with PSA/digital rectal examination (DRE). Men with previous Bx or with recent urine infections were excluded. Men with abnormal DRE and/or PSA > 3 ng/ml were submitted for PCA3. All men with PCA3 ≥ 35 underwent an initial biopsy (IBx) —12cores—. Men with PCA3 < 35 were randomized 1:1 to either IBx or observation. Re-biopsy (16–18 cores) criteria were PSA increase > .5 ng/ml at 4-6months or PSAv > .75 ng/ml/year.
Results: With a median follow-up (FU) of 10.1 months, PCA3 was performed in 321/2366 men (13.57%), 289 at first visit and 32 during FU. All 110 PCA3+ men (34.3%) were biopsied and PCa was identified in 43 men in IBx (39.1%). In the randomized arm, 110 were observed and 101 underwent biopsy, finding 12 PCa (11.9%), showing a statistically significant reduction of PCa detection rate in this cohort ($p < .001$). Global PCa detection rates were 40.9% and 9.5% for the PCA3+ and PCA3− branches, respectively ($p < .001$). Area under the curve for PSA and PCA3 were .601 and .74, respectively. This is an ongoing prospective study limited by its short follow-up period and still limited enrolment.

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Conclusions: PCA3 as a second-line biomarker within an opportunistic dual screening protocol can potentially avoid 65.7% and 50.1% biopsies at first round and at median FU of 10.1 months, respectively, just missing around 3.2% of high grade PCa.

PALABRAS CLAVE
Cáncer de próstata;
Cribado;
PCA3;
Biopsia prostática

Optimización de un programa de cribado oportunista de cáncer de próstata; ensayo aleatorizado prospectivo del papel del PSA y del PCA3 en uso secuencial

Resumen

Objetivos: Reducir el número de biopsias (Bx) innecesarias en un programa de cribado oportunista en cáncer de próstata (CaP).

Material y métodos: Estudio prospectivo y aleatorizado evaluando el PCA3 como biomarcador de segunda línea. De septiembre de 2010 a septiembre de 2012 2.366 hombres con edad en rango 40–74 años, y más de 10 años de expectativa de vida, fueron estudiados mediante PSA y tacto rectal (TR), excluyendo los biopsiados previamente o con infección urinaria reciente. Ante un TR sospechoso y/o PSA > 3 ng/ml se les realizó un PCA3. A todos aquellos con PCA3 ≥ 35 se les realizó una Bx inicial (IBx) —12 cilindros—. Con PCA3 < 35 fueron aleatorizados 1:1 a IBx u observación. Los criterios de rebiopsia (16–18 cilindros) durante el seguimiento fueron un incremento de PSA > 0,5 ng/ml a 6 meses o PSAv > 0,75 ng/ml/año.

Resultados: Con un seguimiento medio de 10,1 meses se testó el PCA3 en 321/2.366 hombres (13,57%), 289 en la primera visita y 32 durante el seguimiento. Entre los 110 hombres con PCA3+ (34,3%) se identificó CaP en 43 en IBx (39,1%). En el brazo aleatorizado 110 se observaron y 101 se biopsiaron, encontrando 12 CaP (11,9%), mostrando un reducción en la detección de CaP estadísticamente significativa en esta cohorte ($p < 0{,}001$). Las tasas de detección global de CaP fueron de 40,9 y 9,5% para las ramas PCA3+ y PCA3− respectivamente ($p < 0{,}001$). AUC para PSA y PCA3 fueron 0,601 y 0,74. Este es un protocolo abierto en este momento, limitado por su seguimiento insuficiente.

Conclusiones: El PCA3 como biomarcador de segunda línea en un programa de cribado oportunista podría potencialmente evitar un 65,7% de IBx y 50,1% a 10 meses de seguimiento, dejando de diagnosticar 3,2% de CaP de alto grado.

Background

The results of the European Randomized Study of Screening for Prostate Cancer (ERSPC) after 11 years of follow-up have shown that a lower number of patients required screening and a lower number of patients required life-saving treatment. Compared with previous publications, the study also showed an improvement in cancer-specific survival compared with the control arm. However, this result was at the expense of too many negative biopsies (Bx).[1] In addition to optimizing the age ranges to be included and the chronology of the screening, we all seek to avoid unnecessary biopsies, given the undesirable morbidity (infections, bleeding, urinary obstruction, etc.)[2,3] and emotional stress that biopsies inflict on healthy men.[4,5]

PCA3 is an FDA-approved biomarker of prostate cancer (PC) for the indication of repeat biopsies with a cutoff point of 25. After an initial internal validation was conducted on the data provided by the commercial kit and the data published to date with a cutoff point of 35, we began using PCA3 in January 2009, along with other clinical variables to optimize our clinical judgment for indicating an initial biopsy (IBx) or a rebiopsy. Prior to this date, we biopsied all our patients regardless of their PCA3. We achieved the best performance of PCA3 for IBx.[6] In this context, nomograms that consider PCA3 significantly improve the baseline clinical models that lack PCA3 ($p < .001$), improving their predictive capacity by 4.5–7.1% after including PCA3, leaving only 2% of high-grade PCa undiagnosed.[7]

Given that the role of PCA3 in the framework of opportunistic screening is yet to be established, we designed a prospective randomized study using PCA3 as a second-line biomarker after PSA and rectal examination (DRE) performed by a urologist. Our primary objective was to assess our preliminary result in terms of the potential reduction in the number of biopsies, without undermining the inherent benefits of opportunistic screening. Our secondary objectives were to evaluate the false negative rates for PCA3 and their prognostic value within opportunistic screening.

Material and methods

Our opportunistic screening program for PCa consists of a prospective randomized study (Fig. 1) with the following inclusion criteria: healthy men aged 40–75 years, with more than 10 years of life expectancy, with no prior Bx, who freely committed to the protocol and signed the informed consent for that purpose (opportunistic screening and nonpopulational). The study was approved by the Ethics Committee of the Valencian Foundation Institute of Oncology (no. ref. 2010-20).

During the initial visit, a specialist nurse reviewed the general medical history with an emphasis on dietary habits and obtained the PSA, after which a urologist evaluated the

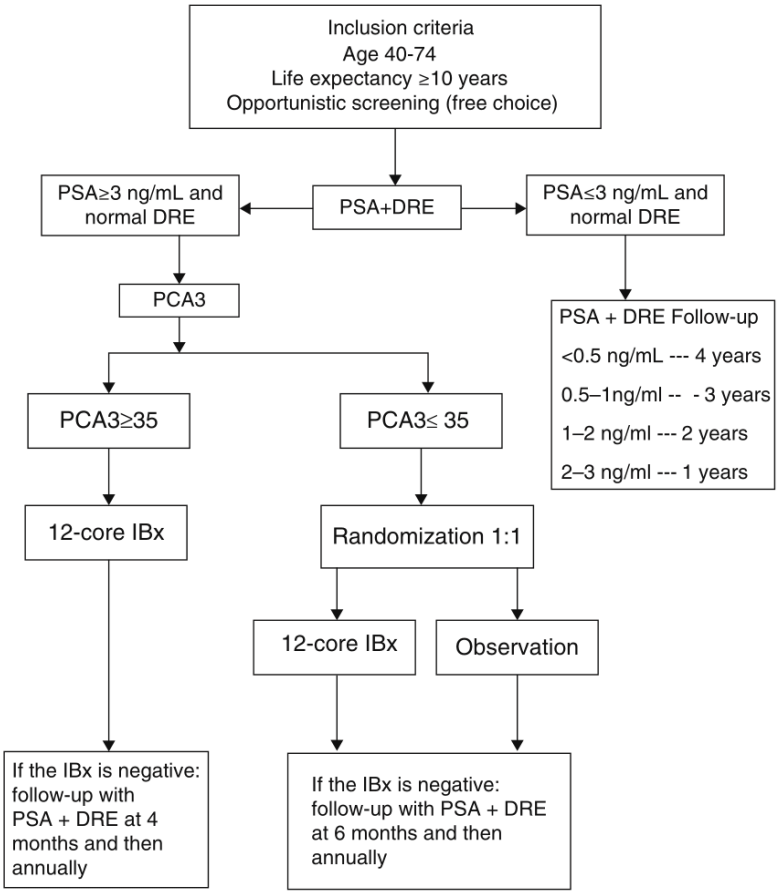


Figure 1 Study scheme. *Abbreviations*: IBx, initial biopsy; PSA, prostate-specific antigen; DRE, digital rectal examination.

urological, sexual and family history of PCa and performed the DRE. Men who had already been biopsied or who had a history of prostatitis or urinary infections during the previous year were excluded. Men with normal DRE and PSA results (<3 ng/dL) proceeded to PSA and DRE monitoring at 1, 2, 3 or 4 years, based on whether the PSA level was <0.5, 0.5–1, 1–2 or 2–3 ng/mL, respectively. The rest of the candidates or study group (PSA ≥ 3 ng/mL and/or abnormal DRE results) underwent a second DRE by another urologist, and their PCA3 levels were determined (Progensa™ PCA3 test; Genetics Probe-Hologic, San Diego, USA).

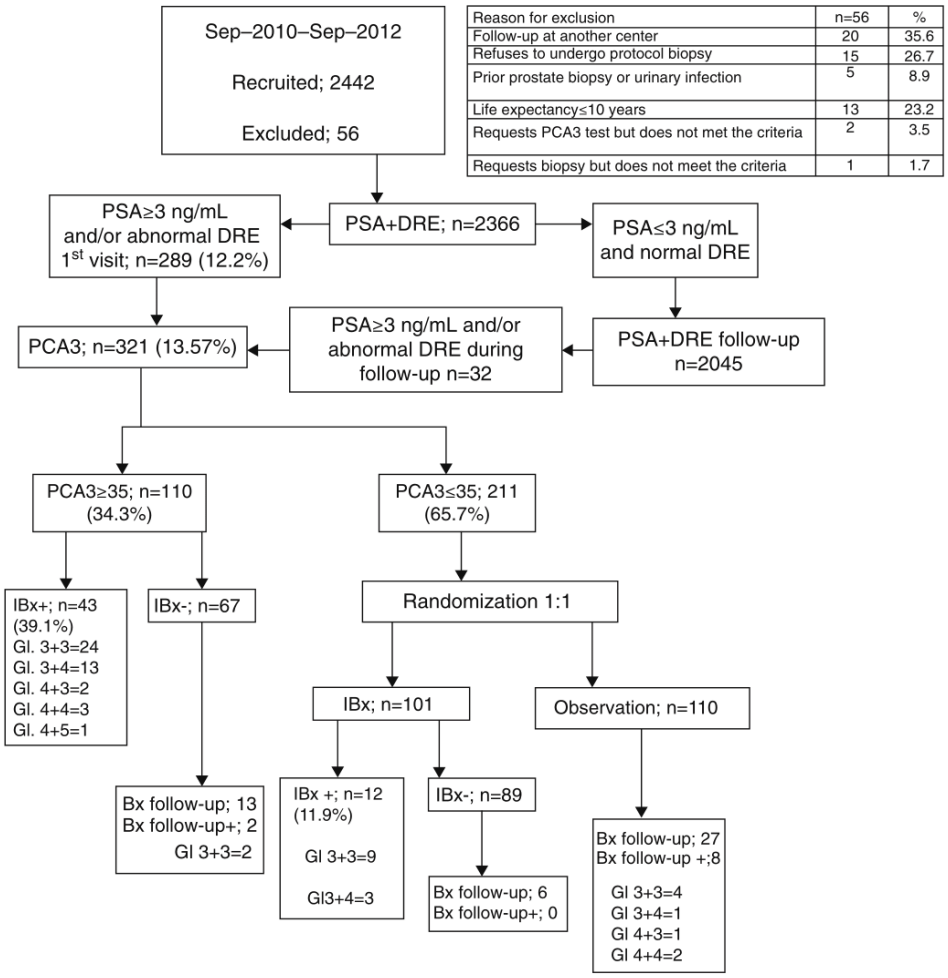
All men with PCA3 levels ≥ 35 were recommended to undergo a 12-core IBx. Participants with PCA3 levels < 35 were blindly randomized 1:1 (using a software application) to IBx or observation (Fig. 1). Participants with positive biopsies for PCa were withdrawn from the study.

The men with negative PCa Bx were scheduled for 4-month follow-ups if their PCA3 levels were ≥35 or 6-month follow-ups if their PCA3 levels were <35, due to a recommendation by the center's ethics committee, and then annually, always performing PSA determinations and DREs during each visit. Rebiopsy (16–18 core) was proposed during the follow-up according to the following criteria: increased PSA levels > 0.5 ng/mL since the last visit, PSAv levels > 0.75 ng/mL/year or persistent or de novo abnormal DRE results.

The initial sample size was established at 1065 men; however, given the initial results and in accordance with the ethics committee and health authorities, we continued recruiting volunteers and the protocol remained open. The data were analyzed with the SPSS and R statistical packets. The frequency comparisons were performed with the χ^2 test (Fisher's exact test for 2×2 contingency tables) for categorical variables. For the continuous variables, the differences between means were studied with Student's t-test when the associations were acceptable. When the normality was not acceptable, the samples were compared using the Mann–Whitney *U* test. The areas under the curve (AUC) of the receiver operating characteristic (ROC) were compared with the De Long test. We used a two-sided test, and a *p*-value ≤ .05 was considered statistically significant. The randomization was automatic using a computer program blind to the study.

Results

The screening began in September 2010, and from then until September 30, 2012, 2422 men requested PCa screening (Fig. 2). We included 2366 participants and rejected 56 cases for various reasons (Fig. 2). Their mean age was 57.5 (SD, 6.2) years, with a median of 57 years and a range of 40–74



Reason for exclusion	n=56	%
Follow-up at another center	20	35.6
Refuses to undergo protocol biopsy	15	26.7
Prior prostate biopsy or urinary infection	5	8.9
Life expectancy≤10 years	13	23.2
Requests PCA3 test but does not met the criteria	2	3.5
Requests biopsy but does not meet the criteria	1	1.7

Figure 2 Results of the study. *Abbreviations*: Gl. Gleason score; IBx, initial biopsy; PSA, prostate-specific antigen; RE, rectal examination.

years. The median follow-up time for the entire group was 10.1 months (range: 1–33 months).

At the initial visit, 289 men (12.2%) entered the study for an abnormal PSA and/or DRE. Another 32 joined during the follow-up, ultimately resulting in 321 (13.6%) men tested for PCA3 in second line. This study group had a mean age of 60.8 years (SD: 5.9; range: 43–74 years), significantly greater than that of the overall group, with a mean age of 57 years (SD: 6.1; range: 40–74 years) ($p < .001$). The mean PSA level of the study group was 4.63 ng/mL (SD: 2.25) with a median of 4.04 ng/mL (0.37–19.50). A suspicious DRE was detected in 20 men, this being the only factor of inclusion for 15 of the men.

In the study group, the mean and median PCA3 levels were 33.7 (SD: 39) and 23, respectively (range: 1–371). A weak relationship was detected between PSA and PCA3, which was not significant ($p = .377$). A total of 110 men had PCA3 levels ≥ 35 (34.3%), with these men being a mean of 2 years older than those with PCA3 levels < 35 ($p = .006$), but without showing differences in their PSA readings ($p = .122$).

A total of 211 IBx were performed; 110 of these men were PCA3+ and 101 were PCA3−, all of whom were randomized to this procedure. Table 1 shows the results of the IBx and the statistically significant relationship between a PCA3+ and the diagnosis of PCa in IBx ($p < .001$). The mean and median of the PCA3 levels among the patients with PCa were 71.5 (SD, 67.2) and 54, respectively (range, 11–371), while these statistics for negative IBx were 33.1 (SD, 27.8) and 28 (range, 1–189), respectively. Fig. 3 shows the ROC curves for PSA and PCA3. The AUC for PSA was 0.601 (95% CI: 0.514–0.689) and 0.748 (95% CI: 0.677–0.819) for PCA3, showing statistically significant differences ($p < .008$). The cutoff of 35 for PCA3 achieved 78.2% sensitivity and 57.1% specificity. Table 2 shows these statistics for other cutoff points. Regarding the prognostic variables of IBx, there were no differences in the number of affected cores or in the percentage of involvement of the cores among the specificity with PCA3+ and PCA3−, with both groups having a median of 2 affected cores and a range of 1–10. Sixty percent of the PCa diagnosed with IBx was Gleason 3 + 3, while 75% of PCa were diagnosed with PCA3 < 35, a group in which only 3 Gleason 3 + 4 PCa were diagnosed. The 6 cases detected with primary Gleason 4 in IBx had PCA3 levels ≥ 35 (Fig. 2 and Table 1).

The remaining 266 men of the study group with no IBx or negative IBx were monitored. We performed protocol biopsies in follow-up in 13, 6 and 27 men in the groups of men

Table 1 Results of the initial biopsy and follow-up biopsies and Gleason score according to PCA3 branch.

	PCA3 < 35		PCA3 ≥ 35 $n = 110$
	Randomized IBx $n = 101$	Randomized observation $n = 110$	
IBx (n)	101	0	110 (100%)/
PCa detection	12 (11.9%)[a]		43 (39.1%)[b]
Bx during follow-up (n)	6/89 (6.7%)	27/110 (24.5%) 8/110	13/67 (19.4%) 2/67 (2.9%)[d]
PCa detection	0	(7.3%)[c]	
Detection of overall PCa up to the last follow-up	20/201 (9.5%)	45/110 (40.9%)	

Abbreviations: Bx, biopsies; PC, prostate cancer; IBx, initial biopsy.
[a] Nine cases of Gleason 3 + 3 and 3 Gleason 3 + 4.
[b] Twenty-four cases of Gleason 3 + 3, 13 Gleason 3 + 4, 2 Gleason 4 + 3, 3 Gleason 4 + 4 and 1 Gleason 4 + 5.
[c] Four cases of Gleason 3 + 3, 1 Gleason 3 + 4, 1 Gleason 4 + 3 and 2 Gleason 4 + 4.
[d] Two cases of Gleason 3 + 3.

Table 2 Statistics for various PCA3 cutoff points.

PCA3	Sensitivity (%)	Specificity (%)
≥10	100.0	26.9
≥15	94.5	33.3
≥20	87.3	41.0
≥25	83.6	47.4
≥30	80.0	53.2
≥35	78.2	57.1
≥40	70.9	63.5
≥45	63.6	70.5
≥50	56.4	74.4

with PCA3+, PCA3− with negative IBx and PCA3− randomized to follow-up, respectively. In the same 3 groups, we diagnosed 2, none and 8 additional cases of PCa (Fig. 2). When we compared the PCA3+ and PCA3− study group arms, we found PCa detection rates of 40.9% and 9.5%, respectively (Table 2), showing clear and statistically significant differences ($p < .001$). Up to this review, only 3 cases with primary Gleason 4 had been detected in the PCA3− arm (1.4%).

As previously mentioned, we found statistically significant differences in the rates of PCa detection in IBx among the men with PCA3 levels ≥ 35 vs. PCA3 levels < 35 (39.1 vs. 11.9% [Table 1]). However, if we had only biopsied the men with PCA3+, we would not have diagnosed 11.9% of the latent PCa in the IBx, although none of them were primary Gleason 4. Extrapolating these results to a possible implementation of this protocol, we could avoid a total of 65.7% of IBx (211 men with PCA3− out of a total 321 candidates). With the current mean follow-up of 10.1 months, we had to perform follow-up biopsies for 27 (24.5%) men with PCA3− following our rebiopsy criteria.

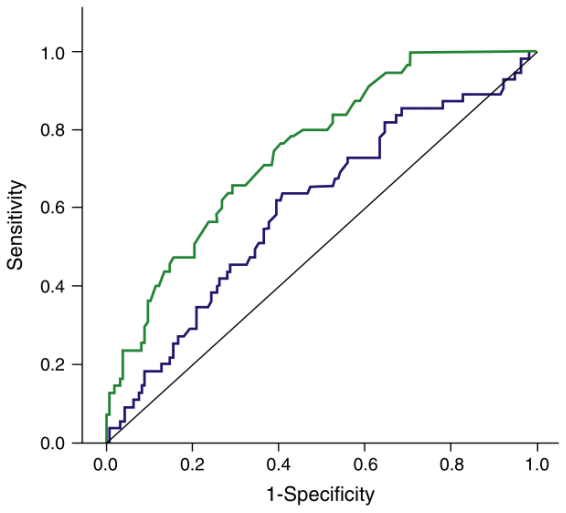


Figure 3 ROC curves for PSA (blue curve) and PCA3 (green curve).

Discussion

Although populational and opportunistic screening has not been formally compared, the former has not been accepted by various health authorities due to the overdiagnoses it entails.[8] However, there is no turning back the use of opportunistic screening in primary medicine and in our society, which is a system that has resulted in reduced mortality due to PCa. In the epidemiological registry, sponsored by the Spanish Urological Association in 25 public hospitals, the incidence of PCa in Spain, adjusted for age, was 70.75 cases per 100,000 inhabitants. Of these, 40% were of low risk and the majority were detected by opportunistic screening,[9] reliable reflections of the sociodemographic problem that confronts public health.

The latest results of the Finnish, Dutch and Swedish branches of the ERSPC (with median follow-ups of 12, 12.8 and 16 years) were recently presented at the UAE-2013 Congress. The results showed a reduction in cancer-specific mortality in its study groups, with rates between 0.44 and 0.67. The number of men necessary for screening was between 208 and 1199, and the number of diagnoses necessary to save a life was between 9 and 25, readings much lower than those published with shorter follow-up periods.[1,10] The differences between these results could

be justified by the differences in strategies and follow-ups allowed by the ERSPC, which points to the possibility of optimizing the scheduling of PCa screening and the need for developing tools for this screening. Age ranges and baseline PSA are examples of these differences and have been proposed as guidelines for the scheduling of screening during follow-up.[11] Other variables such as family history, the DRE, prostate volume and the presence of prior negative biopsies also form part of the risk calculations for experiencing PCa within a screening program.[12,13]

The optimal cutoff for PCA3 is still controversial, as is the cutoff for PSA after more than 25 years of use, and might not be the same in follow-up IBx or Bx. However, this has still not been tested clearly and prospectively within the framework of opportunistic screening.[12] PCA3 has been tested as a first-line marker with a cutoff of 10 in a pilot study within the Dutch branch of the ERSPC, comparing it with PSA. However, we believe that such a low cutoff point is the reason there would have been no significant differences in the AUC for detecting PCa between the PCA3 (0.64) and the PSA (0.58) ($p = 0.143$), thus requiring 75% of the IBx men, and therefore, not resolving the problem of excessive number of IBx.[14]

Our approach was to assess whether the combination of PSA (with its acceptable sensitivity) as a first-line marker and PCA3 (with its better specificity) as a second-line marker[12,15–17] could provide us with a better selection process for IBx, thereby avoiding unnecessary biopsies. We observed that the detection rates in IBx showed significant differences among men undergoing screening in the traditional manner with PCA3 $\geq$ 35 vs. PCA3 < 35 (39.1 vs. 11.9%) (Table 1). Assuming that we would have only biopsied those men with PCA3+, we would not have detected 11.9% of latent PCa in the IBx, none with primary Gleason 4 (Fig. 2). Therefore, a potential savings of 65.7% of IBx should be weighed with the rate of false PCA3 negatives with a cutoff of 35. We believe, however, that our results require us to continue analyzing the regimen we are proposing.

It is interesting to recognize that more PCa has not been diagnosed in the group of repeat biopsies with PCA3−. However, with a 10-month follow-up, we can see how 24.5% of the men in the group subjected to observation required an IBx (27/110) invoking our rebiopsy criteria, which we considered strict but necessary when faced with a healthy man who wants to know if he has PCa. Therefore, if they had not been randomized and assuming this rate in the entire PCA3− arm, we would have saved 50.1% of Bx at 10.1 months of follow-up.

We recognize various weaknesses in our study. The first is the apparently insufficient recruitment period (24 months) and especially the mean follow-up (10.1 months). However, we believed it necessary to analyze our preliminary results to justify or discard the protocol. We now believe that a longer follow-up would enable us to compare detection rates, biopsy avoidance rates and cost-effectiveness studies of the protocol with traditional screening schemes, calculators of risk and nomograms, tools that improve the isolated use of PSA as a decision element for Bx.[18,19] We found 3 cases of potentially lethal PCa (primary Gleason 4) with this short follow-up. Therefore, the big question to be answered with longer follow-ups is whether the savings in initial biopsies would delay the detection of these 3.2% Gleason PCa $\geq$ 7 not diagnosed in the PCA3− branch of the protocol, and to what extent it could worsen the cancer-specific and overall survival rates of these patients. This rate is slightly greater than the 1% of undiagnosed Gleason PC $\geq$ 7 when the PCA3 is added for better clinical judgment in a scenario of repeat Bx with PSA levels between 2.5 and 10 ng/mL.[20]

We also recognize that a man with a suspicious DRE could be randomized to observation if his PCA3 level is less than 35. Despite this situation not having occurred so far and knowing its high positive predictive value,[21,22] when the case does present itself we will offer a biopsy outside of the protocol if the patient does not wish to wait for a reassessment at 6 months when, if the suspicious DRE persists, a follow-up Bx would be performed per protocol.

We recognize that repeating the PSA measurements within a prudent time before the performance of the PCA3 would probably have reduced its indication.[23] We therefore did not include men with urinary tract infections or prostatitis in the year prior to their consultation. With a longer follow-up, we can assess pharmacokinetic parameters of PSA for the indication of PCA3, as well as other biomarkers in multimodal panels form,[24–26] as other authors have done. We agree with these authors that this is the road towards improving the AUC for the detection of PCa.[26–28]

Conclusions

Our initial data show that the use of PCA3 at a cutoff of 35 as a second-line biomarker within an opportunistic screening program could entail a potential savings in initial biopsies of 65.7% in the first visit and 50.1% at 10.1 months of follow-up, leaving approximately 3% of Gleason PCa $\geq$ 7 undetected. In this context, we should accept a rate of false negatives for PCA3 of approximately 12% and its possible diagnostic delay, knowing that the majority is low-grade PCa. We need a longer follow-up to understand its true value as a diagnostic and prognostic tool for our protocol and thereby weigh the rate of biopsy savings and its cost.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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NUEVOS BIOMARCADORES PARA OPTIMIZAR SELECCIÓN Y SEGUIMIENTO DE PACIENTES CON CÁNCER DE PRÓSTATA EN VIGILANCIA ACTIVA

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Resumen.- *La identificación de biomarcadores que, en el momento del diagnóstico del CaP, se asocian con la presencia de enfermedad o de un comportamiento más agresivo del CaP transformará el manejo clínico de esta enfermedad. Si tanto los pacientes como los clínicos contaran con herramientas reproducibles y válidas para estimar el riesgo específico de la morbilidad asociada al CaP, entonces muchos pacientes optarían y se adherirían a los protocolos de vigilancia activa (VA), y consecuentemente se reducirían los costes y comorbilidades asociados al sobretratamiento actual del CaP. Así un biomarcador, o un panel de biomarcadores, con elevada especificidad para identificar pacientes con riesgo de progresión en protocolos de VA, identificaría a aquellos hombres que pudieran beneficiarse de protocolos de VA menos intensos con menos biopsias de repetición, reduciendo así el riesgo y los costes de estos procedimientos invasivos. En esta revisión se pretende ofrecer una visión de los nuevos biomarcadores identificados por técnicas genómicas y discutir su posible papel en un contexto de VA. Por otra parte, el protocolo de VA, ofrece un marco adecuado para la validación de biomarcadores asociados a la progresión de la enfermedad.*

Palabras clave: *Cáncer de próstata. Vigilancia activa. Biomarcadores.*

Summary.- *Identification of biomarkers that, at the time of diagnosis of prostate cancer (PCa) , are associated with presence of disease or a more aggressive behavior will transform the clinical management of this disease. If both patients and clinicians would have reproducible and valid tools to estimate the specific risk of morbidity associated with PCa, then many patients would opt to and join active surveillance (AS) protocols, and consequently costs and comorbidities associated with the current overtreatment of prostate cancer would be reduced. Thus, a biomarker, or a panel of biomarkers, with high specificity to identify patients at risk for progression in AS protocols, would identify those men who could benefit from less intensive AS protocols with less repeated biopsies, so reducing the risk and cost of these invasive procedures. In this review we try to offer*

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an overview of the new markers identified by genomic techniques and to discuss their potential role in an AS context. Moreover, the AS protocol offers an adequate setting for validation of biomarkers associated to disease progression.

Keywords: *Prostate cancer. Active Surveillance. Biomarkers.*

INTRODUCCIÓN

La era del cribado con el antígeno prostático específico (PSA) se ha asociado claramente con la migración del estadio del cáncer de próstata (CaP), en el sentido de que una alta proporción de los nuevos casos diagnosticados presentan características que se asocian con un riesgo muy bajo de invasión, metástasis, y consiguientemente de morbilidad y mortalidad (1). Algunos estudios han examinado la historia natural de estas neoplasias de bajo riesgo, mostrando que la vasta mayoría de los hombres con este diagnóstico muere por otras causas a las del CaP, incluso si ellos ya se encuentran en un régimen primario de tratamiento con intención curativa (2). Sin embargo, la asignación de un CaP de bajo riesgo no equivale a una ausencia completa de riesgo, de hecho la mayoría de los pacientes contemporáneos con CaP de bajo riesgo eligen someterse a un tratamiento con intención curativa como la cirugía o la radioterapia con el coste y los efectos secundarios que se asocian a este tipo de medidas (1, 3). Este tipo de prácticas ha suscitado grandes debates en relación al sobrediagnóstico y sobretratamiento y la conveniencia de un cribado basado en la determinación del PSA (4-6).

Para resolver el problema del sobretratamiento, se plantea la estrategia terapéutica de la vigilancia activa (VA) como alternativa en el manejo del paciente con CaP de bajo riesgo. La VA incorpora medidas seriadas del PSA, exámenes físicos, y biopsias prostáticas repetidas para monitorizar la posible presencia de enfermedad agresiva oculta o de la progresión hacia un fenotipo más comúnmente asociado a la metástasis y a la mortalidad. Sin embargo, la aceptación de la VA se ve limitada por varias razones entre las que se incluyen la falta de consenso sobre los criterios óptimos de selección e indicadores de intervención, la falta de seguimientos largos en las series de VA publicadas, así como inconsistencia en sus diseños, y por último, miedo tanto en pacientes como clínicos en perder el margen de curabilidad. Es importante también destacar, como se expondrá más adelante con mayor detalle, que el CaP exhibe en la mayoría de los casos un patrón de multifocalidad que puede manifestarse como lesiones independientes con diferentes grados patológicos y distintas características moleculares (7).

Un muestreo inadecuado en la próstata mediante las técnicas convencionales de biopsia, la falta de conocimiento en relación a los ratios de progresión de la enfermedad junto con la ausencia de diferentes modalidades de diagnóstico por imagen capaces de determinar con precisión el volumen y la histología tumoral han promovido la incorporación biopsias de repetición en los protocolos de VA (8-10). Sin embargo, y aunque la morbilidad asociada al programa de VA es baja, la disconformidad, el coste y el problema del muestreo inadecuado inherente a la práctica de la toma de biopsias recomiendan el desarrollo de biomarcadores no invasivos capaces de reflejar los cambios que se producen en la glándula prostática con el tiempo gracias a la posibilidad de poder medirlos en el transcurso de la VA.

Con esta revisión pretendemos describir y remarcar qué biomarcadores desde el punto de vista genético pueden ser de utilidad en un contexto de VA en CaP tanto por su especificidad como por que predicen un comportamiento más agresivo del tumor. Por otra parte, también proponemos el contexto de la vigilancia activa como un entorno ideal para la validación de estos biomarcadores.

Complejidad genómica del CaP

La revolución biotecnológica de los últimos años con técnicas como las ómicas (genómica, proteómica, epigenómica, metabolómica) y la secuenciación de nueva generación o NGS (Next Generation Sequencing), junto con los nuevos conocimientos de la biología del cáncer, han cambiado el concepto que teníamos del CaP y hemos pasado de tener una enfermedad pobremente entendida y heterogénea desde el punto de vista clínico a una colección de subtipos homogéneos inidentificables por criterios moleculares (11). En este sentido, y al igual que sucede en otras neoplasias como las leucemias, linfomas o el cáncer de mama, la clasificación molecular del CaP constituirá un paso crítico y fundamental tanto en el desarrollo de biomarcadores que diferencien formas agresivas e indolentes de la enfermedad como el desarrollo de terapias dirigidas a determinados biotipos tumorales (12).

Gracias a estos estudios, sabemos que los genomas del CaP muestran relativamente pocas pérdidas y/o ganancias cromosómicas, siendo la pérdi-

da focal más común la del gen supresor de tumores *PTEN*, y en total también presentan una baja frecuencia de mutaciones comparado con otros tumores (alrededor de una mutación por Megabase de ADN) (13). En cánceres localizados, las mutaciones puntuales son más comunes en *SPOP*, que codifica para el componente del substrato de reconocimiento de una ubiquitin ligasa, *TP53* y *PTEN*, aunque con relativa baja frecuencia (solo entre el 5-10% para cada uno de los genes) (14-17). Sin embargo, los estudios más recientes nos revelan que la mayoría de los CaP son portadores de fusiones génicas recurrentes en los que está implicado alguno de los miembros de la familia de factores de transcripción ETS (18) y que los análisis tanto transcriptómicos como genómicos muestran que los CaP pueden clasificarse en función de los perfiles de expresión o de las aberraciones somáticas en cuanto a variaciones del número de copias génicas (15, 19).

Por tanto, el espectro de lesiones genómicas específicas en CaP es diversa, con una considerable heterogeneidad molecular entre los tumores. Pero a pesar del esfuerzo que se ha llevado a cabo a la hora de catalogar estas alteraciones en CaP, el significado pronóstico de la mayoría de estas alteraciones permanece incierto. En el marco del CaP podemos encontrarnos con una serie de retos a la hora de establecer estas asociaciones, por ejemplo, la larga historia natural del CaP, la necesidad de un tiempo de seguimiento largo en cohortes de pacientes bien establecidas, aspectos de la recogida de muestras y la multifocalidad tumoral, y las complicaciones a la hora de definir y comprender las lesiones iniciantes de la lesión tumoral de aquellas que se asocian con la progresión y la mortalidad (13). También constituye un reto trasladar estos conocimientos en el contexto de la VA, en la que no existen programas específicos diseñados para la validación de estos biomarcadores.

La misma heterogeneidad del CaP complica la estratificación del riesgo y la selección de los pacientes para su manejo clínico. Sin embargo, la clasificación molecular del CaP tiene la premisa de identificar subclases específicas de CaP asociadas con distintos patrones de anormalidades genómicas. Comprender bien estas diferencias en el contexto biológico y clínico, evaluar su impacto pronóstico y establecer unas herramientas adecuadas para su identificación harían fácilmente trasladables estos conocimientos a un programa de VA.

A continuación vamos a exponer aquellos biomarcadores recientemente identificados que por su implicación biológica en la patología y progresión del CaP podrían jugar un papel relevante en el contexto de la VA.

PCA3

El desarrollo de nuevos métodos diagnósticos en CaP poco invasivos basados en suero u orina suponen una verdadera necesidad clínica para mejorar el estándar actual basado en PSA sobre los que evaluar la presencia de nuevos biomarcadores [para revisión consultar ref. Rubio-Briones J et al. (20)]. Algunos de estos biomarcadores tienen más recorrido que otros, pero muy pocos se han testado en el contexto de la VA. Así, en febrero del año 2012, la FDA aprobó un test diagnóstico (PROGENSA® PCA3 Assay; Hologic-GeneProbe) basado en la detección en orina del transcrito no codificante PCA3 [un gen específico del CaP que se expresa en el 95% de los CaP (21)] que ha mostrado su utilidad no solo en el diagnóstico del CaP (22, 23), sino también en la identificación de CaP con un comportamiento más agresivo (22). Sirva como ejemplo el estudio llevado a cabo por Ploussard y cols. (24) sobre una serie de 106 hombres con CaP de bajo grado (PSA <10ng/ml, cT1c-cT2a y Gleason de la biopsia de 6) tratados con prostatectomía para comprobar si el PCA3 puede identificar pacientes candidatos para VA. El análisis multivariable de este estudio, a pesar de que el tamaño muestral se puede considerar bajo, muestra que el score del PCA3>25 se asocia con un incremento de 5.47 veces a tumores con volúmenes >0.5cc (p=0.01) y con un incremento de 12.74 veces a CaP 'significativos' (p=0.003). Basados en estos datos, los autores concluyen que el PCA3 podría ser útil en la selección de pacientes candidatos a VA. Sin embargo, y a pesar de esto, tenemos pocos datos consistentes que confirmen el papel del PCA3 en el contexto de la VA. De hecho, un estudio llevado a cabo en el marco del programa de VA de la Johns Hopkins sobre 294 pacientes no demostró ninguna diferencia en valores de PCA3 entre los pacientes que progresaron y no progresaron en las biopsias de repetición de vigilancia (p=0.131) (25).

Pro-PSA e índice PHI

Recientemente se han descrito distintas formas moleculares del PSA libre (PSAl), precursores inactivos que se expresan diferencialmente en HBP como el PSA-benigno y el PSA-intacto, o el proPSA, que se expresa más en CaP y que es enzimáticamente inactivo. Una de las isoformas del proPSA, el -2proPSA se ha erigido en un potencial biomarcador al comprobarse su mayor concentración en tejido canceroso a diferencia de otras isoformas (26). El desarrollo clínico de este nuevo biomarcador ha permido integrarlo en un modelo matemático junto al PSAt y al PSAl para generar el Beckman Coulter prostate health index, más conocido como índice PHI. Es importante recordar que el -2proPSA tiene mínimo

valor como marcador único y sin embargo, al igual que con el PSAI, su ratio frente a éste y su conjugación en la fórmula del índice PHI son las que ofrecen resultados diferenciales entre CaP y próstata benigna y en su relación con el índice Gleason.

En una cohorte prospectiva reciente de hombres incluidos en un programa de VA, los niveles de suero -2pro-PSA al mo-mento del diagnóstico eran significativamente más altos (0.87 ± 0.44 versus 0.65 ± 0.36 pg/mL; P = 0.02) en aquellos pacientes que tuvieron criterios de progresión en las biopsias de seguimiento y se asociaron significativamente con la ocurrencia de una biopsia desfavorable mediante los análisis de Cox y Kaplan-Meier (hazard ratio, 2.53 (1.18-5.41); P = 0.02) (27).

Genes de fusión

Como se ha indicado anteriormente, un porcentaje significativo de CaP son portadores de fusiones génicas en las que están implicados un gen en la posición 5' con elementos reguladores dependientes de andrógeno (*TMPRSS2, SLC4A3* y *NDRG1*) con miembros de la familia de factores de transcripción *ETS* (*ERG, ETV1, ETV4*, etc.) (18, 28). La aplicación de tecnología NGS ha revelado que estos eventos son más frecuentes de lo que inicialmente se pensaba y aunque su impacto biológico no está bien establecido, parece ser que juegan un papel en el desarrollo y progresión del CaP.

Los reordenamientos ETS pueden detectarse empleando FISH (29, 30). Sin embargo, la detección inmunohistoquímica de la proteína ERG sobreexpresada como consecuencia de la fusión génica se correlaciona significativamente con la presencia de la fusión *TMPRSS2-ERG* (T2E) detectada mediante FISH (>95% concordancia) (31). Además, ERG es altamente específica de CaP y la neoplasia intraepitelial de alto grado (HG-PIN) (13, 31), y como consecuencia, la utilidad clínica de la determinación de ERG está emergiendo significativamente para el diagnóstico específico del CaP en glándulas prostáticas atípicas sospechosas (32).

La prevalencia de los reordenamientos ETS oscila entre el 27%-79% en muestras de biopsia y piezas de prostatectomía, que por lo general representan pacientes preseleccionados mediante PSA (18). Como el evento molecular más frecuente del CaP, son muchos los estudios que han intentado esclarecer el valor pronóstico del reordenamiento T2E. Los datos en este sentido no son concluyentes, de hecho se han asociado tanto comportamientos agresivos como indolentes, lo que es indicativo de la heterogeneidad de las series analizadas, metodologías empleadas, criterios de evaluación de seguimiento, etc (13). Sin embargo, llama la atención, que los estudios poblacionales no preseleccionados mediante PSA y diagnosticados mediante resección transuretral de la próstata y clínicamente manejados de forma conservadora han mostrado una asociación significativa entre la presencia del reordenamiento ERG con factores clinicopatológicos de pronóstico adverso, presencia de metástasis o muerte específica de enfermedad (33, 34). En una cohorte de pacientes en VA, T2E correlaciona con un mayor volumen tumoral y un mayor grado de Gelason (35).

El efecto de las fusiones ETS sobre las características agresivas del CaP o sobre el seguimiento tras la prostatectomía radical es menos claro, con muchos estudios mostrando una asociación entre las fusiones ETS y las características agresivas del CaP, mientras que otros no encuentran estas asociaciones (13). El estudio prospectivo con más casuística y más reciente que incluye un total de 1100 pacientes tratados con prostatectomía radical, muestra que los reordenamientos T2E o la sobre expresión de ERG se asocia con el estadio tumoral, pero no con la recaída o la mortalidad (36). En conjunto podríamos decir que las fusiones ETS se asocian con mal pronóstico en estudios poblacionales o en cohortes de VA, mientras que en series de prostatectomía radical los resultados son más contradictorios en relación a la agresividad del CaP y el pronóstico. También se podría concluir que hay una variedad de variables de confusión que complican la interpretación entre los diferentes estudios y que entre estas variables destacan la variación en cuanto a técnicas para determinar los reordenamientos T2E o la sobreexpresión de ERG, así como la ausencia de la preselección de pacientes mediante PSA.

Dado que los reordenamientos ETS constituyen el evento genético más frecuente en CaP, la caracterización molecular del CaP a menudo empieza con la subclasificación entre tumores ETS-positivos (ETS+) y ETS-negativos (ETS-), dada la reproducibilidad de las técnicas empleadas para la detección de estos reordenamientos y su prevalencia (29). Del mismo modo, los estudios basados en tecnologías –ómicas apoyan el concepto de que los tumores ETS+ son entidades biológicas diferentes comparadas con los tumores ETS- (30, 37-39). Algunas alteraciones moleculares, como las mutaciones o deleciones en *PTEN* y *TP53*, así como las deleciones de 3p están enriquecidas en tumores ETS+, mientras otras mutaciones en genes como en *SPOP*, deleciones en *CHD1*, o sobreexpresión de *SPINK1* se presentan exclusivamente en los tumores ETS- (11, 13, 40) (Figura 1).

Una de las características fundamentales de estas huellas moleculares es que son específicas

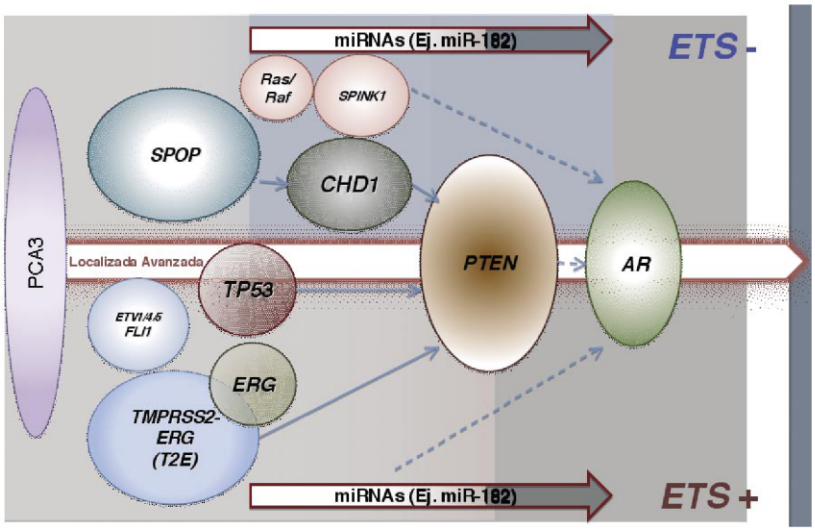

Figura 1. Esquema de los principales biomarcadores identificados mediante tecnicas –omicas que podrían jugar un papel en el manejo clínico del paciente en VA. Conviene reseñar que desde el punto de vista genómico los CaP se diferencian en dos grandes grupos: ETS+ (parte inferior del diagrama), portadores de reordenamientos específicos de genes que codician para factores de transcripción ETS (ERG, ETV1/4/5, FLI1) con un gen con elementos de respuesta a los andrógenos; y ETS-, que presentan alteraciones específicas de este subtipo de tumores, como son las alteraciones de SPOP o CHD1. También hay elementos comunes a ambos subtipos, como es la sobreexpresión de PCA3, que se produce en prácticamente la totalidad de los CaP, o en menor medida las alteraciones de PTEN, TP53 o la desregulación de miRNAs.

del CaP, y para algunos tipos de fusión son prácticamente exclusivas de determinados tipos tumorales que pueden constituir biomarcadores personalizados para seguir la progresión de la enfermedad de forma altamente sensible empleando la tecnología necesaria (11). En este sentido, estudios previos empleando procedimientos basados en RT-PCR han mostrado que el transcrito T2E puede detectarse también en orina (41) y que su detección se asocia con peor pronóstico (42).

T2E+PCA3

Recientemente se han publicado los resultados de un test basado en la amplificación mediada de transcripción (TMA) para cuantificar los niveles de mRNA de T2E basado en la misma tecnología que el PCA3 (43). El transcrito T2E se determinó de forma prospectiva en orina total recogida de 218 pacientes sometidos a prostatectomía radical y a 1094 hombres a los que se le realizó una biopsia diagnóstica de CaP. Los niveles de T2E se asociaron a indicadores de CaP clínicamente significativo tanto en biopsias diagnósticas como en prostatectomía, incluyendo el tamaño tumoral y el Gleason score (43). Además, la combinación de la determinación de T2E con PCA3 mejoró significativamente la capacidad predictiva de CaP en biopsia (área bajo la curva (AUC) = 0.79 versus 0.64; p<0.001). De forma llamativa, en las cohortes de biopsias, utilizando un sistema de estratificación en tres clases, se mostró que hombres en el mayor y menor grupo de T2E+PCA3 escore presentan diferencias significativas en cuanto a la tasa de detección de CaP (69% vs. 21%, p<0.001), cáncer clínicamente significativo por el criterio de Epstein (44) (61% vs. 15%, p<0.001) y cáncer de alto grado (40% vs. 7%, p<0.001) en biopsia. Estos autores demostraron que la determinación en orina de T2E en combinación con PCA3, mejoraba considerablemente la capacidad predictiva de la presencia de CaP clínicamente significativo en biopsia en comparación con el PSA.

En el contexto de la VA nos encontramos con el estudio Canario, multicéntrico y prospectivo, PASS (Prostate Active Surveillance Study) (35), en el que el objetivo primario es confirmar los biomarcadores que predigan la presencia o la progresión de un CaP agresivo (45). En este estudio se recogieron muestras de orina de 387 hombres tras masaje prostático en los que se determinó la presencia de los biomarcadores PCA3 y T2E mediante TMA en el momento de su inclusión en el estudio. Se observó que cada uno de estos biomarcadores se asoció significativamente y de forma directa con el volumen del tumor y la carga tumoral de las biopsias de repetición (p<0.005), así como con la presencia de enfermedad de alto grado (p=0.02 para PCA3 y p=0.001 para T2E). El análisis multivariable de este estudio tomando el score de cada uno de estos biomarcadores de forma continua, mostró que la OR para una biopsia en la que se detectara cáncer frente a una biopsia de repetición negativa (referencia) era de 1.41 (95% IC: 1.07-1.85; p=0.01) para PCA3 y de 1.28 (95% IC: 1.10-1.49; p=0.001) para T2E. El estudio concluye que especialmente PCA3, pero también T2E, parecen estratificar a los pacientes según el riesgo de tener un CaP agresivo (definido por volumen tumoral y Gleason) en el momento de la inclusión en el programa de VA.

Tabla I. Potenciales biomarcadores a validar en un programa de VA.

Biomarcador	Prevalencia en CaP	Muestra biológica	Técnica	Pronóstico
PCA3	>95%	Orina	TMA	Mayor score se asocia a CaP clínicamente significativos
Pro-PSA	>95%	Suero	Bioquímica	Mayor score se asocia a CaP clínicamente significativos
Genes de fusión	27-79%	Biopsia	FISH/RT-PCR	-Discutido en poblaciones seleccionadas mediante PSA -Asociada a CaP más agresivos en población no seleccionada.
		Orina	RT-PCR/TMA	Mayor score se asocia a CaP de mayor grado y mayor volumen tumoral.
ERG	Hasta un 80% (>95% concordancia con fusión génica)	Biopsia	IHQ	Presente en los CaP con la fusión T2E. Muy útil en el diagnóstico de HG-PIN.
SPOP	5-15% de los CaP ETS-	Biopsia Orina/plasma	Secuenciación Hibridación +/- PCR digital	Se desconoce el valor pronóstico. Se pueden identificar herramientas diagnósticas para mutaciones de SPOP específicas (sondas de hibridación) muy sensibles y emplearlas en el seguimiento personalizado de la enfermedad.
CDH1	10-25% de los CaP ETS-	Biopsia Orina/plasma	IHQ/ secuenciación	La pérdida de expresión se asocia a progresión.
SPINK1	10% de los CaP ETS-	Orina	RT-PCR	Se asocia con una enfermedad más agresiva y un mayor riesgo de recaída bioquímica
PTEN	25-70%	Biopsia	IHQ/FISH/ secuenciación	Se asocia a alto grado de Gleason, progresión, enfermedad metastásica. Como en el caso de SPOP, las mutaciones de específicas de PTEN se pueden utilizar en el seguimiento personalizado de la enfermedad.
TP53	25-40% deleciones 5-40% mutaciones	Biopsia	FISH/ secuenciación	No está definido. Las mutaciones de específicas de TP53 se pueden utilizar en el seguimiento personalizado de la enfermedad.
miRNAs	-	Biopsia Orina/suero	RT-PCR	Gleason, estadio y progresión. De momento no hay un método robusto para determinar miRNA en orine de forma reproducible.
CCP score	Mayor en CaP vs tejido prostático normal	Biopsia	RT-PCR	Predice mortalidad cáncer específica.

Abreviaturas: FISH: Hibridación fluorescente in situ; HG-PIN: neoplasia intraepitelial de alto grado; IHQ: Inmunohistoquímica; RT-PCR: Retrotranscripción-reacción en cadena de la polimerasa; T2E: TMPRSS2-ERG; TMA: amplificación mediada de transcripción;

SPOP, CDH1 y SPINK1

Estudios de secuenciación genómica mediante NGS en CaP con un Gleason score >7 han identificado mutaciones puntuales en una serie de genes candidatos a jugar un papel clave en la patogénesis del CaP (11). Por ejemplo, se identificaron mutaciones en los genes *SPTA1, ADAM18*, en miembros del complejo proteico HSP-1 de respuesta al estrés térmico (*HSPA2, HSPA5 y HSP90AB1*) o canales de potasio (*KCNQ3* y *KCNT1*), así como en *PTEN* y *MAGI2*. Sin embargo, las mutaciones no sinónimas más frecuentes que podemos encontrar en CaP implican al gen *SPOP* (37). Este gen codifica para el componente del sustrato de reconocimiento de la ligasa ubiquitina-E3 asociada a la Culina 3 (46). La frecuencia de mutaciones de *SPOP* se ha descrito entre un 6-15% de los CaP(14, 16, 37, 40) y es de resaltar, que todas las mutaciones de *SPOP* se producen en tumores EST- (11) (Figura 1). Se han descrito mutaciones puntuales exclusivamente en el dominio N- terminal (dominio MATH) de *SPOP* que se encarga de reclutar las proteínas que se tienen que degradar (14, 16). Las mutaciones de *SPOP* son mutuamente excluyentes a la presencia de las fusiones T2E y otros reordenamientos ETS, así como también de las mutaciones y deleciones de *TP53* (13, 14). Por último, los tumores con mutaciones en *SPOP* muestran un patrón diferencial de aberraciones genómicas, específicamente, las deleciones de *CDH1* en 5q21 y de la región (14). *CHD1* codifica para un dominio de unión a la cromatina de una proteína de unión al DNA con actividad helicasa implicada en el control transcripcional a lo largo de todo el genoma. *CDH1* está delecionado entre el 10-25% de los CaP tanto primarios como metastásicos (13). También se han descrito reordenamientos y mutaciones puntuales que afectan a este gen y que se presentan exclusivamente en el conjunto de CaP ETS-(47). Interesantemente, los CaP portadores de alteraciones en *CDH1* tienen un incremento de reordenamientos genómicos. En conjunto podríamos decir que aquellos CaP con mutaciones en *SPOP* y deleciones en *CDH1* constituyen las alteraciones precursoras que definen un subtipo ETS- de CaP (11, 13).

SPINK1 es una proteasa que se sobreexpresa específicamente en un subtipo de CaP ETS- (aproximadamente 10% de estos tumores), y se asocia con una enfermedad más agresiva y un mayor riesgo de recaída bioquímica (48).

PTEN

La vía de señalización PI3K se encuentra entre las más comúnmente alteradas en cáncer, incluyendo entre el 25-70% de los CaP, siendo los tumores metastáticos los que significativamente presentan una mayor incidencia. El gen supresor de tumores desactiva esta vía de señalización; las deleciones del locus PTEN ocurren aproximadamente en un 40% de los CaP primarios, mientras que se pueden encontrar entre un 5-10% de mutaciones puntuales en CaP metastático (11, 13, 15). La desregulación de *PTEN* se asocia consistentemente con mal pronóstico en CaP. De hecho hay evidencias que muestran que las deleciones de *PTEN* se asocian con enfermedad localizada avanzada o metastásica, alto grado de Gleason, y un mayor riesgo de progresión, recaída tras terapia y muerte cáncer específica especialmente en los CaP ETS+(49, 50). Sin embargo el valor clínico de la determinación del estatus de *PTEN* todavía es incierto; los estudios todavía no han demostrado que añada información pronóstica cuando se combina con los parámetros clínicopatológicos habituales.

p53

El gen supresor de tumores p53 (*TP53*) es el gen que más frecuentemente se encuentra alterado en cáncer. En CaP sabemos que aproximadamente entre el 25-40% de los casos muestras pérdidas del locus *TP53*, con mutaciones puntuales entre el 5-40% de los casos (13). Los estudios recientes en cuanto a genómica completa del CaP vienen a indicar que la desregulación de *p53* se produce de forma temprana en la patogénesis del CaP y su significación pronóstica todavía no ha sido evaluada (51).

CCP score

Uno de los biomarcadores que puede imbrincarse mejor en los protocolos de VA puede ser el CCP score (Test Prolaris©; Myriad), que normaliza la expresión de 31 genes implicados en el ciclo celular con 15 genes de referencia (o housekeeping genes), y que ha sido testado en varios escenarios clínicos del CaP, entre ellos la observación (52). El análisis se hace sobre el tejido biopsiado, seleccionando el CaP presente en la muestra, y el estudio multivariado muestra que el CCP Score es independiente del Gleason en su capacidad de predicción de mortalidad cáncer específica, objetivo éste que se ha podido testar dada su capacidad de estudio en material archivado. El test Prolaris se está validando actualmente en diferentes escenarios, pero sin duda alguna en uno de los que más valor tendría su confirmación sería en la VA (53).

Ras/Raf/MAPK pathway

La vía de señalización intracelular MAPK juega una papel crítico en una variedad de tumores, si bien su papel en el CaP está menos establecido.

La activación de los componentes de la vía MAPK e intermediarios son más frecuentes en metástasis; sin embargo, las mutaciones en estos componentes son fenómenos relativamente raros (13). De forma interesante, y al igual que sucede con *SPOP* o *SPINK1*, los tumores RAF/RAS+ se producen exclusivamente en los tumores ETS-(13).

miRNAs

Un campo emergente de investigación en los últimos años lo constituyen los microRNAs (miRNAs). Los miRNAs son moléculas de RNA de hebra simple, no codificante, de pequeño tamaño (17-27 nt) que regulan de forma negativa la expresión de genes diana impidiendo que éstos se traduzcan. Se ha visto que estas moléculas se han implicado en la regulación del crecimiento, desarrollo, invasión, metástasis y pronóstico de varios cánceres, incluido el CaP (54). Muchos de estos miRNAs se han asociado con el pronóstico y son muchos los intentos que se han llevado a cabo para detectar estas moléculas en fluidos corporales como fluidos y plasma con el objeto de utilizarlos como biomarcadores en el contexto clínico, especialmente debido a su alta estabilidad en estos medios. Sin embargo, la mayoría de los estudios se han llevado a cabo en series muy cortas y heterogéneas, así como por tecnologías muy poco reproducibles (55).

Un ejemplo de miRNA que pudiera jugar un papel en la selección de la población candidata para VA lo constituye el miR-182. Este miRNA está directamente relacionado con el score de Gleason y se ha asociado con metástasis en CaP (56) (Figura 1). Recientemente, nuestro grupo ha descrito en una serie de 276 pacientes sometidos a prostatectomía radical, que este miRNA en combinación con el score de Gleason es más preciso a la hora de definir las poblaciones con riesgo a desarrollar una recaída bioquímica(57) (Figura 2). Indudablemente, si fuéramos capaces de trasladar estos resultados en el contexto de la biopsia diagnóstica, bien sobre el mismo tejido o en fluidos corporales, este miRNA sería un buen candidato para considerarlo en la selección de población candidata a VA o para evaluar la progresión en las biopsias de repetición durante la vigilancia.

Estudio de los exosomas como fuente de biomarcadores en VA

Una de las limitaciones de los programas de VA, que es bien reconocida y aceptada, es el bajo muestreo de los procedimientos de biopsia actuales, que puede subestimar la proporción de casos con un CaP agresivo y que está muy asociada a la heterogeneidad y multifocalidad de estos tumores. En los últimos años, se está especulando que el estudio de exosomas, como fuente de biomarcadores, podría matizar esta limitación. Los exosomas son pequeñas vesículas membranosas (de 30-100 nm) de origen endocítico secretadas por la mayoría de tipos celulares y de las que se piensa que juegan un papel importante en las comunicaciones intercelulares así como en la progresión y en la capacidad metastásica de algunos tumores (58). Hoy sabemos que estas vesículas contienen mRNA y miRNA que pueden ser traducidos a proteínas (mRNA) o acometer su función de silenciamiento (miRNA) en las células diana, sugiriendo que los exosomas pueden actuar como 'transportadores' de información genética (59). No todos los RNA presentes en las células se encuentran en los exosomas, si no que parece ser que solo se incluyen aquéllos que selectivamente son incluidos en las vesículas (59), de hecho algunos transcritos de RNA se encuentran enriquecidos más de 100 veces en los exosomas en comparación con los de la célula origen, lo que soporta aun más la idea de un mecanismo específico de empaquetamiento (60). Por tanto, los exosomas derivados de células tumorales están enriquecidos de transcritos específicos del tumor que podrían estar incluso por debajo del nivel de detección cuando analizamos el transcriptoma de un tejido tumoral (60, 61).

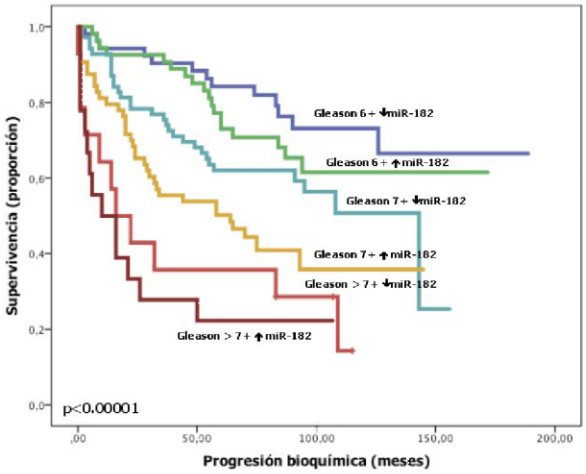

Figura 2. Curvas de supervivencia de Kaplan-Meier correspondiente a una serie retrospectiva de 276 pacientes sometidos a prostatectomía radical en el que se ha determinado la expresión del miRNA miR-182. La combinación del score de Gleason con los niveles de expresión de este miRNA define de forma independiente diferentes grupos de riesgo a la progresión bioquímica, sugiriendo la posibilidad de que un biomarcador de estas características pudiera seleccionar de forma más eficiente a una población candidata a VA (adaptado de Casanova-Salas y cols, 2014).

Además de la incertidumbre en relación de la naturaleza y función biológica de los mRNA y miRNAs que transportan los exosomas, éstos se convierten en biomarcadores ideales para ser testados en un contexto clínico. Su estudio se hace más accesible de lo que podría parecer ya que su encapsulación en las vesículas de membrana los protege de la degradación, convirtiéndolos en sustratos estables, y permite una recuperación eficiente a partir de fluidos corporales.

En CaP se han llevado a cabo estudios proteómicos (62) y genéticos a muy pequeña escala en los que se ha demostrado la presencia en estos exosomas de biomarcadores como PCA3 y T2E(63). Sin embargo, la utilidad clínica de estos biomarcadores debe ser debidamente validada en series prospectivas de pacientes (64).

El programa de VA como modelo para la validación de biomarcadores

Como se ha visto a lo largo de la exposición de esta revisión, existen biomarcadores muy prometedores en la identificación del CaP más agresivo (Tabla I y Figura 1), pero la cuestión clínica relevante es qué pueden ofrecer estos nuevos biomarcadores en la predicción de la presencia de enfermedad agresiva oculta o de la progresión de la enfermedad a lo largo del tiempo tal y como requiere un programa de VA. La realidad es que tenemos poca información al respecto, pero consideramos que el mismo programa de VA constituye un modelo ideal para la validación de dichos biomarcadores. Nos brinda la oportunidad de acceder a muestras biológicas de gran valor asociadas a información clínica en el que la determinación de estos nuevos biomarcadores pueda constituir, una vez validados, un valor añadido en la toma de decisiones.

Evidentemente, esto requiere un esfuerzo y un marco de colaboración entre el paciente y el equipo multidisciplinar de facultativos, que garantice la recogida de muestras biológicas (orina, suero, biopsias de repetición) e información clínica con garantías y de calidad con el objetivo de evitar dispersión entre los posibles resultados. aplicando la tecnología adecuada podemos evaluar esos cambios a lo largo del tiempo en aquellos biomarcadores con mayor capacidad predictiva de riesgo y evaluar su verdadero papel en un contexto clínico como el de la VA.

CONCLUSIÓN

El CaP es muy heterogéneo desde el punto de vista genético y se han definido diversos biotipos en función de las alteraciones genéticas más prevalentes. Cada uno de estos biotipos puede contar con biomarcadores específicos que hacen que el manejo de estos pacientes sea cada vez más personalizado, lo que significa que reducir el criterio de selección de pacientes candidatos a VA en base a uno o dos biomarcadores sea un poco reduccionista y sea más preciso hablar de un panel múltiple que tenga en consideración otras características moleculares del CaP y propias de cada biotipo específico. La inclusión de estos biomarcadores, algunos menos prevalentes, como otras variantes de fusión génica (por ejemplo *TMPRSS2-ETV1*), mutaciones puntuales en genes como *SPOP* o miRNAs en estos paneles complejos mejorarían la sensibilidad del test sin comprometer su especificidad.

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