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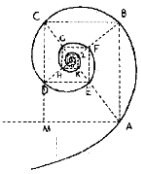

SCUOLA DI DOTTORATO IN MEDICINA MOLECOLARE

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TESI DI DOTTORATO DI RICERCA

Settore disciplinare **MED08**

Expression analysis of microRNA in prostate cancer and identification of novel diagnostic biomarker

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Sommario

Introduzione: Il tumore alla prostata (PCa) è una delle neoplasie più comuni tra gli uomini nel mondo occidentale e, globalmente, rappresenta la sesta causa di morte dovuta a cancro. L'approccio diagnostico attuale si basa sulla misurazione dei livelli sierici dell'antigene prostatico PSA (prostate specific antigen), nonostante recenti studi clinici abbiano dimostrato che questo marcatore non riduce significativamente la mortalità associata a PCa. In questo scenario abbiamo ipotizzato che i microRNA possano essere nuovi potenziali biomarcatori di PCa.

Scopo del lavoro: L'identificazione di microRNA, coinvolti nella progressione neoplastica del tumore prostatico come nuovi biomarcatori diagnostici aggiuntivi al PSA, prognostici e predittivi di aggressività tumorale. La nostra strategia sperimentale ha previsto l'uso di linee cellulari di prostata non tumorigeniche e a diverso grado di malignità, modelli murini di carcinoma prostatico e casistiche di pazienti affetti da PCa.

Materiali e metodi: Linee cellulari commerciali: analisi dell'espressione globale dei miRNA tramite piattaforma low-density array nelle linee di PCa (LNCap, PC3, DU145), normali o iperplastiche (RWPE-1 e BPH-1). Casistiche cliniche: analisi di miRNA selezionati, in un set di pazienti (n=58) nei tessuti di parenchima normale, pre-neoplastico (prostatic intraepithelial neoplasia, PIN) e tumorale. I risultati ottenuti sono stati correlati a parametri clinicopatologici dei pazienti. I potenziali target proteici dei miRNA selezionati sono stati valutati in una casistica più ampia mediante tissue micro-array (TMA). Modello murino transgenico TRAMP: analisi globale di espressione dei miRNA in ghiandole di PIN e tumorali, e nello stroma associato. Linee cellulari primarie: ottenimento di linee di fibroblasti derivate da resezioni chirurgiche di PCa (n=10).

Risultati: Dallo screening nelle linee cellulari abbiamo selezionato 23 miRNA poi valutati nei 58 pazienti. Tredici miRNA hanno mostrato differenze di espressione significative ($p<0.05$). In particolare, nove miRNA

(miR-135b,-193a-5p,-205,-224,-22,-34b,-34c-5p,-452,miR-886-3p) sono risultati progressivamente diminuiti nella progressione neoplastica (N-PIN-PCa). Viceversa, i miR-130a, -218, -532, -542-5p, -489 e let-7c hanno mostrato una diminuzione di espressione nel PIN rispetto al tessuto normale. È noto che i miR-205, miR-218, miR-224 abbiano come bersaglio proteico RUNX2 (Runt-related transcription factor 2), un fattore di trascrizione coinvolto nel tropismo osseo di cellule metastatiche.

La valutazione di RUNX2 nei TMA di prostata ha rivelato che la positività nucleare è specifica delle cellule tumorali ($p<0.0001$) e correla con l'estensione del tumore e con l'invasione capsulare. Inoltre il confronto tra l'espressione dei miRNA e RUNX2 ha mostrato correlazione inversa significativa. Dall'analisi del modello TRAMP abbiamo identificato un pannello di miRNA differenzialmente espressi tra le componenti epiteliali e stromali associate a PIN o PCa (n=52).

Conclusioni: I nostri risultati mostrano una sinergica perdita di miRNA con funzione oncosoppressiva e contemporaneo aumento di RUNX2 nei tessuti di PCa. Questo dato ha importanti implicazioni a livello di progressione di malattia che sarà valutata in una successiva fase del progetto. Le nostre analisi hanno indicato il miR-205 come potenziale marcatore di aggressività di malattia. Hanno inoltre identificato 9 miRNA precocemente persi nelle lesioni precancerose (PIN) rispetto al parenchima sano. Studi in casistiche indipendenti potranno confermare queste molecole come nuovi biomarcatori di neoplasia da affiancare alla valutazione del PSA. Inoltre lo studio dei miRNA "stromali" ha evidenziato una profonda deregolazione di queste molecole nel microambiente tumorale rispetto a quello non neoplastico. Questo risultato sottolinea dal punto di vista molecolare l'importanza dello stroma nel sostenere la sopravvivenza e la crescita tumorale e fornisce una possibile strategia terapeutica alternativa, mirata alle cellule stromali anziché epiteliali per indurre regressione di malattia. Saranno necessari ulteriori studi per valutare il ruolo dei miRNA nell'interazione tra epitelio tumorale e stroma circostante.

Abstract

Introduction: Prostate cancer (PCa) is one of the most common cancers among men and the sixth cause of cancer-related death in men worldwide. The current diagnostic approach is based on serum measurement of prostate specific antigen (PSA) levels, despite recent clinical studies showed that did not considerably reduced mortality incidence in prostate cancer patients. In this scenario, we hypothesized that microRNAs (miRNAs) could be novel biomarkers for PCa disease.

Aim of the study: We propose to identify miRNA signatures associated to PCa progression that could represent a novel generation of diagnostic biomarkers adjunctive to PSA, prognostic and predictive of cancer progression. Our experimental strategy included the use of normal or tumorigenic prostate cell lines, mouse model of PCa and patients' series.

Methods: Prostate cell lines: global miRNA expression analysis using a low-density array platform in PCa (LNCap, PC3, DU145) or non-tumorigenic cells (RWPE-1, BPH-1). Clinical series. Analysis of selected miRNAs in 58 PCa patients for which normal parenchyma and prostatic intraepithelial neoplasia (PIN) was available. Correlation of molecular profiles to clinicopathological characteristics. Potential miRNA targets were investigated using a larger series of PCa patients arranged in tissue micro-arrays (TMAs). TRAMP mouse: global miRNA profiles were obtained from epithelial and stromal compartments of PIN or tumoral lesions. Primary cell lines: fibroblasts were obtained from prostate resection of PCa patients (n=10).

Results: miRNA screening in cell lines provided a panel of 23 miRNAs that were then investigated in the 58 PCa patients. Thirteen miRNA displayed significant deregulation ($p<0.05$) in disease tissues. Specifically nine miRNAs (miR-135b,-193a-5p,-205,-224,-22,-34b,-34c-5p,-452, miR-886-3p) were progressively down-regulated during neoplastic progression (N-

PIN-PCa). Conversely, miR-130a, -218, -532, -542-5p, -489 and let-7c displayed lower levels in PIN compared to normal prostate. A recognized target of miR-205, miR-218 and miR-224 is the Runt-related transcription factor 2 (RUNX2), a protein involved in metastatic dissemination to the bone. In our patients' TMA, RUNX2 was overexpressed in tumoral cell nuclei ($p<0.0001$) and it was related to tumor size and capsular invasion. Moreover RUNX2 was inversely related to miRNA levels. TRAMP mice analysis has provided a signature of miRNAs (n=52) differentially expressed in epithelial and stromal compartments of PIN or PCa cells.

Conclusions: Our results show a simultaneous loss of oncosuppressive miRNAs and increased RUNX2 expression in PCa tissues. This data is particularly relevant in disease progression monitoring, an aspect that will be studied in future project's phases. Our analysis showed that miR-205 loss is a potential biomarker of aggressive disease. Furthermore, we identified nine miRNAs which expression is decreased from early stage of disease (PIN). Validation of this result in independent patients' series could provide novel biomarkers of PCa useful as adjunts to PSA monitoring. Lastly, profound stromal miRNAs deregulation underlines the importance of tumour microenvironment in sustaining cancer cell survival and growth. Moreover this result suggest that targeting tumour stroma could represent an alternative strategy for anti-cancer therapies.

Future studies are needed to shed light on this aspect.

INDEX

AD	Androgen dependent
ADT	Androgen deprivation therapy
AI	Androgen independent
AMO	anti-miRNA oligonucleotide
AR	Androgen receptor
ARE	Androgen responsive element
ARGs	Androgen-regulated genes
AUC	Area under the curve
BCR	Biochemical recurrence
Bim	BCL2-like 11 (apoptosis facilitator)
BPE	Bovine pituitary extract
BPH	Benign prostatic hyperplasia
CAF	Cancer associated fibroblasts
CRPC	Castration-resistant prostate cancer
DGCR8	DiGeorge syndrome critical region 8
DHEA	dihydroepiandrosterone
DHT	dihydrotestosterone
DRE	Digital rectal examination
DU145	AR- PCa cell line derived from brain metastasis
EGF	Human recombinant epidermal growth factor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFPE	Formalin-fixed Paraffin embedded
GRO-seq	Global run-on sequencing
H&E	Haematoxylin and eosin
HGPIN	High grade prostatic intraepithelial neoplasia
IHC	Immunohistochemistry
ISUP	International Society of Urological Pathology
K-SFM	Keratinocyte Serum Free Medium
LBD	Ligand-binding domain
LGPIN	Low grade prostatic intraepithelial neoplasia
LNCap	AR+ PCa cell lines. Lymph Node Carcinoma of the Prostate
NAF	Normal associated fibroblasts
NGS	Next generation sequencing
nt	Nucleotide

PBS	Phosphate-buffered saline
PC3	AR- PCa cell lines derived from bone metastasis
PCa	Prostate cancer
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PNI	Perineural invasion
PSA	Prostate-specific antigen
Rb	Retinoblastoma protein
RISC	RNA-induced silencing complex
RUNX2	Runt-related transcription factor 2
RWPE-1	Noncancerous prostate epithelial cells
SMC	Smooth muscle cells
SRC-1	Steroid receptor coactivator-1
SREs	Skeletal related events
TMA	Tissue Micro Array
TNM	Classification of Malignant Tumors
TRAMP	Transgenic adenocarcinoma of the mouse prostate
TURP	Transurethral resection of the prostate
UTR	Untranslated region

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INTRODUCTION

1. Prostate cancer

Prostate cancer (PCa) is the most common cancer among men (**Figure 1**) with greater mortality among solid cancers (**Figure 2**). In the United States, PCa is expected to account for 28% (238.590) of the total new cancer cases and 10% (29.720) of the total cancer deaths in males in 2013, ranking second to lung cancer in cancer-related deaths. The probability of developing prostate cancer from birth to death is 16,15% (1 in 6) [1]. In Europe, the incidence of PCa is 22,8%. The mortality rate is 9,5% and in Italy it is the third cause of cancer death after lung and colon cancer [2].

Adenocarcinoma is the predominant histological type of prostate cancer and usually originates from the malignant transformation of glandular luminal epithelial cells.

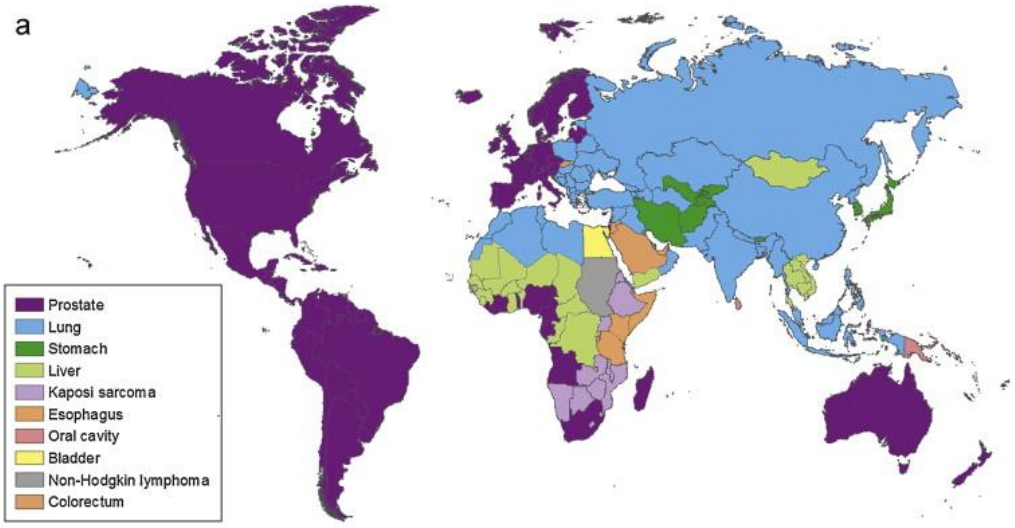

Figure 1: *Most commonly diagnosed cancer among men worldwide, 2008 [3]*

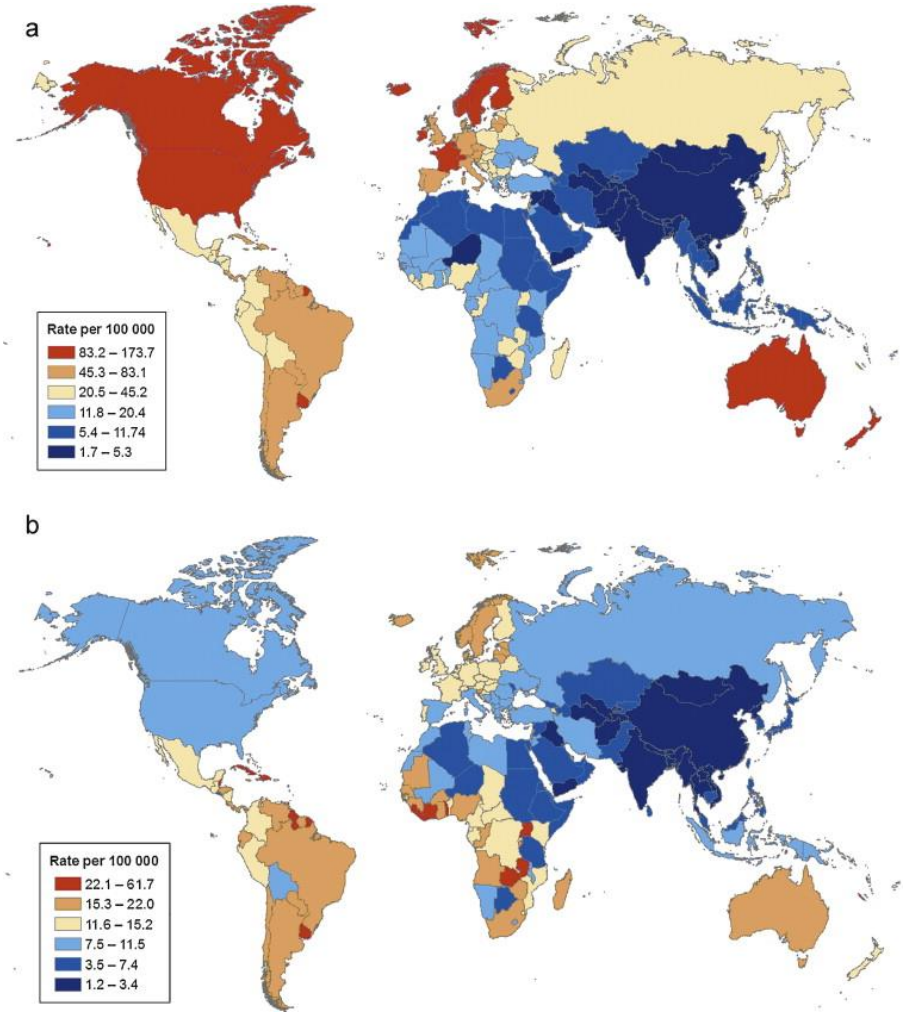

Figure 2: *(a) International variation in age-standardized prostate cancer incidence rates; (b) International variation in age-standardized prostate cancer mortality rates Source: GLOBOCAN 2008 [3]*

1.1. Prostate cancer classification

In order to classify prostate cancer grade, Donald F. Gleason in 1966 created a unique grading system for prostatic carcinoma based on the architectural pattern of the tumor [4]. The Gleason grading system is substantially different from grading systems used for other solid neoplasms.

Most systems examine the morphology of individual cells, focusing on features such as nuclear size and pleomorphism, and the frequency of cells with chromatin aggregation, prominent nucleoli and mitoses. Although individual cells comprising tumors of higher Gleason score demonstrate such morphological changes, these are not integrated into the Gleason scoring system. Rather the Gleason system examines the relationship of cells to each other (a glandular architecture) without incorporating the morphology of individual cells in characterizing the malignancy (**Figure 3**).

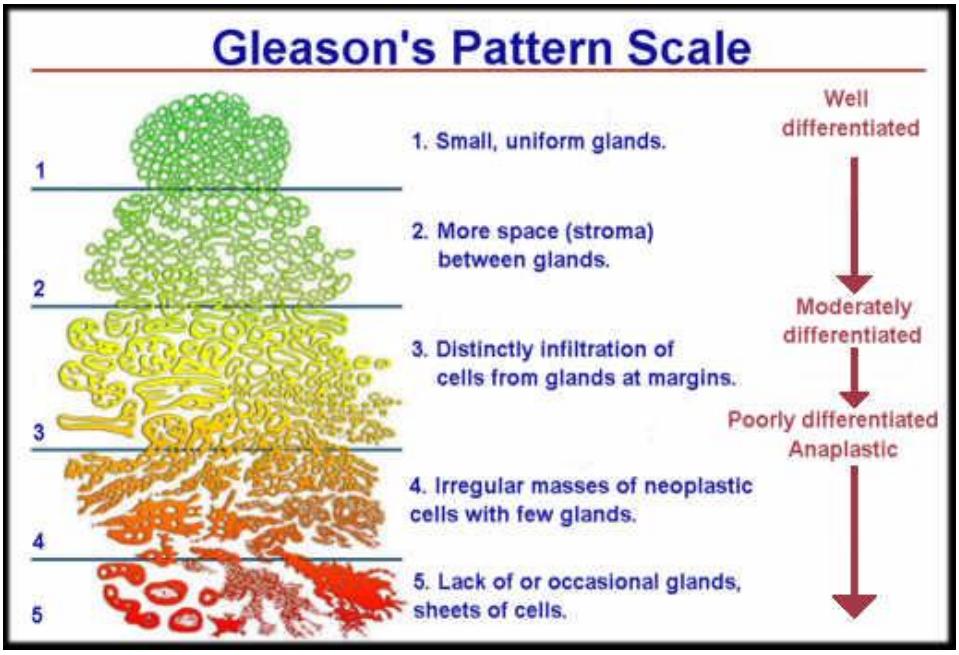

Figure 3: *Gleason grading system*

Another innovative aspect of this system was that rather than assigning the worst grade as the grade of the carcinoma, the grade was defined as the sum of the two most common grade patterns and reported as the Gleason score [5]. The architecture of normal and hypertrophied prostate tissue consists of glands of epithelial cells surrounding a glandular lumen and

resting on a continuous, supportive layer of basal cells, all embedded inconnective tissue stroma. Disruption of the basal cell layer defines prostatic neoplasms, occurring partially in PIN and completely in cancer. Once the basal layer is lost, the Gleason score is used to evaluate the remaining epithelial cell glandular architecture [6].

According to the 2005 ISUP (International Society of Urological Pathology) modifications, Gleason 3 cancer consists of discrete glandular units that vary in size and shape and populate the prostatic stroma in clustered fashion among non-neoplastic prostatic acini [5]. The individual tumor acini have a smooth and usually rounded edge with an intact basement membrane. In contrast, Gleason 4 cancer is comprised of ill defined, fused glands with poorly formed glandular lumina, often with irregular borders that raggedly infiltrate the stroma. Gleason pattern 5 represents the total loss of all glandular differentiation [5]. Although all patterns have in common a loss of their basal cell layer, the difference between Gleason patterns 3 and 4 is the focal loss of a rounded glandular architecture, the fusing of glands and the disruption of an otherwise smooth glandular border. The progressive difference between Gleason patterns 4 and 5 is the total loss of any recognizable rounded glandular form, this being replaced with sheets of cancerous cells [6]

1.2. Prostatic intraepithelial neoplasia (PIN)

High-grade prostatic intraepithelial neoplasia (HGPIN) is the only accepted precursor of prostatic adenocarcinoma, according to numerous studies of animal models and man; other proposed precursors include atrophy and malignancy-associated changes (with no morphological changes). PIN is characterized by progressive abnormalities of phenotype and genotype that are intermediate between benign prostatic epithelium and cancer, indicating impairment of cell differentiation and regulatory control with advancing stages of prostatic carcinogenesis. The only method of detection of PIN is

biopsy because it does not significantly elevate serum prostate-specific antigen concentration and cannot be detected by ultrasonography [7].

PIN is characterized by cellular proliferation within pre-existing ducts and acini, with cytological changes mimicking cancer, including nuclear and nucleolar enlargement (**Figure 4**, **Table 1**). There is an inversion of the normal orientation of epithelial proliferation from the basal cell compartment to the luminal surface, similar to adenomas in the colon [7].

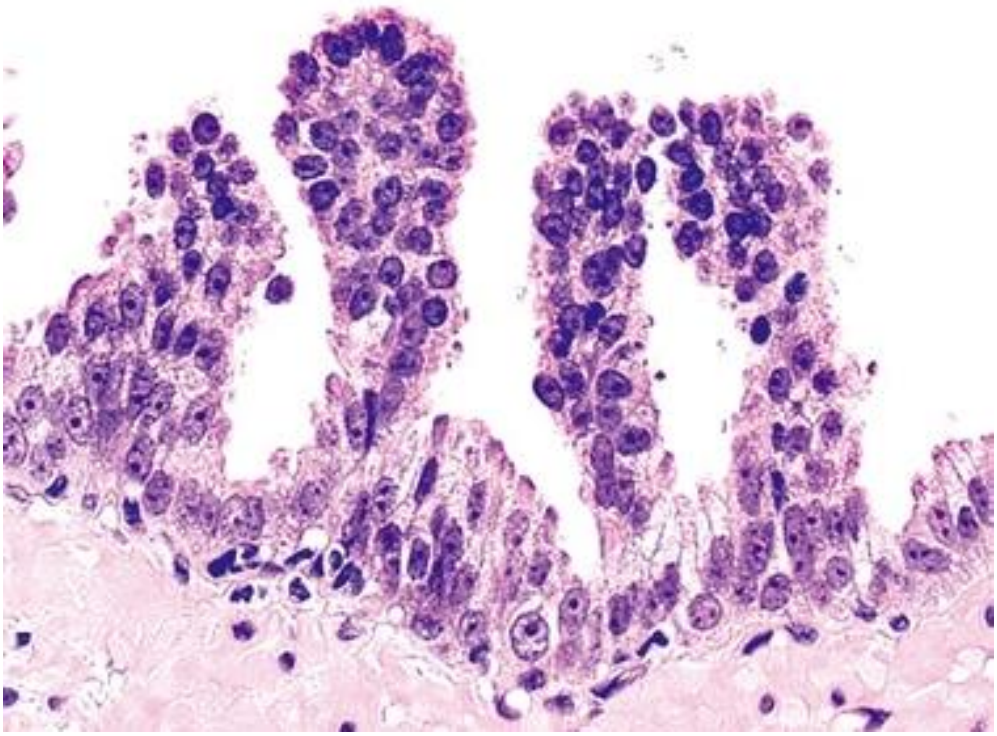

Figure 4: *High-grade PIN, micropapillary pattern* [7].

	Low-grade PIN	High-grade PIN
Architecture	Epithelial cells crowding and stratification, with irregular spacing	Similar to low-grade PIN; More crowding and stratification; four patterns: tufting, micropapillary, cribriform, and flat
Cytology		
Nuclei	Enlarged, with marked size variation	Enlarged; some size and shape variation
Chromatin	Normal	Increased density and clumping
Nucleoli	Rarely prominent*	Prominent
Basal cell layer	Intact	May show some disruption
Basement membrane	Intact	Intact

Fewer than 10% of cells have prominent nucleoli.

Table 1: *Diagnostic criteria of PIN [7]*

In its original description, PIN was subcategorized into three grades, with grade 1 equating to LGPIN (low-grade PIN) and grades 2 – 3 combined into HGPIN (high-grade PIN).

Prostatic intraepithelial neoplasia is found predominantly in the peripheral zone of the prostate (75 – 80%), rarely in the transition zone (10 – 15%), and very rarely in the central zone (5%). This distribution mirrors the frequency of the zonal predilection for carcinoma of the prostate (PCa). The frequency of HGPIN in needle biopsy series ranges from 5 to 16%. HGPIN is relatively uncommon in TURP (transurethral resection of the prostate), with studies reporting a rate of 2.3% and 2.8%, respectively [8]. The prevalence of HGPIN in radical prostatectomy specimens is remarkably high; it was present in 85 – 100% of specimens, reflecting the strong

association between the lesion and PCa [9]. There are other two possible findings in prostate that may be premalignant: adenosis (atypical adenomatous hyperplasia) and proliferative inflammatory atrophy (PIA). The data for these are much less convincing than those for HGPIN. If adenosis and PIA are removed from the list of precursor lesions of PCa, there remains only one well established precursor to PCa, that is HGPIN [8]**.**

1.3. Diagnosis

Currently, the diagnostic method is based on the quantification of PCa serum prostate-specific antigen (PSA), supported by digital rectal examination (DRE), subsequently confirmed by ago biopsy. The PSA test measures the blood level of the enzyme (<4 ng / mL normal,> 4 ng / mL increased risk of cancer), however, blood levels of PSA may vary for many reasons other than cancer. Two common causes of increased levels of PSA are the benign prostatic hyperplasia (BPH) and the prostatitis. Although the method of screening with PSA is widely used, recent studies have shown that screening with PSA did not significantly reduce mortality associated with PCa. This is due to the low specificity of this method [10]. In order to have a clear diagnosis, the biopsy supports the evaluation of the PSA, to perform a histological sample analysis. In the event that has been diagnosed PCa, current therapeutic approaches include prostatectomy and radiation therapy, for localized PCa, and androgen deprivation therapy (ADT) in advanced stages treatment. Currently is not yet available a targeted therapy for advanced disease, and androgen-independent cytotoxic chemotherapy is the current choice of treatment in spite of his limited effectiveness. The lack of available treatment options for efficiently eradicating advanced PCa makes the development of alternative methods urgent. Understanding the molecular alterations that distinguish progressive disease from non-progressive disease will help identify novel prognostic

markers or therapeutic targets. In fact, new target therapies are developing to damage cancer cells specifically.

1.4. Androgen Receptor

Androgens play a critical role in the development of the male phenotype during sexual differentiation but also in the development and progression of PCa [11]. Androgenic effect in the prostate is primarily mediated by dihydrotestosterone (DHT), which derives predominantly from the reduction of testicular testosterone, but also adrenal dihydroepiandrosterone (DHEA) catalyzed by locally produced 5α-reductase enzymes [12]. The cellular response to androgens is mediated via the AR, a ligand-inducible transcription factor that comprises a C-terminal ligand-binding domain (LBD), a highly conserved DNA-binding domain, a hinge region and N-terminal transactivation domain [11]. Upon ligand binding, cytosolic AR undergoes conformational changes, including interaction of the N- and C-terminal domains and dissociation from heat shock proteins, enabling the AR to interact with co-regulatory molecules such as ARA70 and importin-α, which promote nuclear translocation and dimerization (**Figure 5**) [13]. In the nucleus, AR binds to the promoters of androgen-regulated genes (ARGs), such as prostate-specific antigen (PSA) and recruits various coactivators and RNA polymerase II to induce transcription [14]. This classic genomic mode of AR action promotes the transcription of a variety of genes encoding proteins necessary for the development, growth and maintenance of a healthy prostate [15].

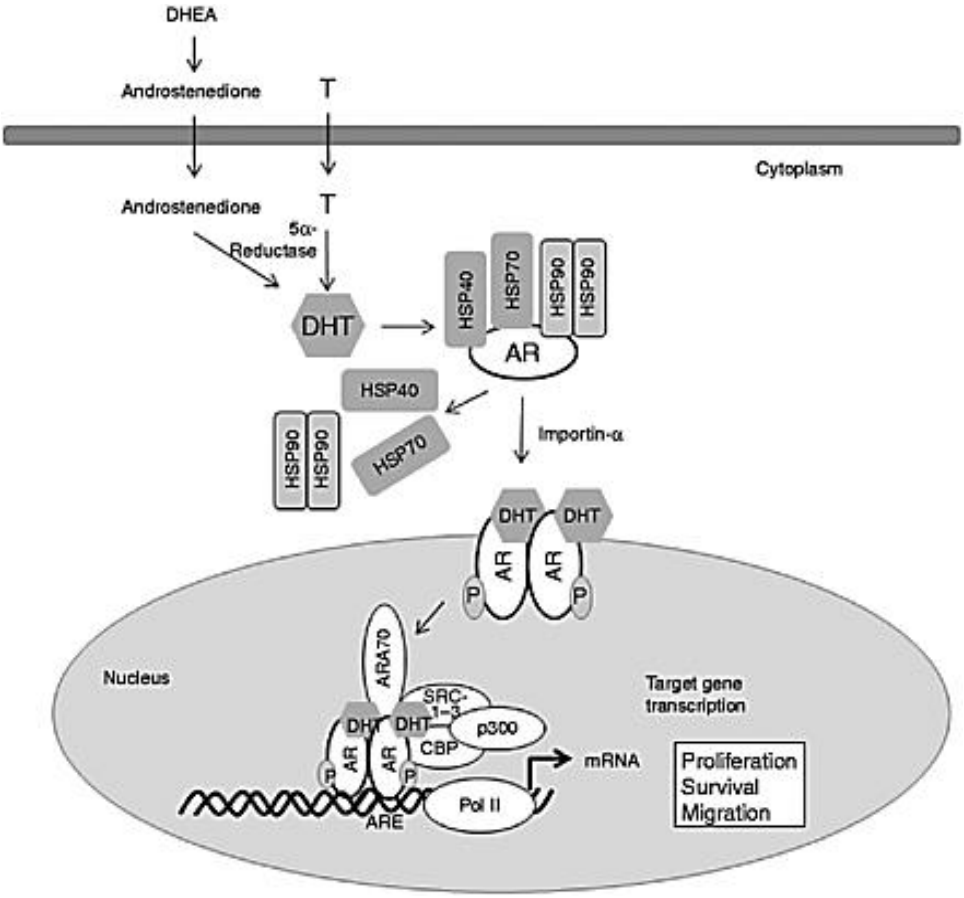

Figure 5: *Classic androgen receptor (AR) genomic activity via androgen. Androgens derive predominantly from the testis (90–95%) but also to a lesser extent from the adrenal glands (5–10%) and mediate their effects via binding to the AR. Testicular testosterone (T) and adrenal DHEA or androstenedione are converted locally in the prostate into bioactive DHT by the enzymes 5α-reductase 1 and 2. In the classic mode of AR genomic activity, androgen binding to the AR induces a conformational change that leads to the dissociation of chaperone and heat shock proteins (HSP40, HSP90) and its subsequent interaction with co-regulatory molecules and importin-α, which promote nuclear translocation of AR–ligand complexes. In the nucleus, the AR undergoes phosphorylation and dimerization, which permits chromatin binding to androgen-responsive elements (ARE) within androgen-regulated target genes. The AR recruits a variety of coactivators (ARA70, SRC-1, -3, and CBP/p300) and RNA polymerase II (Pol II) to induce gene transcription [15].*

1.5. Therapy and castration resistant prostate cancer (CRPC)

Identification of new therapeutic targets for CRPC has mainly resulted from an improved understanding of tumor biology. Tumor-derived factors, host factors, and tumor microenvironment are all essential contributors in sustaining prostate tumor growth and progression of metastases, triggering clinical development of novel therapeutics, including those targeting hormonal signaling, angiogenesis, bone-derived factors, cell cycle checkpoints, activated tyrosine kinases, and host immune surveillance.

Although patients with CRPC have, by definition, castrate levels of circulating testosterone, most tumors continue to remain dependent on androgen and on signaling from the androgen receptor (AR). This may occur through constitutive activation of the AR gene amplification, alternative splicing [16]

Although <5% of patients present with metastatic disease, up to 40% of men eventually develop metastases despite local treatment [16]. Metastases are frequently osseous, can cause substantial pain, and increase the risk for fractures and other skeletal related events (SREs). Once metastases have developed, PCa is incurable, and all treatment is palliative.

Surgical or medical castration is highly effective in shrinking tumor burden, decreasing prostate-specific antigen (PSA) levels, enhancing the quality of life, and improving survival [16]. However, most patients will eventually experience disease progression despite the castration, with a median duration of response of 12–24 month [16].

Androgen deprivation therapy (ADT) for prostate cancer (PCa) represents one of the most effective systemic palliative treatments known for solid tumors. Although clinical trials have assessed the role of ADT in patients with metastatic disease, the risk–benefit ratio, especially in earlier stages, remains poorly defined. Given the mounting evidence for potentially life-

threatening adverse effects with short- and long-term ADT, it is important to redefine the role of ADT for this disease [17].

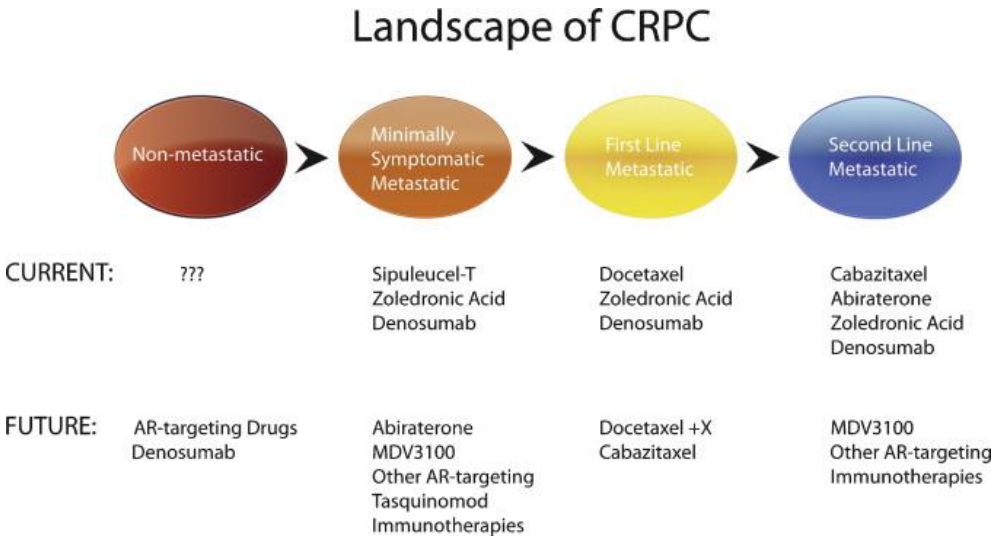

Figure 6: *Landscape of castration-resistant prostate cancer. AR= androgen receptor [16]*

Although some patients will respond initially to secondary hormonal manipulations, castration-resistant prostate cancer (CRPC) will inevitably improve [16]. Following hormonal manipulations, cytotoxic chemotherapy had been the only treatment shown to improve survival for patients with CRPC [18], [19]. In 1996, mitoxantrone was the first chemotherapy to show a palliative benefit for patients with CRPC in combination with steroids compared with steroids alone (29% vs 12%; p = 0,01) [20]. Although no survival benefit was seen with mitoxantrone in two phase 3 trials [20][21], it was the first chemotherapy to be approved by the US Food and Drug Administration (FDA) for the treatment of men with CRPC.

Although docetaxel plus prednisone extended survival compared to mitoxantrone, the overall benefit was modest, with most patients experiencing disease progression within 7 months. Hence, the focus since 2004 has been on trying to improve clinical outcomes by exploring

alternative chemotherapy agents, novel targeted agents, and sequential and combination regimens. Within the past year, several promising agents with widely varied mechanisms of action and therapeutic targets have demonstrated efficacy, and four new drugs were FDA approved for the treatment of patients with CRPC (cabazitaxel, sipuleucel-T, denosumab, and abiraterone acetate). The understanding of advanced PCa has changed in parallel with the expansion of the repertoire of therapeutic options (**Figure 6**).

1.6. TRAMP mice

Historically, a variety of models including cell lines, transgenics, transplantable tumors, and chemically and hormonally induced tumors have been used to test strategies for prevention and treatment of prostate cancer. Each of these model systems has strengths and merit, and each presents specific windows of opportunity for research. The primary advantages of using transgenic mice for pre-clinical testing are: (1) the cancer arises autochthonously within the appropriate microenvironment; (2) mice possess an intact immune system; (3) heterogeneous cancers arise de novo and can change with time or as a consequence of selective pressure in contrast to more static systems where tumors simply grow from clonal cancer cells often derived from late stage disease or metastases. While cancer in mice may only approximate cancer in men, the degree to which cancer in transgenic mice will reflect the natural history of clinical prostate cancerbe related to I) the strain of mice, II) the nature of the transgene construct, III) the site of integration IV) the temporal and spatial pattern of transgene expression. Fortunately, most of these variables can be controlled or modified [22].

The model TRAMP (Transgenic Adenocarcinoma of Mouse Prostate) is a transgenic mouse that, differently from other mouse models, uniformly and spontaneously develops prostate cancer after the onset of puberty [22].

The TRAMP mouse has been established as an excellent mouse model of prostate cancer because spontaneously develop prostatic adenocarcinoma that progresses through multiple stages and that exhibits both histological and molecular features similar to that of human prostate cancer [22]. Male TRAMP mice express a PB-Tag transgene consisting of the minimal −426/+28 bp regulatory element of the rat probasin promoter directing prostate-specific epithelial expression of the simian virus 40 early genes to abrogate the activity of p53 and Rb tumor suppressor genes. Loss of functional p53 and Rb predisposes epithelial cells to enhanced survival signals and allows investigation of molecular pathways and targets. Prostate cancer progresses in this model in an androgen-dependent fashion and is highly reproducible. The transgene is hormonally regulated by androgens, thus temporally, transgene expression correlates with sexual maturity [23]. By approximately 6 weeks, TRAMP mice exhibit low-grade PIN, which progresses to HGPIN by 12 weeks. Focal adenocarcinoma develops between 12 and 18 weeks, and progresses to poorly differentiated carcinoma within 24 weeks. By 28 weeks of age, 100% of these transgenic mice, without any treatment, harbor metastatic prostate cancer in the liver, lymph nodes, lungs, and occasionally in bone [22], [23] (**Figure 7**).

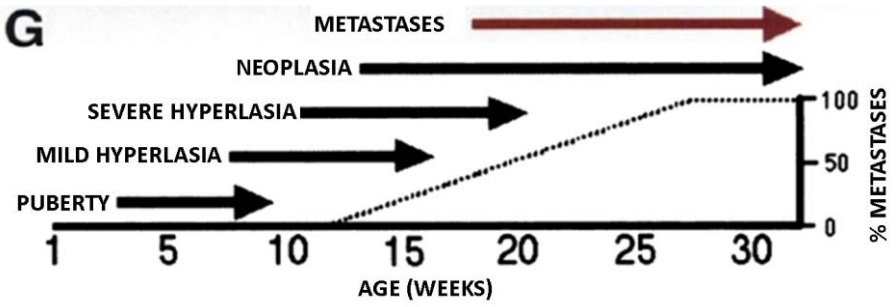

***Figure 7**: Cancer progression in TRAMP mouse*

1.7. microRNAs

MicroRNAs (miRNAs) are small, non-coding, endogenous RNAs that repress translation of genes post-transcriptionally [24], [25]. They are single-stranded RNA molecules of 20-23-nucleotides (nt) length that control gene expression in many cellular processes. These molecules typically reduce the translation and stability of mRNAs, including those of genes that mediate processes in tumorigenesis, such as inflammation, cell cycle regulation, stress response, differentiation, apoptosis, and invasion. miRNA targeting is initiated through specific base-pairing interactions between the 5' end ("seed" region) of the miRNA and sites within coding and untranslated regions (UTRs) of mRNAs; target sites in the 3' UTR lead to more effective mRNA destabilization. Since miRNAs frequently target hundreds of mRNAs, miRNA regulatory pathways are complex.

1.7.1. microRNAs: biogenesis and function

Most miRNAs are transcribed as large primary transcripts (pri-miRNAs) by RNA polymerase II [26]. These structured RNAs are then processed into 60–70 nt hairpin loop precursors (pre-miRNAs) by the nuclear RNase III protein, Drosha, in concert with the enzyme called DGCR8 [27].

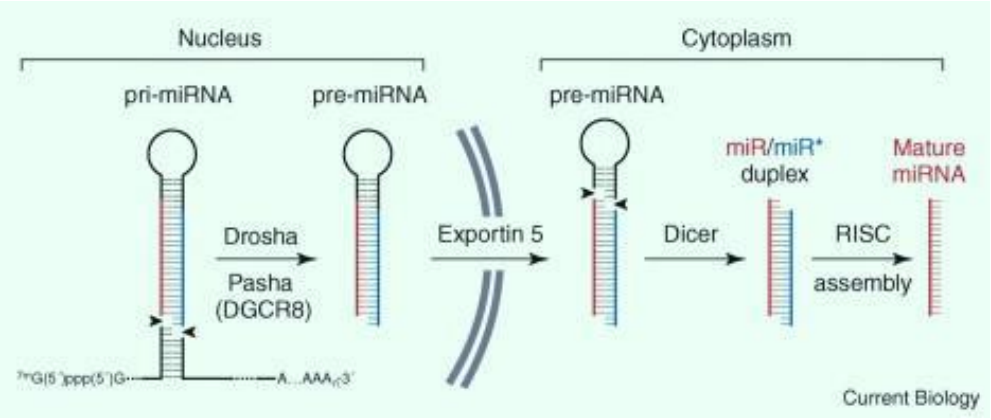

Figure 8: *The miRNA biogenesis pathway in animals [27]*

Pre-miRNAs are exported into the cytoplasm by Exportin-5 (**Figure 8**). Another RNase III enzyme, Dicer, to generate duplex forms of miRNAs, then processes the pre-miRNAs again. Following the maturation of miRNAs, one strand of the duplex is incorporated into the RNA-induced silencing complex (RISC) containing Argonaute 2 (Ago2), creating the inhibitory complex on the target mRNA (**Figure 9**) [28], [29].

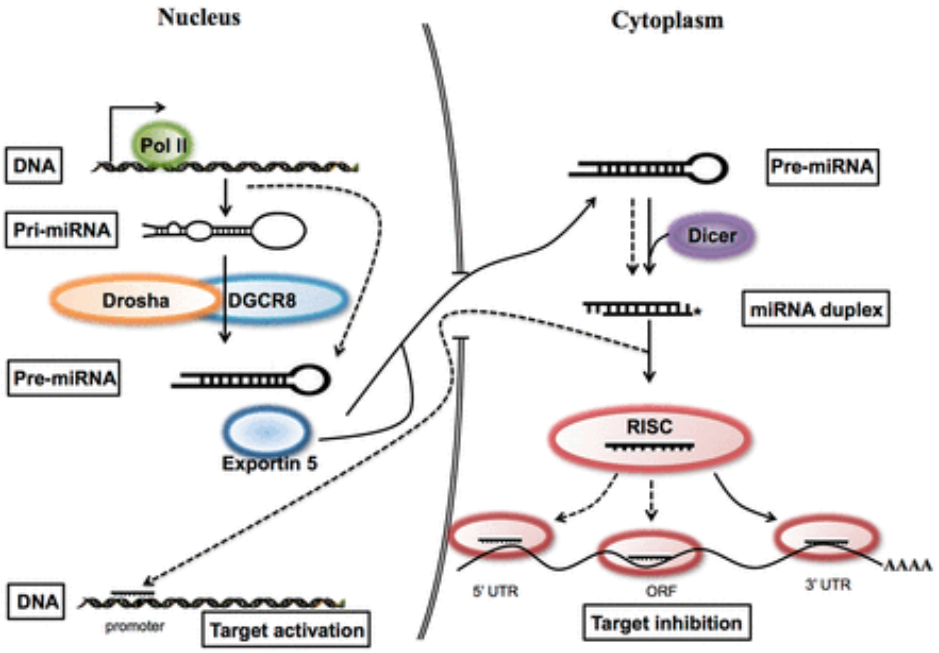

***Figure 9**: Traditional and non-traditional concepts for the biogenesis and function of miRNA. The traditional miRNA biogenesis pathway is designated with a normal arrow while non-traditional pathways are designated with dotted arrow. Canonical animal pri-miRNA is transcribed by RNA polymerase II and processed into pre-miRNA by the Drosha/DGCR8 enzyme complex in the nucleus. Then the pre-miRNA is exported into the cytoplasm in concert with Exportin-5. In the cytoplasm, Dicer cleaves pre-miRNA to a duplex form of miRNAs. Finally, one strand of the duplex is incorporated into the RNA-induced silencing complex to enable inhibition of translation of the target mRNA. Non-traditional miRNAs can be generated via Drosha- or Dicer-independent pathways. Some miRNAs can induce gene expression by targeting the gene's promoter.*

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The first evidence for the existence of miRNAs as well as their important regulatory role has been obtained by the discovery of the small endogenous RNA lin-4 as an essential regulator of cell-fate determination in the C. Elegans larvae. This effect of lin-4 was caused by its ability to downregulate the expression of the transcription factor lin-14, whose temporal decrease is critical during the L1–L3 larval cell stage progression [30] [31]. An additional proof for the ability of miRNAs to regulate gene expression has been obtained by the discovery of second miRNA, let-7, that controls L3-L4 larvae cell stage progression during C. elegans development by targeting lin-41 [32] [32][33]. Since the discovery of let-7, more than 1000 different miRNAs have been described and annotated in various organisms ranging from algae to humans (www.mirbase.org) [34].

miRNA genes are encoded within the genome, suggesting that their transcription might be tightly coordinated with the transcription of other genes including the protein-coding genes that serve either as a source of miRNAs or as their targets. The genomic origin of miRNAs raises the question about the mechanism that either supports or discriminates against simultaneous expression of protein-coding genes and miRNAs. The most common mechanism of transcriptional segregation between specific protein-coding genes and miRNA gene relies on the localization of ~50% of mammalian miRNA-coding genes within the intergenic space. Most of the intergenic miRNAs are autonomously expressed and hold their own enhancer and promoter elements [35] [36] [31]. Similar to protein coding genes, most miRNA genes are transcribed by RNA Polymerase II (RNA Pol II) [37]. It is therefore likely that temporal- and lineage-specific differences in miRNA and protein-coding gene expression are controlled by the expression of specific transcription factors and post-translational chromatin modifications. This possibility is particularly plausible because of the cluster organization of several miRNA genes. miRNA genes, that are clustered within 0.1–50 kb from each other, display common expression patterns [38].

Common expression patterns of clustered miRNAs such as miRNAs from the miR-17-92 and miR-23-27a-24 cluster reflect their generation from a single PolII-dependent polycistronic transcript [26]. The coordinated miRNA gene expression may have functional significance as suggested by the ability of individual miRNAs derived from the miR-17-92 cluster to contribute simultaneously to regulation of cell survival by targeting the pro-apoptotic protein Bim [39]. Opposite to proximally located miRNA genes, miRNA genes that are spaced more than 50 kb apart tend to express in a non-coordinated fashion [38].

Approximately 40% of miRNA genes are localized within gene introns [40]. Initiation of RNA Pol II- or, in some cases, RNA Pol III-dependent transcription [41], [42] within an intron may prevent transcription and splicing of the protein coding genes. However, experimental and bioinformatics data show the ability of numerous intronic miRNAs to coexpress with their host genes [38] [40] [43] [44]. It should be noted that the simultaneous presence of the miRNA and its host mRNA in a given tissue do not automatically imply co-transcription of the miRNA and its host gene(s). It could well be that transcription of the host genes and embedded in them miRNAs does not occur simultaneously but rather in a 'seesaw'-like fashion, thus preventing potential impact of the intronic transcription on host gene splicing. Addressing this question would require GROseq analysis of mRNA and miRNA gene transcription [45].

Approximately 10% of known miRNA genes are situated within exons and, if encoded in sense direction with the coding gene, follow the transcription patterns of their host gene(s). It is plausible that miRNAs localized within the introns or exons of cell lineage- or function-specific genes might contribute to the control of genetic networks according to the expected function of the host gene product. The distinct evolutionary conservation of protein coding host genes and miRNAs suggest that localization of miRNAs within genes emerged in response to the environmental pressure that

required coordination of specific cell functions. In this respect, it would be interesting to determine the evolutionary traits of miRNAs in conjunction with the evolution of their hosts, as well as the appearance of novel regulatory networks in multicellular organisms [36].

Regardless of the genomic location, production of mature miRNAs occurs in a highly conserved fashion that involves the processing of the primary miRNA transcript in the nucleus to the mature product in the cell cytosol. The primary miRNA transcripts (pri-miRNAs) present themselves to the processing machinery not merely as specific sequences but rather as particularly shaped structures. This mode of RNA recognition reflects the ability of the pri-miRNA sequence to fold into an imperfectly paired, double-stranded stem loop structure. The pri-miRNA transcripts can spawn a highly complex structure containing several multiple stem loops in a row. The nuclear microprocessor complex that contains two core proteins, Drosha and DGCR8, recognizes the imperfect pri-miRNA stem loop structures. DGCR8 recognizes and binds to the stem region of the pri-miRNA hairpin followed by the recruitment of Drosha and ensuing cleavage of pri-miRNA and generation of the precursor-miRNA (pre-miR) [46] [47]. The efficiency of pri-miRNA processing depends on the structural characteristics of single pri-miRNA sequences. In case that miRNAs that derive from large polycistronic clusters, such as miR-17-92, the miRNAs inside the core of the highly compact tertiary structure are processed less efficiently than miRNAs on the surface of the structure [48]. The processing of pri-miRNAs occurs co-transcriptionally and rapidly produces a pool of ~60–70-nt-long stem-loop pre-miRNAs [49]. The nascent pre-miRNAs are exported to the cytoplasm by the karyopherin protein family member Exportin-5 in a GTP-dependent manner [50]. Once in the cytoplasm, the pre-miRNA is incorporated into the RISC Loading Complex (RLC) where it is processed by the type III ribonuclease Dicer into a ~21-nt-long miRNA/miRNA* duplex [51]. The miRNA/miRNA* duplex is further

processed/unwound by members of the Argonaute family, giving raise to the mature, single-stranded ~21-nt-long miRNA [52]. The miRNA-generating process described above is currently viewed as the canonical pathway and contributes to the production of most mammalian miRNAs.

1.7.2. miRNA and cancer

Cancer is caused by uncontrolled proliferation and the inappropriate survival of damaged cells, which results in tumor formation. Cells have developed several safeguards to ensure that cell division, differentiation and death occur correctly and in a coordinated fashion, both during development and in the adult body.

Many regulatory factors switch on or off genes that direct cellular proliferation and differentiation. Damage to these genes, which are referred to as tumor-suppressor genes and oncogenes, is selected for in cancer.

Most tumor- suppressor genes and oncogenes are first transcribed from DNA into RNA, and are then translated into protein to exert their effects. Recent evidence indicates that small non-protein-coding RNA molecules, called microRNAs (miRNAs), might also function as tumor suppressors and oncogenes [53]. They have been shown to control cell growth, differentiation and apoptosis; consequently, impaired miRNA expression has been implicated in tumor suppressors and oncogenes. miRNAs, as previously described, regulate gene expression at post-transcriptional level, by binding to the 3'UTR targets messenger RNA, thus inhibiting their translation into proteins (**Figure 10**). miRNAs play fundamental roles in organisms development and differentiation. Strong evidence documents that miRNAs unbalances are involved in many diseases, including cancer. A rapidly increasing number of platforms have been developed for miRNA expression profiling and microarray analysis was the most common method carried out to identify tumor-specific miRNA signatures [54]. However, the arrivals of next generation sequencing (NGS) technologies have offered a

new approach in the identification of previously unknown miRNAs [55]. 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.

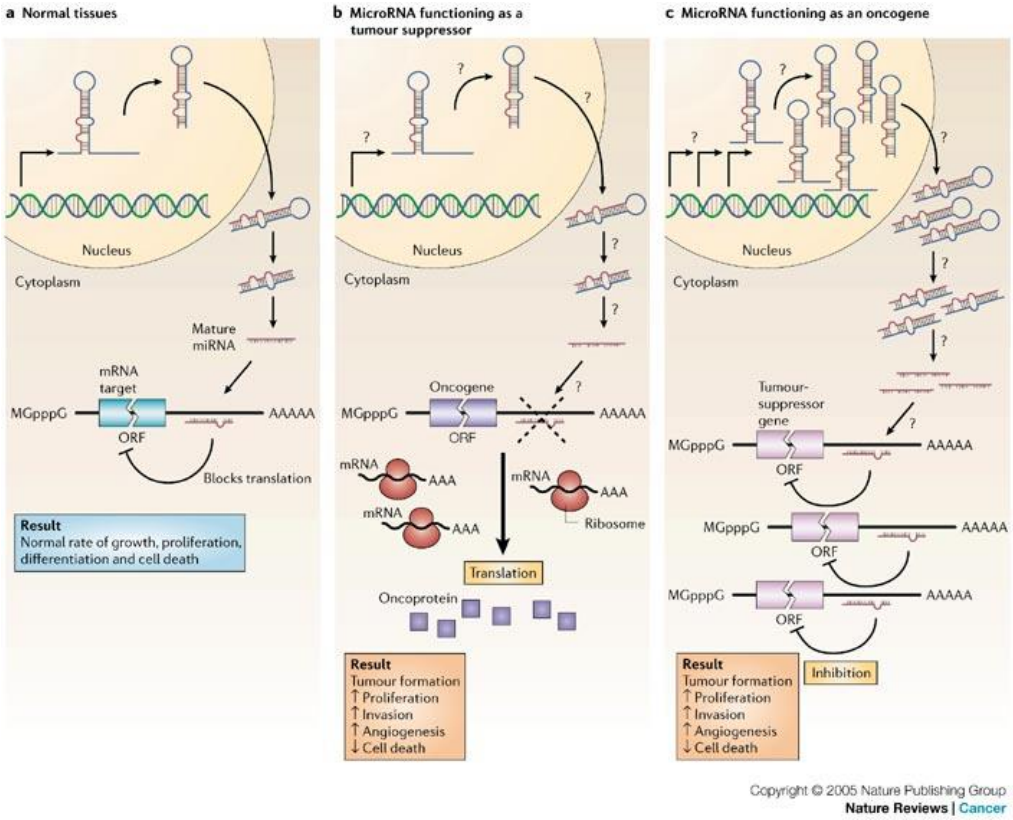


Figure 10*: miRNAs can function as tumor suppressors and oncogenes* ***a*** *–in normal tissue proper microRNA (miRNA) transcription, processing and binding to complementary sequences on the target mRNA results in the repression of target-gene expression through a block in protein. The overall result is normal rates of cellular growth, proliferation, differentiation and cell death.* ***b****- The reduction or deletion of a miRNA that functions as a tumor suppressor leads to tumor formation.* ***c*** *- The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumor formation. In this situation, increased amounts of a miRNA, which might be produced at inappropriate times or in the wrong tissues, would eliminate the expression of a miRNA-target tumor-suppressor gene (pink) and lead to cancer progression[53].*

1.7.3. miRNA and prostate cancer

The first miRNA expression profile in PCa was carried out by Porkka et al [56]. 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 [56]. Further studies confirmed some of the results achieved by these authors, while others showed different expression profiles or identified new altered miRNAs.

Table 2 describes those miRNA involved in the pathogenesis of PCa.

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 [57]. 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 the outcome for individual patients, and identification of molecular indicators of aggressiveness is still needed. Androgen signaling has been associated with miRNA expression, since some miRNAs have been found to modulate the androgen pathway and further classified prostate carcinomas according to castration resistance [56]. For instance, the expression of miR-125b [58], miR-21 [59] and miR-141 [60] is regulated by an androgen responsive element (ARE) which controls the upregulation of these miRNAs and consequently the inhibition of their targets. miR-331-3p is also associated with regulation of androgen receptor (AR) pathway since overexpression of its target, ERBB-2, has been related to disease progression and AR signaling [61]. Some miRNAs play an important role in PCa because modulate the expression of genes that regulate cell growth and apoptosis, others correlate with metastatic PCa (down-regulation of miR-16, miR-34a, miR-126*, miR-205, miR-146a and the up-regulation of miR-301 and miR-125b) [62]. Some miRNAs such as miR-141, miR-143

and miR-145, have been found to be involved in cancer-related cell migration. miR-141 expression, up-regulated in metastatic PCa, correlated with Gleason score [43], [62]. Loss of expression of miR-143 and miR-145 is related to development and progression of PCa [63] and metastasis and also correlate with Gleason score [64]. MiR-200 family regulate the epithelial-mesenchymal transition (EMT) and is down-regulated in tumor tissues [65]. In fact, miR-203 is progressively lost in advanced metastatic PCa showing a linkage between its expression and an antimetastatic role [66]. 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) as show

Table 3 [43].

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 [43]. 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. 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 [67]. Multiple approaches have been designed to achieve miRNAs down-regulation. One of these approaches consists 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. 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 [68]. In recent studies, they 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 were also screened to find a potential inhibitor of miRNA function. Therefore, miRNA-based therapeutics offers promising results for cancer treatment although they are still far away from clinical application Several reports describe PCa-specific miRNA expression signatures, however, the kind of regulated miRNAs is diverse, and there is no agreement in which would be the miRNA-profiling signature of PCa.. Agreement exists among these studies in that the majority of miRNAs are down-regulated in the PCa samples [55], [56], [60], [69], [70]. Although a correlation to tumor stage and grade was described for several miRNAs their relevance as prognostic markers to predict hard clinical endpoints, like clinical failure or cancer-related death, remains limited [55], [71]–[73] [65]. However, there are promising approaches to correlate the altered expression of specific miRNAs and progression of the disease. These miRNAs represent potential factors for PCa diagnosis and prognosis and promising therapeutic tools.

miRNA	Expression	Predicted/validated target(s)	Altered function
miR-10a	Up-reg.	*HOXA1*	Gene expression, cell differentiation
miR-20a	Up-reg.	*E2F1-3*	Apoptosis
miR-21	Up-reg.	*MARCKS, PDCD4, PTEN, TPM1, SPRY2, TIMP3, RECK*	Apoptosis, castrate resistant (CR)
miR-24	Up-reg.	*FAF1*	Apoptosis
miR-25	Up-reg.	*PTEN*	Cell proliferation, cell cycle
miR-31	Up-reg.	*Bcl-w, E2F6*	Apoptosis, cell cycle
miR-32	Up-reg.	*C9orf5, Bim*	Apoptosis
miR-34b	Up-reg.	*CDK6, CREB, c-MYC, MET*	Cell cycle, cell proliferation
miR-96	Up-reg.	*FOXO1, hZIPs*	Apoptosis
miR-106a	Up-reg.	*RB1*	Cell cycle
miR-125b	Up-reg.	*BAK1*	Apoptosis, AR, metastasis
miR-141	Up-reg.	*Clock*	AR, metastasis
miR-148a	Up-reg.	*CAND1, MSK1*	Cell cycle, cell proliferation
miR-181a-1	Up-reg.	*RB1, RBAK*	Cell cycle, tumor progression
miR-182	Up-reg.	*FOXO1, FOXO3, BRCA1, hZIP1*	Apoptosis
miR-194	Up-reg.	*DNMT3a, MeCP2*	Genomic instability
miR-200a/b	Up-reg.	*β-catenin, SIRT1*	EMT, cell growth
miR-200c	Up-reg.	*SEC23A, JAGGED1*	Cell growth, apoptosis, metastasis
miR-210	Up-reg.	*EFNA3, MNT, HOXA1, APC, ELK3*	Hypoxia, cell proliferation, migration
miR-214	Up-reg.	*EZH2, N-Ras, PTEN*	Cell cycle, cell proliferation
miR-218	Up-reg.	*RAS, c-myc, Laminin 5 β3, THAP2, SMARCA5, and BAZ2A*	Cell proliferation, apoptosis
miR-224	Up-reg.	*KLK1, API-5*	Apoptosis, cell proliferation, invasion

miR-296	Up-reg.	*HMGA1*	Cell proliferation, invasion
miR-345	Up-reg.	*BAG3*	Apoptosis, invasion, metastasis
miR-375	Up-reg.	*Sec23A*	Cell proliferation
miR-521	Up-reg.	*CSA*	DNA repair
miR-26a	Up and down-reg.	*PLAG1, EZH2*	Apoptosis, cell proliferation, invasion
miR-30c	Up and down-reg.	*BCL-9, MTA1*	Metastasis
miR-100	Up and down-reg.	*RAS, c-myc, Laminin 5 β3, THAP2, SMARCA5, and BAZ2A*	Cell proliferation, apoptosis
miR-125a	Up and down-reg.	*ERBB2, ERBB3*	Cell proliferation, apoptosis
miR-195	Up and down-reg.	*CDK4, GLUT3, WEE1, CDK6, Bcl-2*	Cell cycle, cell proliferation, apoptosis
miR-221	Up and down-reg.	*p27kip1*	Cell cycle
miR-222	Up and down-reg.	*p27kip1*	Cell cycle
miR-30b	Up and down reg.	*GalNAc, Snail1*	Invasion, immunosuppression
let-7-family	Down-reg.	*Ras, Cdc25A, Cyclin D1*	Apoptosis, cell proliferation, cell cycle
miR-1	Down-reg.	*Exportin-6, Tyrosine kinase 9, PNP*	Cell proliferation, invasion
miR-7	Down-reg.	*ERBB2*	Cell proliferation, tumor progression
miR-16	Down-reg.	*Bcl-2, cyclin D1 and D3, CDK1, CDK2*	Apoptosis, cell cycle, metastasis
miR-22	Down-reg.	*PTEN*	Cell proliferation, cell cycle
miR-23a/b	Down-reg.	*Mitochondrial glutaminase*	Advantage in growth
miR-27b	Down-reg.	*CYP1B1, Notch1*	Hormone metabolism, cell proliferation
miR-29a	Down-reg.	*Dkk1, Kremen2, sFRP2, B7-H3*	Cell differentiation, immune response

miR-34a	Down-reg.	*BCL-2,SIRT1,E2F3, N-MYC, MET, CDK4-6, DLL1*	Apoptosis, proliferation, survival
miR-34c	Down-reg.	*CDK4, E2F3, MET, c-MYC*	Apoptosis, cell proliferation
miR-92	Down-reg.	*Bim*	Apoptosis
miR-99a	Down-reg.	*SMARCA, SMARCD1, mTOR*	Apoptosis, cell cycle
miR-101	Down-reg.	*EZH2*	Cell proliferation, invasion
miR-106b-25	Down-reg.	*MCM7*	Cell cycle, cell proliferation
miR-107	Down-reg.	*Granulin*	Cell proliferation
miR-126	Down-reg.	*CRK, Spred1, PIK3R2/p85-beta*	Cell proliferation, invasion, tumor progression
miR-126*	Down-reg.	*Prostein*	Metastasis
miR-128a	Down-reg.	*GOLM1, PHB, TROVE2, TMSB10*	Tumor progression, invasion
miR-143	Down-reg.	*MYO6, ERK5, KRAS*	Cell proliferation, migration, metastasis
miR-145	Down-reg.	*MYO6, MYC, BNIP3*	Cell migration, metastasis, apoptosis
miR-146a	Down-reg.	*CXCR4, ROCK1*	CR, metastasis
miR-203	Down-reg.	*ZEB2, Bmi, survivin, Runx2*	EMT, metastasis
miR-205	Down-reg.	*ErbB3, E2F1, E2F5, ZEB2, Protein Kinase Cε, IL24, IL32*	Cell cycle, cell proliferation, apoptosis,EMT
miR-223	Down-reg.	*NFI-A*	Cell differentiation
miR-301a	Down-reg.	*FOXF2, BBC3, PTEN, COL2A1*	Cell proliferation
miR-320a	Down-reg.	*ETS2*	Tumor progression
miR-330	Down-reg.	*E2F1*	Apoptosis
miR-331-3p	Down-reg.	*ERBB2*	Cell cycle
miR-449a	Down-reg.	*HDAC-1*	Cell cycle, apoptosis

Table 2: *miRNAs altered in PCa, their target and function [54]*

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

***Table 3**: miRNAs implicated in PCa prognosis [54]*

1.7.4. miRNAs as circulating biomarkers of PCa

The biological function and the impact of circulating miRNAs on the tumor microenvironment and tumor progression remain to be investigated, and mechanisms of selective miRNA export from cells to the circulation system also remain to be uncovered. In one of the preliminary studies, they found that miR-21 and -221 levels in the patients were higher than in healthy controls, while, for the miR-141, no difference was observed [74]. In a recent review, the author underlines some lack in studies about circulating miRNA.. The first problem shown is that, both serum and plasma samples collected and processed under very different or partly indefinitely described conditions (various blood collection devices, time interval between phlebotomy and centrifugation, storage temperature during this period, centrifugation conditions, e.g. speed, duration, and temperature etc.) were uniformly defined as sources of 'circulating' miRNAs [75]. The pre-analytical differences of samplings alone give rise to distinctly interfering effects by varying levels of miRNAs from leukocytes, erythrocytes, and platelets, respectively. The true cell-free circulating miRNAs could be confounded by cellular miRNAs from blood cells released either from them or as contaminating cellular particles insufficiently removed by inappropriate centrifugation. Indeed only four miRNAs (miR-21, miR-141, miR-221, miR-375) were found to be changed in at least two studies, but not always consistently in the same direction [75].

Others studies started to describe the expression profile of miRNAs also in serum [74]. In a recent study, they analyzed five miRNAs highly abundant in the sera of patients with metastatic disease (miRNA-375, miRNA-9*, miRNA-141, miRNA-200b and miRNA-516a-3p). Circulating miRNA-375 and miRNA-141 turned out to be the most pronounced markers for high-risk tumors [63]. Their levels also correlated with high Gleason score or lymph-node positive. In addition, the expression levels of miRNA-375 and miRNA-141 were monitored in 72 prostate tissue samples (36 tumors vs. 36

benign). Both miRNAs were highly expressed in all samples and significantly upregulated in the tumors compared to normal tissues [63]. Overall, their observations suggest that miRNA-375 and miRNA-141 expression is enhanced in prostate cancer specimens, and their release into the blood is further associated with advanced cancer disease. Another study evaluated three miRNAs (miR-141, miR-146b-3p and miR-194) high in patients who subsequently experienced BCR (biochemical recurrence) in the screening study. MiR-146b-3p and miR-194 were also associated with disease progression in the validation cohort. Multivariate analysis revealed that miR-146b-3p possessed prognostic information beyond standard clinic-pathological parameters. Analysis of tissue cohorts revealed that miR-194 was robustly expressed in the prostate, high in metastases, and its expression in primary tumors was associated with a poor prognosis [76].

In **Table 4** are summarized recent studies about circulating miRNA as biomarkers of PCa.

Urine is also easily available source for molecular markers; therefore, detection of miRNAs in urine of patients with PCa would represent an ideal non-invasive diagnosis approach.

Study design	Upregulated miRNAs	Downregulated miRNAs	Sample type	Method	Patient numbers	Reference
Cancer vs benign	100, 125b, 141, 143,		Serum	qRT-PCR	25 metastatic PCa vs25 controls	Mitchell et al, 2008
	16, 92a, 103, 107, 197, 34b, 328, 485-3p, 486-5p, 92b, 574-3p, 636, 640, 766, 885-5p		Serum	MiR microarray	5 PCa vs 8 controls (other cancers tested)	Lodes et al, 2009
	221		Plasma	qRT-PCR	28 PCa vs 20 controls	Zheng et al, 2011
	141, 298, 346, 375		Serum	qRT-PCR	25 metastatic PCa vs25 controls	Selth et al, 2012
	346, 622, 940, 1285	let-7e, let-7c	Plasma	Illumina miR microarray	25 PCa vs 17 BPH, then validation in 80 PCa, 44 BPH and 54 HI	Chen et al, 2012
	375, 141	181a-2	Plasma	qRT-PCR	78 PCa vs 28 controls, then 16 metastatic vs55 non-metastatic	Bryant et al, 2012
Localized cancer vs metastatic cancer	9*, 141, 200b, 375, 516a		Serum	qRT-PCR	7 metastatic PCa vs 14 localised PCa	Brase et al, 2011
	21		Serum	qRT-PCR	50 PCa (20 localised, 20 ADPC, 10 HRPC) vs6 BPH	Zhang et al, 2011
	375, 141	181a-2	Plasma	qRT-PCR	78 PCa vs 28 controls, then 16 metastatic vs55 non-metastatic	Bryant et al, 2012
Cancer at different stage/risks vs benign	21, 221		Plasma	qRT-PCR	51 PCa (18 localised, 8 locally advanced, 25 metastatic) vs 20 HI	Yaman Agaoglu et al, 2011
	93, 106a, 874, 1207	24, 26b, 30c, 223	Serum	Multiplexed qRT-PCR	36 PCa (12 low risk, 12 medium risk, 12 high risk) vs 12 controls	Moltzahnet al, 2011
	26a, 195, Let7i, 16		Serum	qRT-PCR	45 (37 localised, 8 metastatic) PCa vs 38 controls (18 BPH, 20	Mahn et al, 2011
	20a, 21		Plasma	qRT-PCR	82 PCa assessing association with CAPRA score	Shen et al, 2012

Table 4: *Results of published studies investigating the potential of circulating miRNAs as biomarkers of prostate cancer* [111]

1.8. Prostate cancer cell lines

Most of the studies on prostate cancer were done in cell lines that have been widely used for research purposes and proved the useful tool in the genetic approach, and its characterization shows that they are, in fact, an excellent model for the study of the biological mechanisms involved in cancer.

Table 5 lists the non-neoplastic and tumor cell lines used in studies on prostate cancer**.**

Name	Origin	Characteristics
Benign cell lines		
BPH-1	Immortalized with SV40	AR-
PWR-1E	Immortalized with human papilloma virus 18	AR+, androgen responsive, express PSA, nontumorigenic in nude mice
RC-165N/hTERT	Immortalized with human telomerase reverse transcriptase	AR+, androgen responsive, express PSA, nontumorigenic in nude mice
RWPE1	Immortalized with SV40	AR+, androgen responsive, express PSA, nontumorigenic in nude mice
AR+ PCa cell lines		
ARCaP	Ascites fluid of the same patient as MDA PCa cells	Low levels of AR and PSA, growth inhibited by androgens
DUCaP	Brain metastasis	Wild-type AR, androgen sensitive
E006AA	Primary PCa from an African-American patient with hormone naïve localized PCa	Mutated AR, do not express PSA, insensitive to androgens
LAPC	Locally advanced or metastatic PCa	Wild-type AR, express PSA, different sublines available
LNCaP	Lymph node metastasis	Mutated AR, produce PSA, androgen responsive
MDA PCa cells	Bone metastasis	Mutated AR, produce PSA, less responsive to androgens
PC346	Transurethral resection of localized advanced PCa	Wild-type AR, different sublines available
22Rv1	Primary PCa	Mutated AR, low levels of AR and
VCaP	Bone metastasis	Wild-type AR, androgen sensitive
AR- PCa cell lines		
DU145	Brain metastasis	AR-, do not respond to androgens
PC3	Bone metastasis	AR-, do not respond to androgens

Table 5*: Origins, characteristics and culture conditions of prostate epithelial cell lines*

The first human prostatic tumor epithelial cell lines to be spontaneously established were LNCaP, PC3 and DU145, which were derived from PCa lymph node, bone and brain metastases respectively and remain the most commonly used PCa cell lines [77]. Of these three cell lines, only LNCaP expresses significant levels of AR and consequently is the most widely used AR+ cell line. DU145 and PC3 cells are generally considered being AR− and thus usually used as AR− controls or to study androgen signaling by ectopic AR overexpression. Although LNCaPs are androgen responsive and produce PSA, it should be noted that they express a mutated AR (T877A), which results in altered AR signaling [15].

In contrast to the abundance of PCa cell lines, there are relatively few cell lines derived from benign prostatic epithelium suitable for investigating AR signaling. This is primarily due to difficulties in in-vitro immortalization and the terminally differentiated nature of the androgen-dependent luminal epithelium such that primary epithelial cultures predominantly exhibit an androgen-independent but proliferative basal/intermediate phenotype [78]. Nonetheless, there are currently three main AR+ normal prostate epithelial cell lines PWR-1E, RWPE1 and RC-165N/hTERT, which were immortalized using SV40, human papilloma virus 18 or human telomerase reverse transcriptase respectively [79]. These cell lines are androgen responsive, express AR and PSA but do not form tumors when injected into nude mice. This latter characteristic has been exploited to investigate the role of putative oncogenes and carcinogens on tumorigenesis indicating the suitability of these cell lines as a potential model system to study processes of oncogenic transformation. It should be noted, however, that the process of immortalization itself can result in genetic alterations and/or mutation [80]. To date, these cell lines have predominantly been used to compare gene expression levels and drug efficacy with PCa cell lines. In spite of the essential role of cancer cell lines in biomedical research, there is a debate

among the scientific community on the fact whether they are or not representative of the original tumor.

1.9. RUNX2 expression in prostate cancer

Runt-related transcription factors (RUNX) determine cell fate and regulate lineage-specific proliferation and differentiation [81],[82]. The RUNX gene family, which has been studied extensively, includes RUNX1, RUNX2 and RUNX3, closely related transcription factors that have crucial roles in both oncogenic and tumor-suppressive functions. RUNX1 is important for hematopoiesis, and its ablation leads to leukemia; RUNX3 is a critical regulator and its promoter hypermethylation is associated with several types of cancer [83][84]. These findings strongly suggest that RUNX1 and RUNX3 function as tumor suppressors. The RUNX2 gene, on the other hand, is a unique member of the RUNX family that was shown to have a dual function in several studies. RUNX2 is a transcription factor essential for osteoblast differentiation [85] and its ablation results in deregulated cell proliferation and immortalization, supporting its function as a tumor suppressor [84]. However, several studies report that RUNX2 is upregulated in prostate and breast cancer cells that metastasize to bone, indicating a correlation with cancer progression [86] and therefore an oncogenic function for RUNX2.

RUNX2 is closely related to bone formation, and prostate cancer (CaP) is the most common cancer to metastasize to bone. Therefore, several studies focus on the role of RUNX2 in the tumor and bone tissue environments by using cell lines derived from metastatic bone lesions. Only a few studies have used human tissues to investigate the function of RUNX2 in CaP, and these studies report contradictory results [81][87][88].

1.10. Cancer associated fibroblasts (CAF)

The prostate is composed of a number of different cell populations. The interaction between them is critical for the development and proper function

of the prostate. However, the effect of the molecular cross talk between these cells in the course of carcinogenesis is still unclear.

The prostate is made of the epithelium and the stroma, which consists of smooth muscle cells (SMC), fibroblasts, nerves, and lymphatics [89]. The stroma plays an important role during embryonic development of diverse structures in the prostate. In adult tissues, stromal cells are responsible for the maintenance of homeostatic equilibrium and in controlling cell size and cell functions of the epithelium they surround. This is achieved through modifications of the extracellular matrix [90], [91]. In the case of disturbed homeostasis regulation, stromal cells can contribute to the initiation and progression of cancer [92]. During carcinogenesis, the stroma undergoes several changes. Altered fibroblasts, termed cancer-associated fibroblasts (CAF), appear in the proximity of the tumor, the amount of SMCs decreases, the bioavailability of growth factors increases, inflammatory cells are infiltrated, angiogenesis increases, and stromal protease inhibitors are lost [93], [94].

In a study, employing an approach wherein immortalized epithelial cells and immortalized human fibroblasts were cocultured, they show that normal associated fibroblasts (NAF) and cancer-associated fibroblasts (CAF) differentially influenced the growth and proliferation of immortalized human prostate epithelial cells [95]. Whereas NAFs inhibited the growth of immortalized epithelial cells but promoted the growth of metastatic PC-3 cells, CAFs promoted the growth of immortalized epithelial cells but not of PC-3. Cytokine arrays revealed that NAFs secreted higher levels of tumor necrosis factor-alpha compared with CAFs whereas CAFs secreted higher levels of interleukin-6 (IL-6) compared with NAFs. The growth-inhibiting effects of NAFs were counteracted by the addition of IL-6, and the growth-promoting effects exerted by the CAFs were counteracted by tumor necrosis factor-alpha. Furthermore, CAFs induced the migration of endothelial cells in an IL-6-dependent manner. Therefore, they show that

normal fibroblast cells have a protective function at very early stages of carcinogenesis by preventing immortalized epithelial cells from proliferating and forming new blood vessels, whereas CAFs aid immortalized epithelial cells to develop later. [95]

Gandellini research group recently have focused on the involvement of microRNAs in tumor-stroma interplay. They found that miR-205, as already reported, is the most down-modulated miRNA in PCa cells upon CAF stimulation, due to direct transcriptional repression by HIF-1 (Hypoxia-inducible factors), a known redox-sensitive transcription factor. Rescue experiments demonstrated that ectopic miR-205 overexpression in PCa cells counteracts CAF-induced EMT, thus impairing enhancement of cell invasion, acquisition of stem cell traits, tumorigenicity, and metastatic dissemination. In addition, miR-205 blocks tumor-driven activation of surrounding fibroblasts by reducing pro-inflammatory cytokine secretion. Overall, such findings suggest miR-205 as a brake against PCa metastasis by blocking both the afferent and efferent arms of the circuit between tumor cells and associated fibroblasts, thus interrupting the pro-oxidant and pro-inflammatory circuitries engaged by reactive stroma. The evidence that miR-205 replacement in PCa cells is able not only to prevent but also to revert the oxidative/pro-inflammatory axis leading to EMT induced by CAFs, sets the rationale for developing miRNA-based approaches to prevent and treat metastatic disease [96]. This is the only work that addresses the issue of the role of miRNA on the interactions between fibroblasts and epithelial cells. Then it becomes essential to deepen the study of this kind of interaction.

AIM OF THE STUDY

2. AIM OF THE STUDY

The current diagnostic approach of PCa is based on measurement of PSA serum levels. Although the method of screening with PSA is widely used, recent studies have shown that screening with PSA did not significantly reduce mortality associated with PCa. This is due to the low specificity of this method. In order to have a clear diagnosis, the biopsy supports the evaluation of the PSA, to perform a histological sample analysis.

The lack of specific prostate cancer diagnosis methods shows the need to identify novel additional diagnostic and prognostic biomarkers. miRNA are involved in the regulation several physiological and pathological processes; therefore, the aim of this study is the identification of microRNA involved in PCa development from PIN in different study models.

We propose to identify miRNA signatures associated to PCa progression that could represent a novel generation of diagnostic biomarkers adjunctive to PSA, prognostic and predictive of cancer progression. Our experimental strategy included the use of normal or tumorigenic prostate cell lines, mouse model of PCa and patients' series.

The next purpose will be to study in depth the interaction between epithelial cells and tumor associated fibroblasts, analyzing the role of miRNAs in the modulation of pathological processes.

MATERIALS AND METHODS

3. MATERIAL AND METHODS

3.1. Cell lines

First, we analyzed the miRNA expression profile in 5 prostate cell lines. We choose 1 normal epithelial cell line (RWPE-1), 1 cell line of Benign prostatic hyperplasia (BPH-1) and 3 cancer cell lines (LNCap, DU145, PC3). The normal cell line (RWPE-1) is androgen sensitive and was cultured in K-SFM (Keratinocyte Serum Free Medium) with addition of 5 ng/ml of EGF (human recombinant epidermal growth factor) and 0.05 mg/ml of BPE (bovine pituitary extract). Other cell lines were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep and were maintained at 37°C and 5% CO2. We choose one cancer cell line androgen sensitive (LNCap) and the others two androgen insensitive, with high metastatic potential as shown in

Table 6.

Cell line	Description	Derived from metastatic site
RWPE-1	epithelial normal prostate cell line	
BPH-1	Benign prostatic hyperplasia	
LNCap	prostate cancer cell line androgen sensitive	left supra clavicular lymph node
DU145	prostate cancer cell line- metastatic androgen insensitive	brain
PC3	prostate cancer cell line- metastatic	bone

***Table 6**: Prostate cancer and normal prostate cell lines*

3.2. Human samples

We selected 192 human samples of prostatic acinar adenocarcinoma, undergone radical prostatectomy from 2004 to 2006, 58 of which showed PIN. The clinical stage and histopathological variables of PCa diagnosis are displayed in **Table 7**. We also selected 8 patient with high grade prostate cancer (T4, Gleason score >7, N1) in order to analyse trough Laser capture microdissection (see below) the stromal component.

192 patients -Mean Age 65 (range 44-77)

Grading % (n)	
T1	0.5 (1)
T2	**64.1** (123)
T3	33.9 (65)
T4	1.6 (3)

Regional Lymphnodes %(n)	
N0	**86.9** (167)
N1	8.4 (16)
Nx	4.6 (9)

Perineural invasion %(n)	
Yes	**58.3** (112)
No	29.2 (56)

Gleason score % (n)	
5	2.6 (5)
6	29.7 (57)
7	**50.5** (97)
7a (3+4)	(68)
7b (4+3)	(29)
8	11.5 (22)
9	3.6 (7)

Capsular invasion %(n)	
L0	26.6 (51)
L1	7.8 (15)
L2	27.1 (52)
L3	**30.2** (58)
nd	8.3 (16)

***Table 7**: Clinical stage of 192 prostate cancers.*

3.2.1. Tissue microarray

Whit this case study we built a Tissue Micro Array (MiniCore Alphelys ®). TMAs utilise cores, collected from multiple paraffin-embedded tissue 'donor' blocks that are inserted (arranged in columns and rows of potentially hundreds of cases) into a single 'recipient' block. In **Figure 11** are shown all passages of TMA construction. Once the TMAs have been

constructed, they allow analysis of very large numbers of samples more quickly, efficiently and in a more cost-effective manner than examination of whole tissue sections for each marker.

In each paraffin block, we put 1 core of non-neoplastic tissue, 3 cores of cancer, in order to reproduce tumor heterogeneity and different Gleason score in each patient, and 1 core, when present, of PIN. We built 16 paraffin blocks and each core was 2mm diameter. In each block, we spotted cores from 12 patients for a total of 192 patients. From each TMA block, a 4µm section was cut and stained with haematoxylin and eosin stain (H&E) and subsequently with a specific immunohistochemical stain.

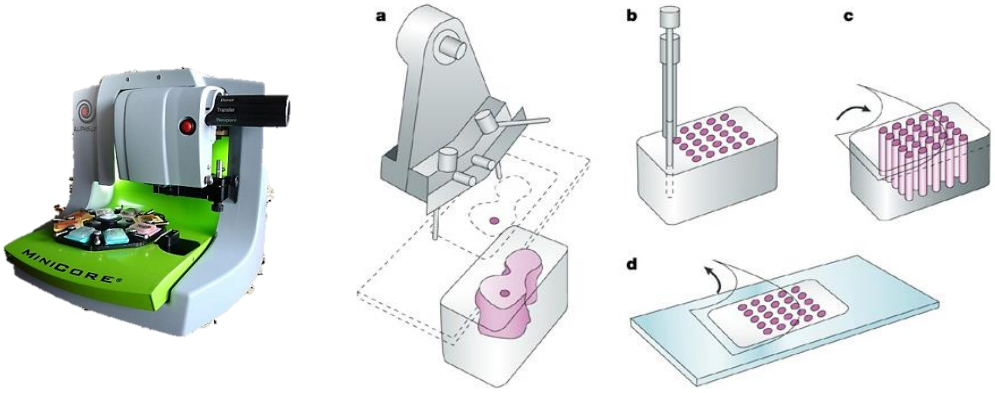


Figure 11: *TMA building* ***a)*** *Area selection and core pick up from donor block* ***b)*** *Core insertion into the recipient block* ***c)*** *4 µm section cut* ***d)*** *TMA section on the slice*

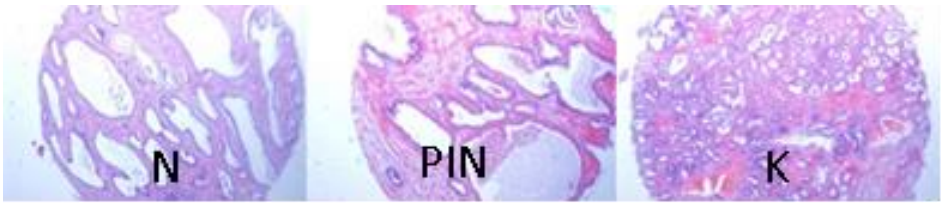


Figure 12: *PCa Tissue micro-array*

3.3. TRAMP mouse

Simultaneously we examined PIN and cancer in the TRAMP mouse model. In this part of the study, we examined a series of 5 transgenic mice, sacrificed at 24 weeks, which have all developed the adenocarcinoma. The animals were sacrificed and the urogenital tract, including the bladder, seminal vesicles and prostate, was collected and then formalin fixed and paraffin embedded.

3.3.1. Laser-capture microdissection (LCM)

The enrichment of stromal and epithelial component in TRAMP mice was accomplished through the LCM (Laser-Capture Microdissection, Leica LMD6000, **Figure 13**). Indeed, we collect from TRAMP mouse three sections 6 μm thick of FFPE (formalin-fixed, paraffin-embedded) tissues. The sections were stained only with haematoxylin in order to preserve nucleic acid stability. Moreover, Leica slides are made of a particular membrane that allows tissues to be cut from the laser without interfering with RNA/DNA purification.

Each slice were deparaffinised and dehydrated following this steps:

- Xylene 1’
- Absolute Ethanol 1’
- Ethanol 95% 1’
- Ethanol 75% 1’
- DEPC water 1’
- Haematoxylin Carazzi 1’
- DEPC water 1’
- Ethanol 75% 1’

Then we allow the section to dry for about 2 hours to follow with the laser microdissection. In **Figure 14** are displayed examples of laser microdissection on TRAMP mouse tissues.

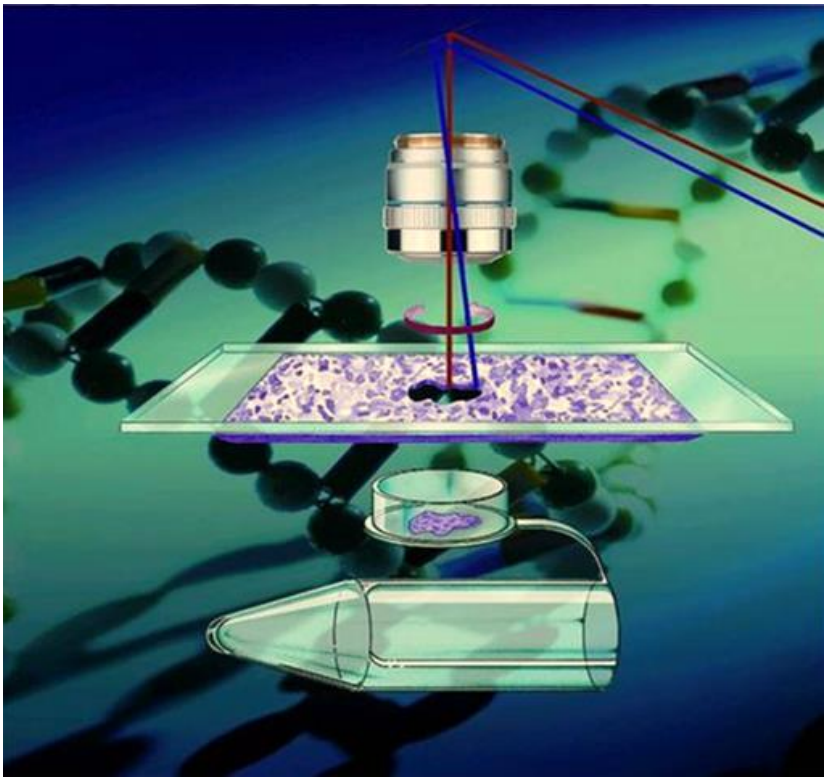


Figure 13: *Laser capture microdissection (Leica microsystem)*

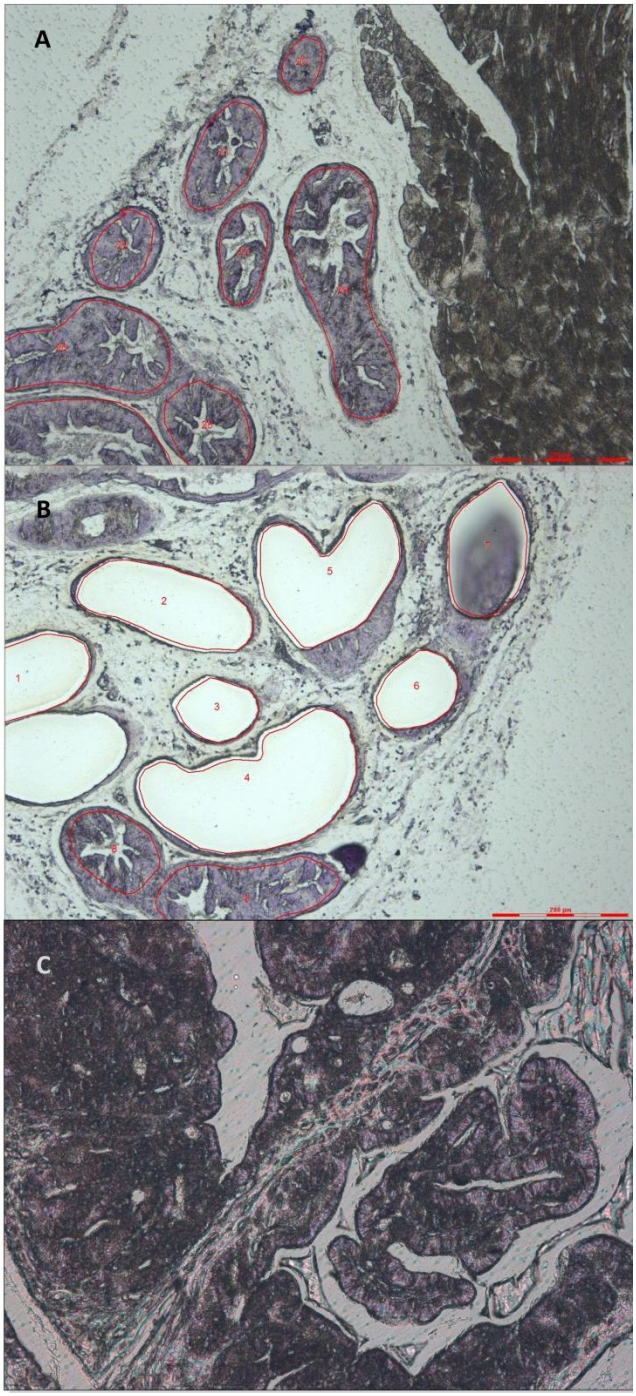


Figure 14: *TRAMP mouse laser microdissection. Panel A: PIN epithelium before laser cut. Panel B: PIN After laser cut. Panel C: Cancer at 24 weeks of age*

3.4. RNA purification

For molecular investigation, macrodissection of the tissues was performed by punching the archival blocks with a needle of 1 mm of diameter in order to achieve 80% epithelial cell purity in each sample. We isolated with the MiniCore puncher 1 core of cancer, 1 core of non-neoplastic tissue and 1 core, when present, of PIN. Then total RNA was isolated with MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer's instructions. From samples obtained with the LCM, we extracted and quantified total RNA as previously described. RNA was quantified spectrophotometrically and stored at -80°C in bioPur safe lock tubes. We collected in all RNA from 442 human samples (192 patients), 5 cell lines and 5 mice.

3.5. Micro Fluidic Card and qRT-PCR

From samples obtained with the LCM, we polled RNA from 5 mice and performed a low-density array platform (TaqMan® Array Rodent MicroRNA, Micro Fluidic Card Applied Biosystems, ABI PRISM® 7900HT) that allows to detect simultaneously about 700 miRNAs including endogenous small RNAs (snoRNA). We evaluated the differential expression of epithelial and stromal component of adenocarcinoma and PIN.

Figure 16 summarizes qRT-PCR chemistry. We performed retro-transcription with 40 ng (TRAMP mouse samples) of RNA using Megaplex RT primer poll A and B Rodent and the Taqman MicroRNA Reverse Transcription kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). RT product was amplified with Pre-amplification primers pool. We analyzed with the same platform (TaqMan® Array Human MicroRNA) the miRNA expression profile in the cell lines (RWPE-1, BPH-1, LNCap, DU145 and PC3) and reverse transcription was performed with 1µg of RNA. For the validation, we ordered a custom RT primers pool and 19 microRNA

Card custom (Micro Fluidic Card Applied Biosystems) designed with 23 miRNA spotted. This platform allowed analyzing 23 miRNA in 8 samples per card simultaneously (**Figure 15**). 500 ng of RNA were reverse transcribed, and RT products were amplified with Pre-amplification primers pool. Housekeeping genes were snoRNA135 and snoRNA202, for TRAMP mouse analysis, MammU6, RNU44, RNU48 for cell line analysis, and MammU6 for human samples.

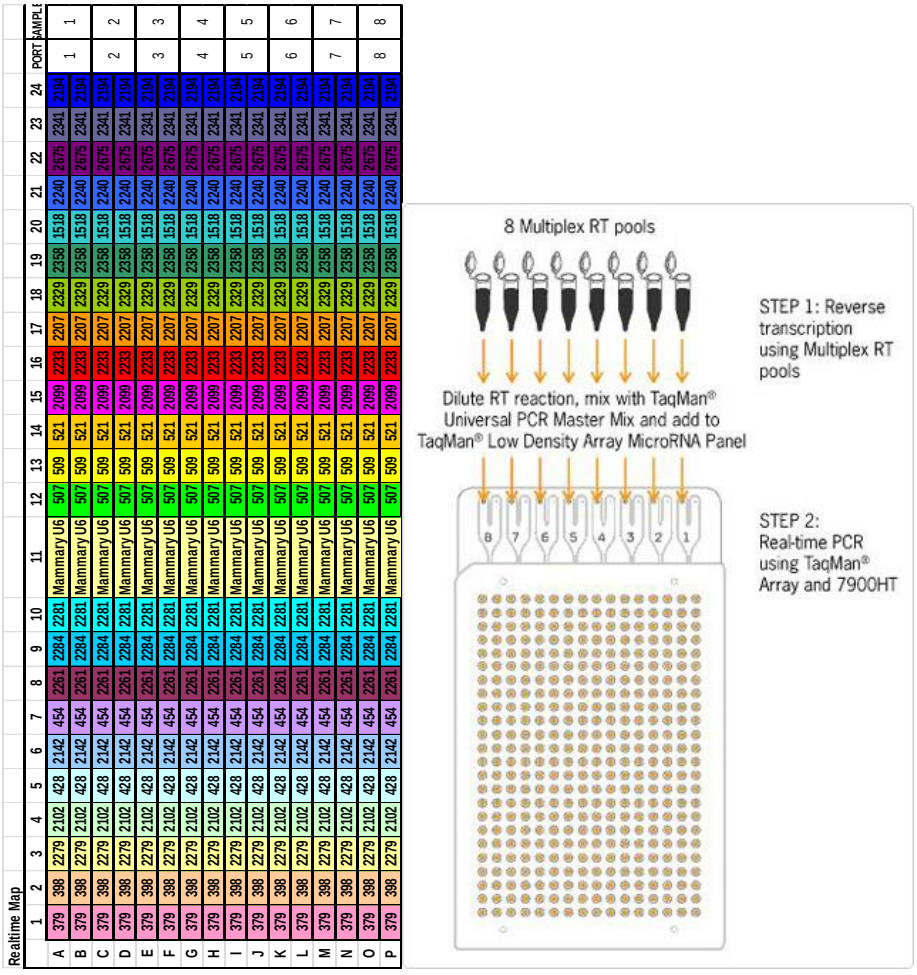


Figure 15 *: Card custom design with miRNA assay ID (left) and Microfluidic Card procedure (right)*

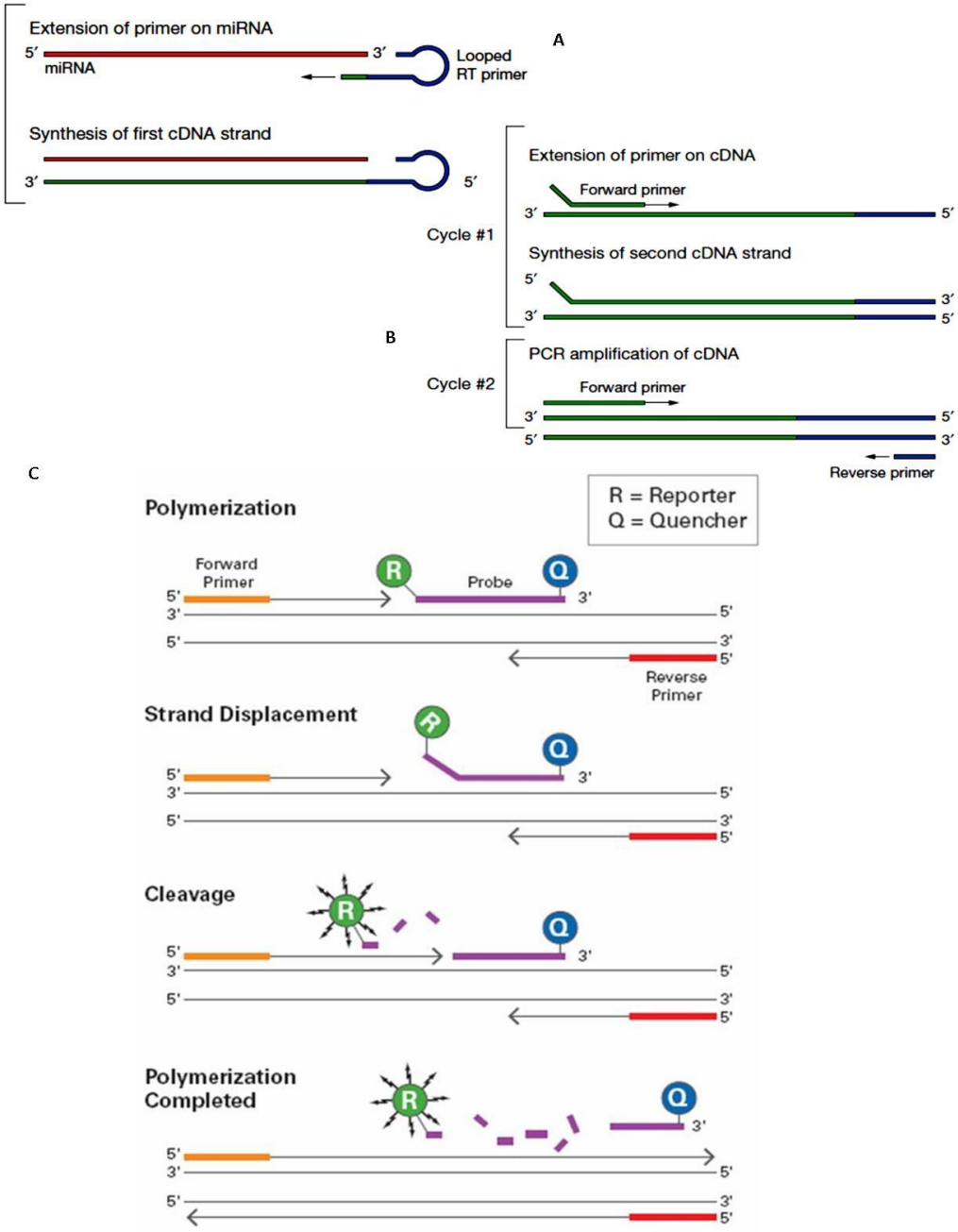


***Figure 16**: qRT-PCR scheme. A: reverse transcription B: Pre-amplification reaction C: Real Time PCR*

3.6. Immunohistochemistry (IHC)

For each TMA block, 4µm-thick sections were stained with RUNX2 primary antibody (Novus Biologicals NBP1-01004) 1:1000 using citrate buffer in antigen retrieval step. Immunohistochemistry was performed using Benchmark Ultra Roche Ventana immunostainer. Endogenous peroxidase activity was blocked by hydrogen peroxide (0,3%) for 10 minutes. The chromogen diaminobenzidine (DAB) was incubated for 8 min at room temperature. All slides were counterstained with haematoxylin.

A pathologist blinded to clinical data evaluated immunohistochemical results. Percentage and localization of immunoreactive cells were calculated by averaging out replicates of tumor cores.

3.7. Derivation of fibroblasts

We collected from radical prostatectomy tissue samples in order to derivate fibroblasts associated with the tissue. After surgery, we immediately collected the sample. A pathologist selected and area of interest and then we cut with at cryostat a frozen sections in order to identify normal and cancer tissues. Once identified the tissue of interest we transferred the piece under a laminar flow hood reserved to tissue culture. We cut up the prostate, normal or cancer, in small pieces in a plate with a sterile blade and let dry for 1 hour in incubator at 37°C 5% CO_2. When the tissue adhered to the plate, we added 5mL of tissue culture medium mixture (HAM F12 GIBCO with 20% FBS, 10% Pen/Strep, 1% amphotericin B, 0,2% Kanamycin) and incubated at 37°C 5% CO_2. The day after we changed the media adding DMEM supplemented with 10% FBS and 1% Pen-Strep.

After several days, fibroblasts associated with the tissue started growing on the plate. Once at confluence, we remove the tissue and split fibroblasts in a new plate with fresh media and we treated for 3 times every change of media with 1% of mycoplasma removal.

In all, we collected and cryopreserved 5 CAF lines and 3 NAF lines. For each line, we extracted RNA with the protocol above.

3.8. Data analysis

Expression data from qRT-PCR were first analyzed with DataAssist™ Software (Applied Biosystem). Mammary U6 was the housekeeping gene. The comparative Ct method ($2^{-\Delta Ct}$) was used for the relative quantification (RQ) and miRNA RQs were median normalized and log2-transformed. In order to compare tissue classes, we used BrB Array tool. In cell lines, we compared miRNA expression data of cancer, hyperplastic and normal cell lines. Comparison was performed also between AR sensitive and insensitive cancer.

MiRNAs expressed in at least one sample group were imported in dChip software (DNA-Chip Analyzer, www.dchip.org) for unsupervised hierarchical clustering.

For human samples (n=58 patients), data were analyzed by means of two-sided unpaired t tests using a GraphPad Prism© 5 software package for Windows. Data are expressed as the mean ± SD or mean ± SEM of multiple independent experiments. A p value of ≤0.05 was considered statistically significant.

IHC evaluation of percentage and localization of immunoreactive cells was calculated by averaging out replicates of tumor cores. Data analysis was performed thought ROC curves (MedCalc© software), in order to discriminate between three categories (cancer normal and PIN). Evaluation was performed considering separately nuclear and cytoplasmic staining. ROC analysis was also performed with 4 miRNA of interest in order to define a cut-of for high and low protein or miRNA expressors as described in result section. Chi-square analysis was performed to compare tissue classes and histopathological parameters. A p value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. Cell lines

To identify miRNA potentially involved in prostate cancer progression we first compared the PCa cell lines (LNCap, DU145 and PC3) with the non-malignant ones (RWPE-1 and BPH-1). In all, we found 23 miRNAs differentially expressed (**Table 8**). miRNA selection for validation in human samples was based on differences of expression levels in more aggressive cancer cell lines and androgen sensitive cancer cell line. **Figure 17** shows miRNA clustering in cell lines and **Figure 18** show dendrogram of cell lines clustering, using centered correlation and average linkage.

miRNA	RWPE-1	BPH1	LNCap	DU145	PC3
hsa-let-7c	1.26	-1.61	6.65	-6.22	-5.88
hsa-miR-100#	4	0.12	4.03	-1.95	-2.82
hsa-miR-130a	1.26	-1.08	-10.15	-1.26	-1.56
hsa-miR-135b	1.26	-1.83	-4.68	-0.81	-0.41
hsa-miR-138	1.26	1.79	-1.8	4.32	5.24
hsa-miR-193a-5p	1.26	-8.64	-0.5	-3.56	-3.97
hsa-miR-203	1.26	-4.06	-0.54	-5.77	-6.89
hsa-miR-205	1.26	-1.57	-13.92	-14.77	-14.88
hsa-miR-218	1.26	-7.91	-2.93	-4.27	-4.7
hsa-miR-224	1.26	-3.23	-6.36	-13.09	-13.19
hsa-miR-22	1.26	-7.26	-0.08	-2.25	-1.33
hsa-miR-31	1.26	-1.48	-18.06	0.53	0.03
hsa-miR-331-5p	1.26	5.22	2.32	9.11	11.33
hsa-miR-34b	-5.55	0.12	-2.11	3.3	3.49
hsa-miR-34c-5p	1.26	-2.7	-3.78	3.43	4.52
hsa-miR-450b-5p	1.26	2.2	3.42	6.55	4.84
hsa-miR-452	1.26	-0.85	-9.98	-10.83	-10.93
hsa-miR-489	1.26	-3.6	-4.51	6.07	4.03
hsa-miR-532-5p	1.26	-6.84	2.34	0.46	0.9
hsa-miR-542-5p	1.26	2.2	1.12	7.18	7.89
hsa-miR-577	-3.84	0.12	-4.5	1.11	3.09
hsa-miR-708	1.26	-4.79	-12.88	-13.73	-13.83
hsa-miR-886-3p	1.26	2.2	1.82	13.01	13.04

Table 8: *23 miRNA selected in cell lines. Expression data are Log2 transformed*

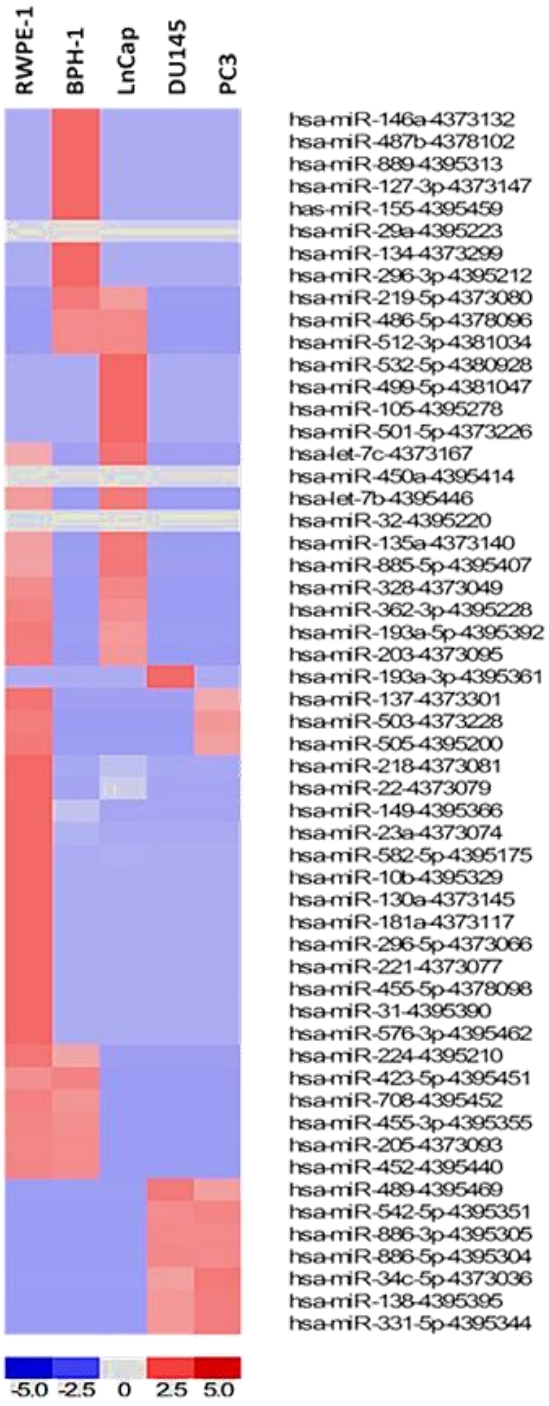


Figure 17: *miRNA clustering*

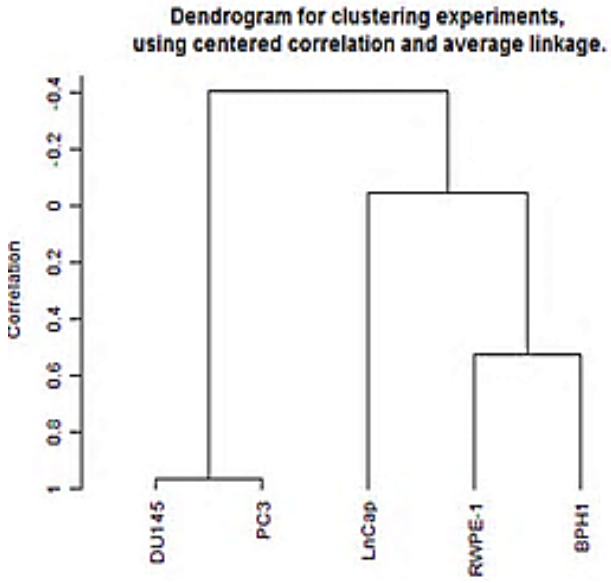


Figure 18: *Dendrogram of cell lines clustering*

Several studies described miRNA profiling in prostate cancer cell lines, with controversial results.

In an early study, Jiang et al.[97] analyzed the abundances of selected miRNAs in PCa cell lines using real-time PCR. They found that androgen independent (AI) cell lines exhibited 4-fold increased expression of miR-100 and androgen-dependent (AD) LNCaP cells had 53-fold increased levels of let-7c, relative to control cells, according with our results (**Table 8**). Kore et al.[98] employed locked nucleic acids (LNA)-labelled miRNA probes to detect let-7c expression in human PCa samples. They found that let-7c was clearly present in PCa cells. Lin et al. [99] compared miRNA expression profiles between AI and AD PCa cells. MiR-205 was the most described in PCa and in a recent study [100], Boll at al. observed that miR-130a, miR-203 and miR-205 simultaneously interfere with two primary oncogenic pathways in prostate carcinoma, AR and MAPK signaling. They found

these miRNAs simultaneously downregulated in the disease and suggest a synergistic effect as tumor suppressors in PCa [100].

4.2. Human samples

To assess the in-vivo relevance of these results, we analyzed this 23 miRNA selected in a larger case study of 58 patients to confirm our results and to find a correlation between miRNA expression profile, tumor staging and patients' follow up. 13 miRNA were confirmed statistically, with a p value <0.05. We compared normal, cancer and PIN samples. MiR-130a, miR-218, miR-532, miR-542-5p, miR-489 and let-7c showed downregulation in PIN compared with matched normal tissue. MiR-135b, miR-193-5p, miR-250, miR-224 and miR452 showed downregulation in PCa compared with normal. Finally, we observed a loss miR-31 in PCa compared to precancerous lesions (PIN). Statistical analysis was performed with GraphPad Prism5 software, and p values are displayed in **Table 9.** All data are Log2 transformed and the comparison of expression levels was a paired t test (p value <0.05 was considered significant).

MiR-130a, miR-203 and miR-205 were previously reported as downregulated in prostate cancer. These miRNAs interfere with two major oncogenic pathways in prostate carcinoma, AR and MAPK signaling [100]. They suggest a synergistic effect of these three miRNA as tumor suppressors in PCa. In our sample set we found that miR-130a is down regulated in PIN compared with normal (p=0.006), miR-203 didn't show differences statistically significant, and miR-205 was down regulated (p=<0.0001), as reported in several papers [72], [101], [102], [103]. As previously reported, downregulation of miR-205 correlate with tumor stage and Gleason score. Indeed, we compared the expression level with clinicopathological parameters and, as shown in

Figure 19, miR-205 is strongly downregulated in high Gleason score PCa. In this validation set, we re-analyzed 2 cell lines (RWPE-1 and PC3) that followed the same trend as in TaqMan® Array Cards.

	N	PIN	K	P value	
hsa-let-7	**0.3734**	**-0.1716**	0.1403	**	0.004
hsa-miR-100*	0.1406	**0.2299**	**-0.2898**	*	0.0390
hsa-miR-130a	**0.4637**	**-0.0004**	0.0737	**	0.0060
hsa-miR-135b	**0.5862**	0.3187	**-0.1269**	**	0.0037
hsa-miR-138	-0.2806	**0.4694**	**-0.8872**	**	0.0075
hsa-miR-193a-5p	**0.5593**	0.2186	**-0.1394**	**	0.0021
hsa-miR-203	0.2715	-0.0315	0.0189		ns
hsa-miR-205	**0.9579**	0.2795	**-1.7323**	***	<0.0001
hsa-miR-218	**0.4573**	**-0.0370**	0.0070	**	0.0016
hsa-miR-224	**0.3706**	0.1592	**-0.6047**	***	0.0008
hsa-miR-22	**0.1844**	0.0756	**-0.1334**	*	0.03
hsa-miR-31	0.0552	**0.4096**	**-1.2884**	**	0.0019
hsa-miR-331-5p	0.3598	0.1458	0.1880		ns
hsa-miR-34b	**0.2750**	-1.1440	**-1.2460**	*	0.0197
hsa-miR-34c	**0.5571**	0.0044	**-0.1630**	*	0.0340
hsa-miR-450b-5p	0.5069	-1.4900	-0.5654		ns
hsa-miR-452	**0.4472**	0.1259	**-0.3982**	***	<0.0001
hsa-miR-489	**0.3966**	**-0.3393**	0.0258	***	0.0004
hsa-miR-532	**0.3736**	**-0.1836**	0.1109	**	0.0018
hsa-miR-542-5p	**0.3554**	**-0.0502**	0.1076	**	0.0406
hsa-miR-577	-0.7200	0.1883	-0.1767		ns
hsa-miR-708	**-0.4920**	-0.2250	**-0.1377**	*	0.0275
hsa-miR-886-3p	**0.2312**	0.2119	**-0.2088**	*	0.0256

Table 9: *Average Log2 expression level. 13 miRNA are downregulated in cancer progression (paired t test p <0.005)*

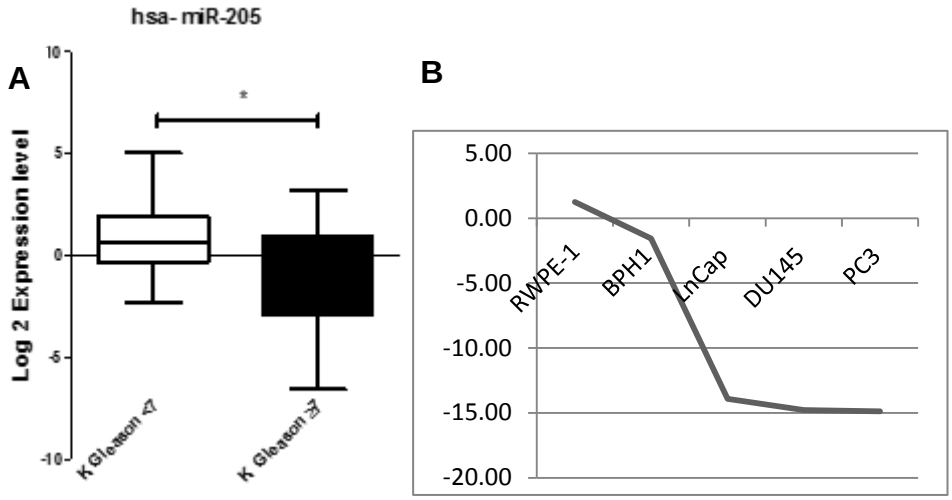


Figure 19: *A: miR-205 downregulation in high Gleason score PCa (p=0.0321)B: miR-205 expression in cell lines*

Our results confirm expression data previously reported in literature but for some miRNA we found differences in expression not previously described. In a recent study, they found a different signature in the high-grade tumors (Gleason score ≥ 8) when compared with tumors Gleason score 6. Up regulation of miR-122, miR-335, miR-184, miR-193, miR-34, miR-138, miR-373, miR-9, miR-198, miR-144 and miR-215 and down-regulation of miR-96, miR-222, miR-148, miR-92, miR-27, miR-125, miR-126, miR-27 were found in the high grade tumors. [104]. In our results about miR-193a, we did not find any differences between high and low Gleason score cancer and we reported a down regulation in tumor (p=0.0021) instead of up regulation. In contrast with these results, we found a downregulation of miR-138 in cancer. Leite et al. [105] observed that miR-let7c, miR-100, and miR-218 were significantly overexpressed in localized high GS, pT3 prostate cancer in comparison with metastatic carcinoma. Our results show only a miR-218 decrease in PIN compared with normal tissue **Table 9**.

MiR-452 was previously described as overexpressed in cancer stem/progenitor cells of prostate [106], but in our results was strongly downregulated (p=<0.0001).

Finally, comparing the expression of 9 miRNAs lost in tumor progression (hsa-miR-135b, -193a-5p, -205, -224, -22, -34b, -34c-5p, -452, -886-3p), for three of these (hsa-miR-34b, -34c-5p and -886-3p) we observed a different expression pattern, because they were overexpressed in cell lines (**Figure 20**).

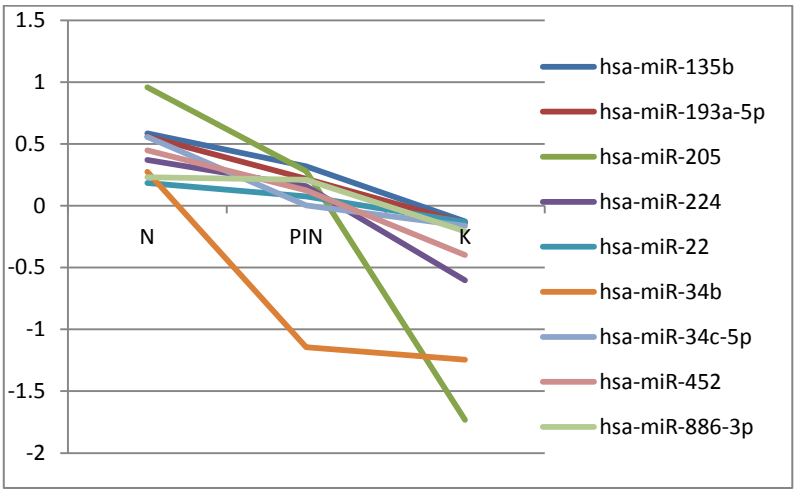


***Figure 20**: 9 miRNA downregulated in PCa human samples*

4.3. RUNX2 expression on TMA

Therefore, in our study we also investigate the expression levels of RUNX2 in human Tissue microarray of prostate tissue (normal, cancer and PIN) and examine the correlation between RUNX2 levels and the clinicopathological characteristics of PCa. Runx2, a key regulator involved in the bone tropism of metastatic cells, when abnormally expressed in tumor cells, has pathological functions that are deregulated compared to normal cells: Runx2 is no longer antiproliferative, and instead appears to have oncogenic properties.

Immunohistochemical staining to evaluate RUNX2 protein levels in PCa yielded results conflicting with those of previous studies. Chua et al. [107] analyzed RUNX2 protein levels in specimens from 39 BPH patients, 8 high-grade prostatic intraepithelial neoplasia cases and 128 PCa tissues by immunohistochemical staining. These authors reported that RUNX2 was undetectable or showed low cytoplasmic expression in BPH (2.6%), whereas the protein was detected in the cytoplasm in 84.4% of PCa tissues. In addition, no correlation between cytoplasmic expression of RUNX2 and prognostic significance was reported in their study. In another study, Akech et al. [86] analyzed a PCa tissue microarray through immunohistochemistry and reported that the majority of non-neoplastic tissues and prostatic intraepithelial neoplasia lesions were negative for RUNX2, whereas 48% of the primary tumors and 46% of metastatic tissues were RUNX2 positive. In contrast to these two reports, Yun et al. [84] observed cytoplasmic RUNX2 protein expression in all BPH tissues with similar patterns of staining with moderate to strong intensity. The correlation was also observed between RUNX2 mRNA expression and the cytoplasmic staining intensity of the protein [84].

Similar immunohistochemical findings to those of the current study were reported by Brubaker et al. [88], who analyzed human prostate tissues obtained from 16 radical prostatectomies and 12 rapid autopsy specimens, and reported the detection of the RUNX2 protein in both PCa cells and normal glands.

Interestingly, nuclear localization of RUNX2 was significantly associated with metastatic disease compared with the cases in which RUNX2 was not detected in the nucleus, which is in agreement with the results reported by Chua et al. [107]. According to their study, RUNX2 nuclear expression was associated with higher Gleason score (greater than or equal to 7), higher PSA levels and an increased risk of metastasis. Yun et al. results

suggested that nuclear expression of RUNX2 could be used as a prognostic marker to predict metastasis in advanced PCa.

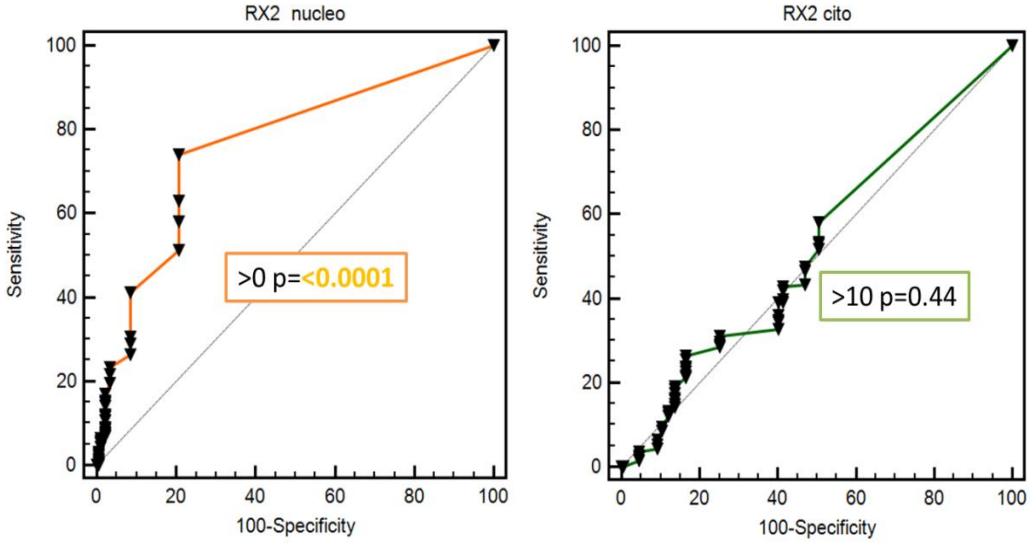
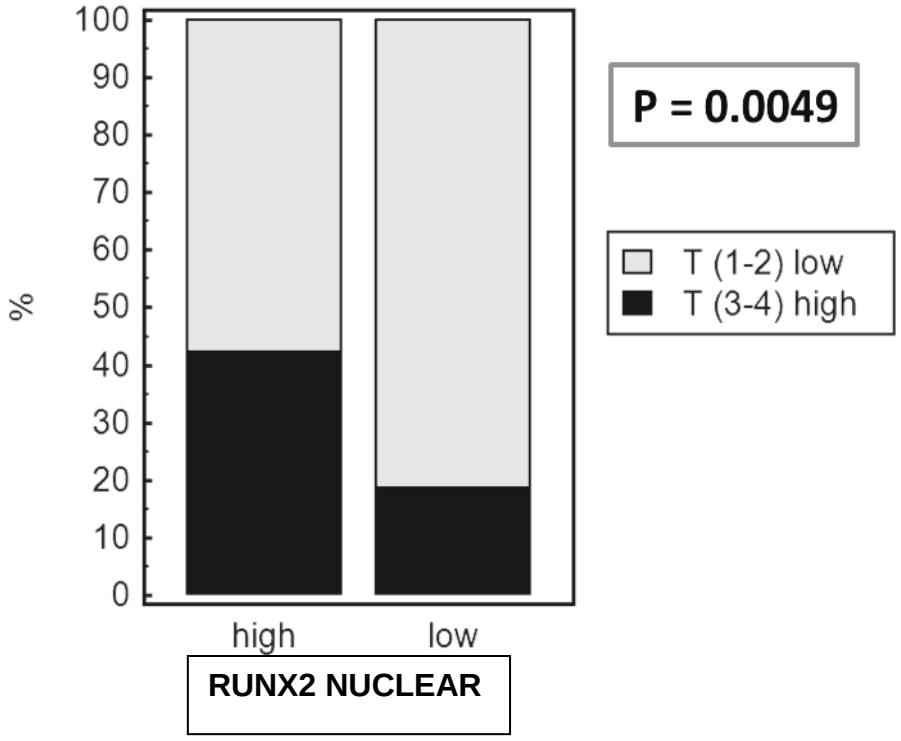


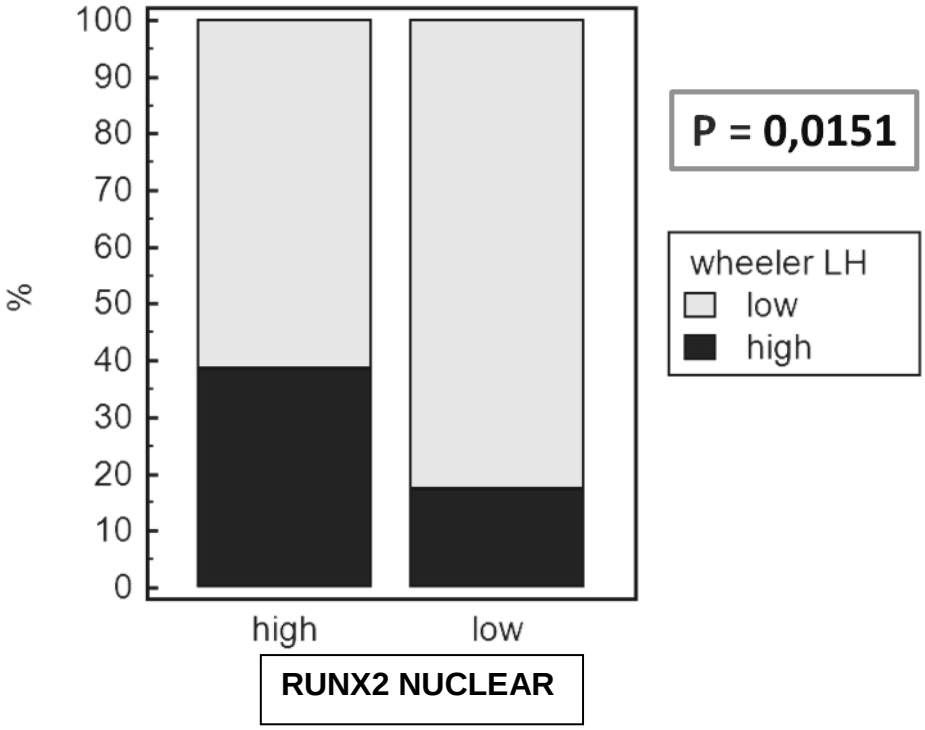
***Figure 21**: ROC curve analysis of nuclear and cytoplasmic expression of RUNX2*

According with Chua and Yun [107][84], evaluating expression of RUNX2 on TMA, we found through ROC curve analysis that only nuclear staining could discriminate between normal and cancer tissue, with a P value <0.0001 (AUC: 0.762, 95%, Confidence interval: 0.715 to 0.805) with 79,3% of specificity and 74,1% of sensitivity (**Figure 21**). Once divided in high- and low- RUNX2 expressors, based on ROC cut-of, we verified correlation between protein expression and clinical data. We found a correlation with tumor extension. RUNX2 expression was lower in low grade cancer (T2) and higher in high grade cancer (T3-T4) with a p value of 0.0049 (**Figure 22**). We also found a correlation statistically significant, considering capsular invasion (Wheeler classification). RUNX2 expression was low in prostate cancer with low capsular invasion (L1-L2) with a p value of 0.0151 (**Figure 23**)



	RUNX2 HIGH	RUNX2 LOW	
T HIGH	59	9	68 (36.0%)
T LOW	81	40	121 (64.0%)
	140	49	189
	-74.10%	-25.90%	

Figure 22: *Chi-square analysis of nuclear expression of RUNX2 correlate with tumor stage (p=0.0049)*



	RUNX2 HIGH	RUNX2 LOW	
Wheeler HIGH	50	8	58 (33,0%)
Wheeler LOW	80	38	118 (67,0%)
	130 (73,9%)	46 (26,1%)	176

Figure 23: *Chi-square analysis of nuclear expression of RUNX2 correlate with capsular invasion (p=0.0151)*

Was also evaluated with Chi-square analysis, the correlation with cellular localization of RUNX2 (nuclear, cytoplasmic and co-localization). We observed a strong nuclear and co-localized expression of RUNX2 in cancer tissue, a cytoplasmic localization in normal and PIN tissue, with a p value <0.0001 (**Figure 24**).

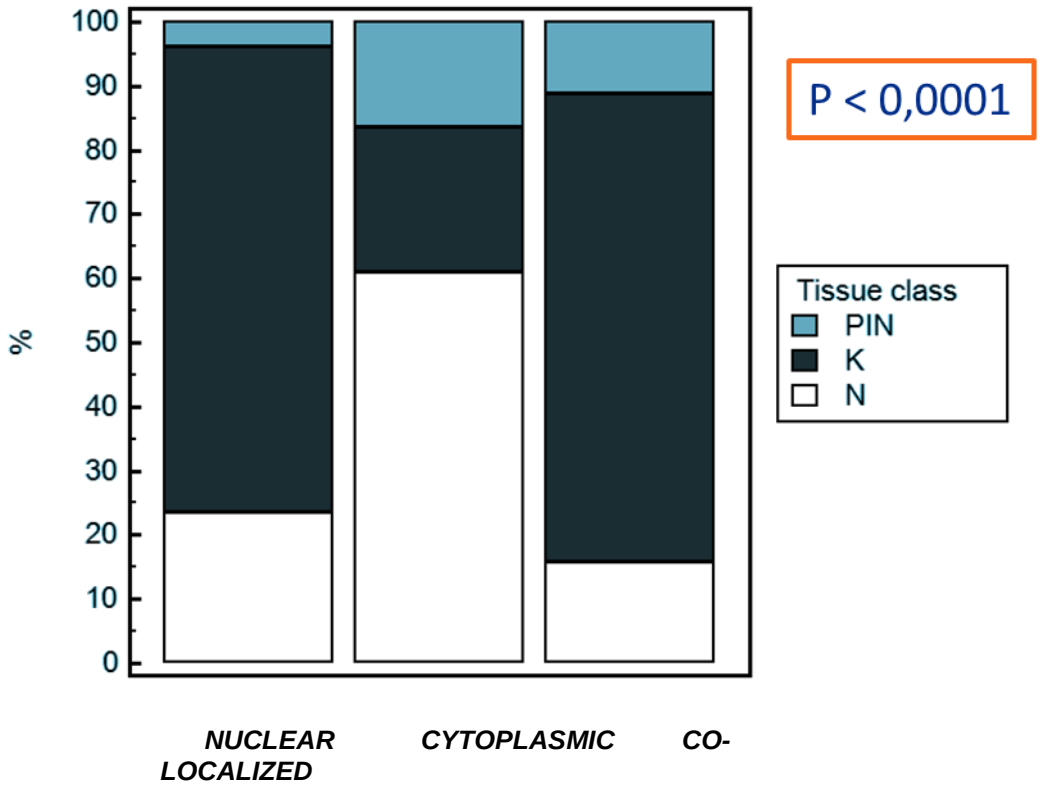


***Figure 24**: RUNX2 localization*

In **Figure 25** and **Figure 26** are displayed illustrative pictures of nuclear staining in cancer and cytoplasmic staining in normal tissue respectively, at 20X magnification.

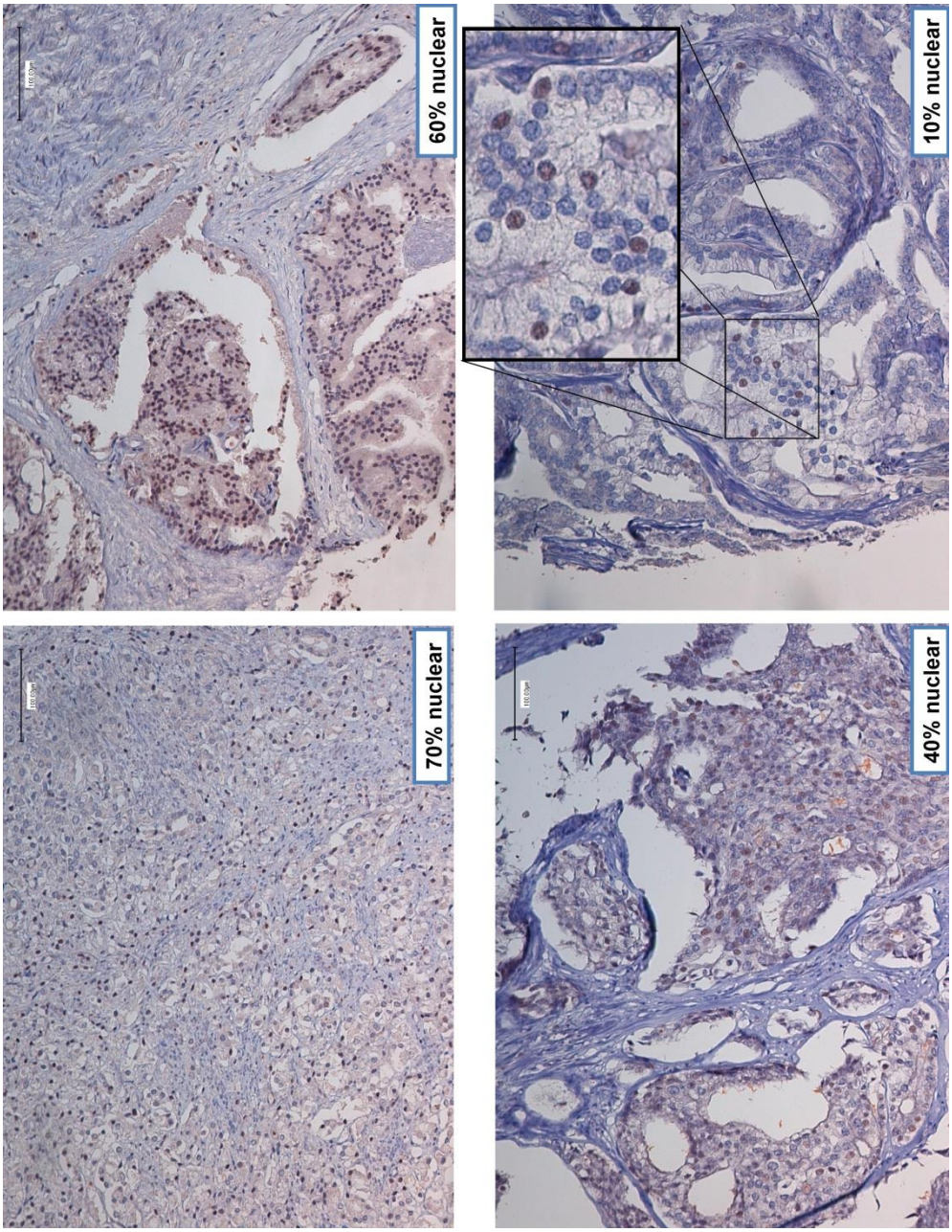


Figure 25: *Illustrative nuclear staining of RUNX2 in cancer (20X magnification)*

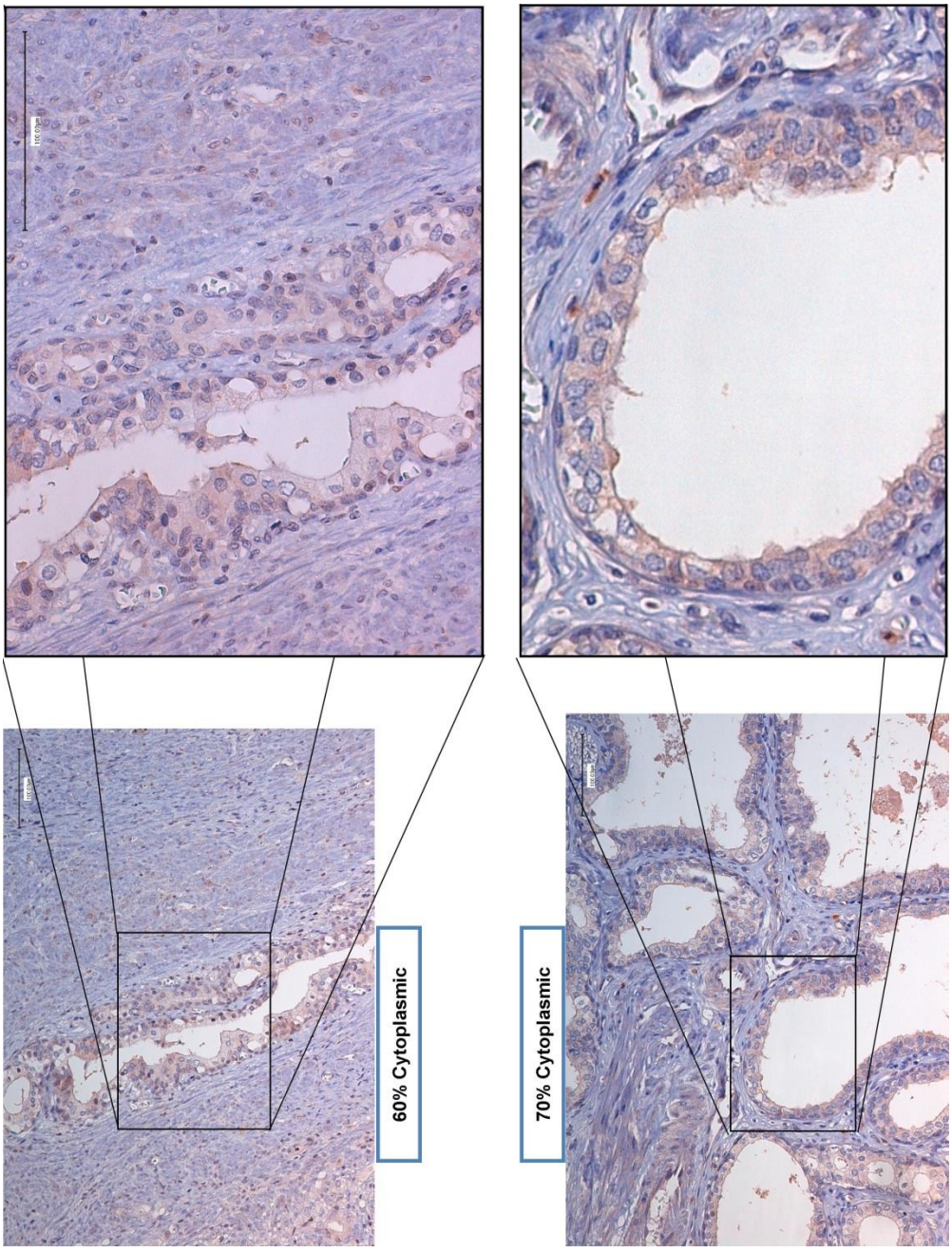


***Figure 26**: Cytoplasmic staining of RUNX2 in normal tissue (20X magnification)*

4.4. Four miRNA candidate target RUNX2 protein

We selected from literature and miRNA database, 4 miRNAs that target RUNX2 (miR-203, -205, -218 and -224).

Saini et al. demonstrate trough luciferase assay, that miR-203 regulates the expression of several bone-specific effectors including Runx2, a master regulator of bone metastasis [108]. Gay et al demonstrate that the human miRNA-218 targets RUNX2 expression [109]. Finally, Zhang et al found, trough luciferase-based miRNA expression reporter assays, seven RUNX2-targeting miRNAs: miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-205, and miR-217 [110].

Mir-224 was only predicted to target RUNX2 (miRDB). This evidences supported our purpose to evaluate miRNA-target interaction

ROC curves analysis shows that 3 miRNA (miR-205, -218, -224) can discriminate between normal and PIN (**Figure 27**,**Table 10**).

miRNA	AUC	95% Confidence interval	Significance level P
miR-203	0.573	0,447 to 0,692	ns
miR-205	0.641	0,516 to 0,754	0.039
miR-218	0.64	0,514 to 0,753	0.0429
miR-224	0.851	0,744 to 0,926	<0.0001

***Table 10**: Values of ROC curves analysis (AUC: Area under curve), discrimination between normal and PIN.*

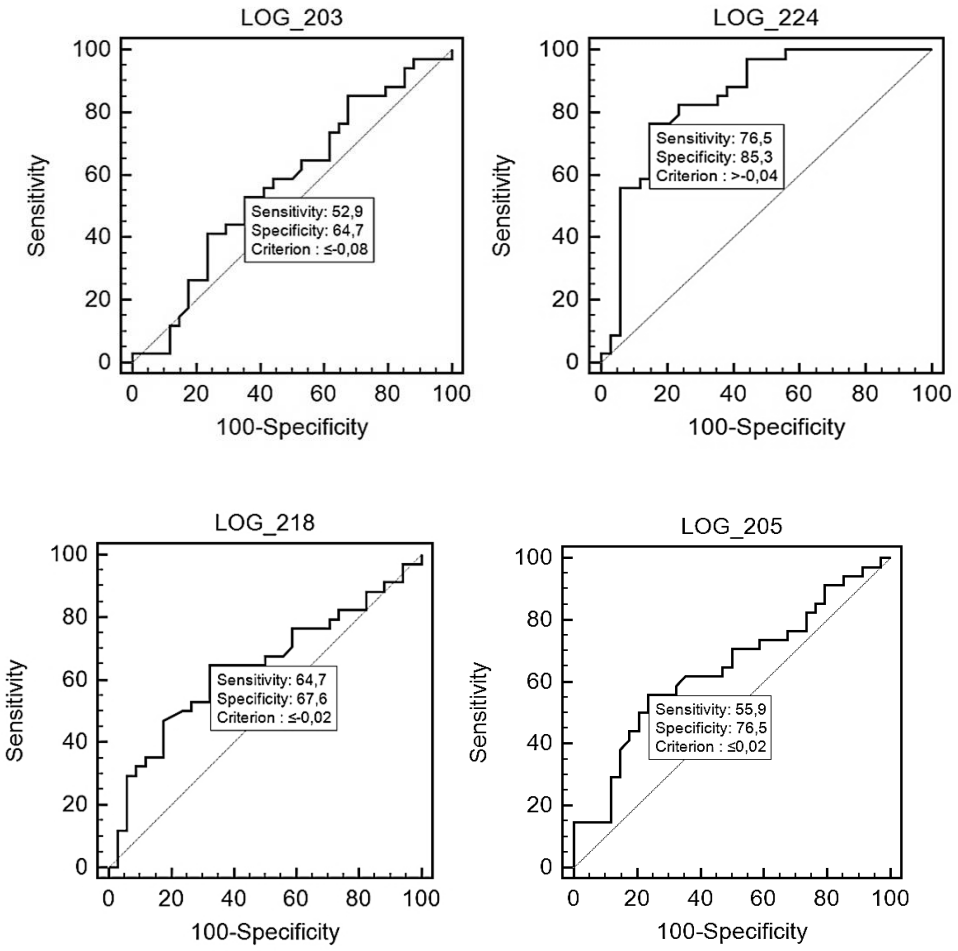


Figure 27: *ROC curve of miR-203, -205, -218 and -224, discrimination between normal and PIN.*

Then we evaluated the correlation between expression of miRNAs and RUNX2 staining on TMA and our results show that miR-205 and miR-218 expression inversely related to nuclear expression of RUNX2. When miRNAs expression decreases, there is an increased nuclear expression of RUNX2, and conversely, when miRNAs are highly expressed, RUNX2 nuclear staining decrease (**Figure 28**). P values of Chi-square analysis were statistically significant (miR-205 p= 0.0011 and miR-218 p=0.0096).

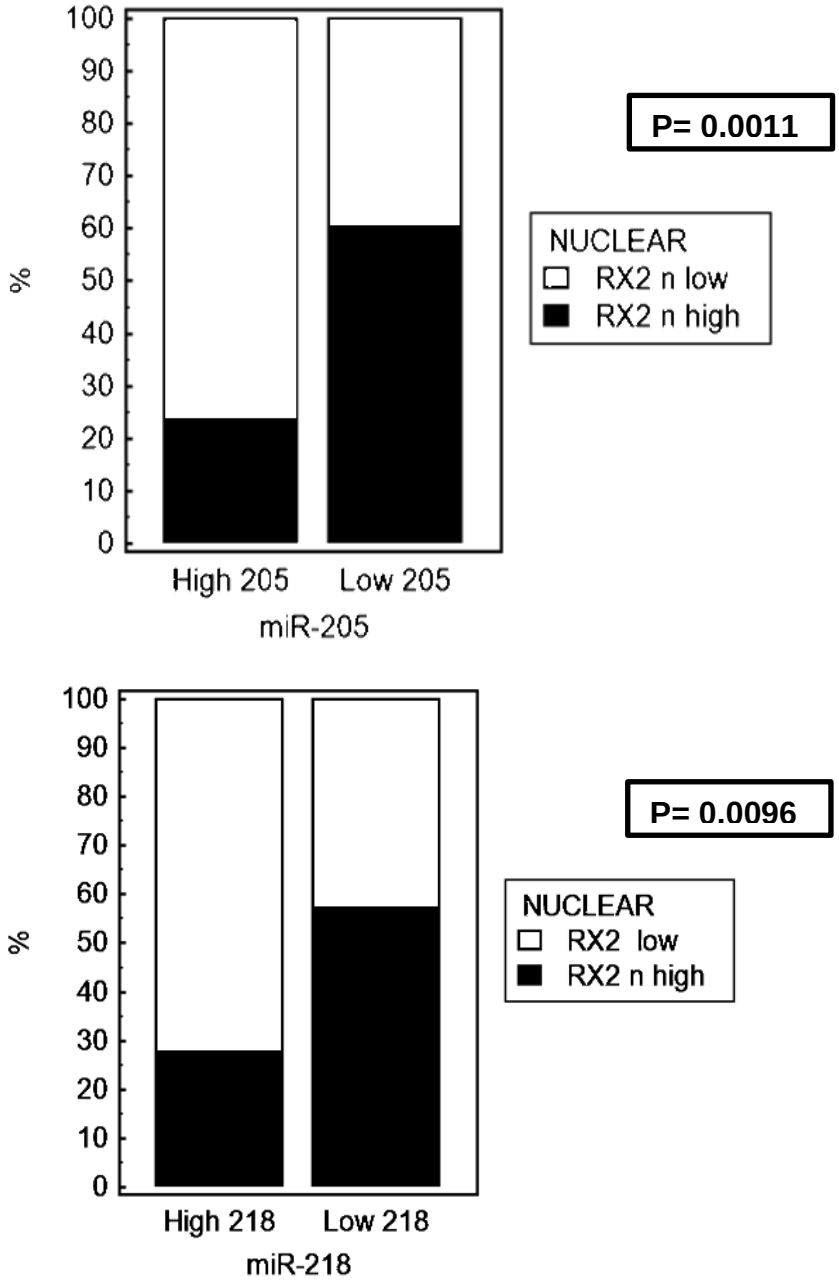


Figure 28: *miR-205 and -218 expression is inversely correlated with nuclear localization of RUNX2 (p value= 0.0011 and 0.0096)*

4.5. TRAMP mouse

Whereas stroma adjacent to tumor cells has gained considerable importance in determining patient response to therapy and disease progression in several human cancers, including PCa, we also evaluate with a statistical analysis different expression of miRNAs in TRAMP model between cancer and PIN epithelial component, in order to find miRNA well conserved that could have a role in cancer therapy.

Figure 29 show a scatter plot of miRNA up and down regulated in the epithelial component between cancer and PIN. MiRNA differentially expressed are listed in **Table 11**. We found 52 miRNAs deregulated, among which 10 miRNAs upregulated in PIN and 42 upregulated in cancer epithelium.

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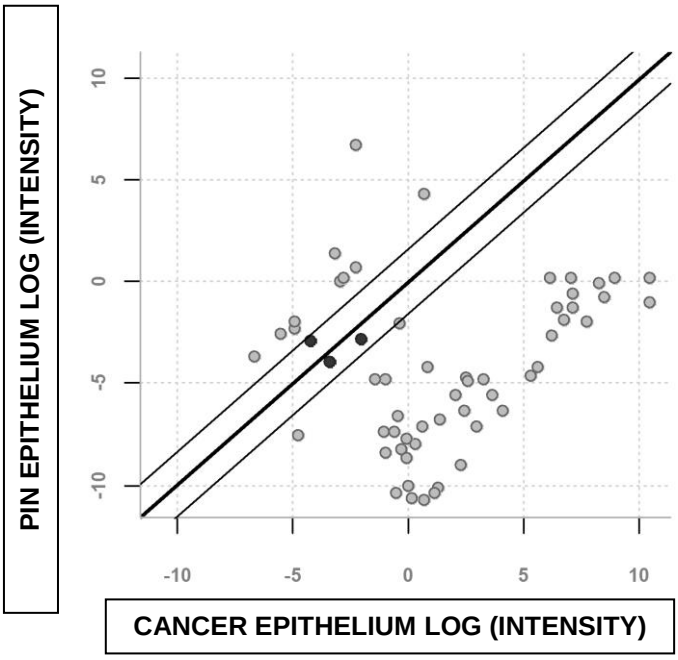

***Figure 29**: Scatter plot of miRNA expression in stromal component of PIN and cancer tissue of TRAMP mouse at 24 weeks*

UP in PIN epithelium	DOWN in PIN epithelium		
mmu-miR-107-4373154	mmu-miR-127-4373147	mmu-miR-344-4373340	mmu-miR-490-4373215
mmu-miR-122-4395356	mmu-miR-129-3p-4373297	mmu-miR-34c-4373036	mmu-miR-495-4381078
mmu-miR-139-3p-4395676	mmu-miR-132-4373143	mmu-miR-370-4395386	mmu-miR-500-4395736
mmu-miR-199a-5p-4373272	mmu-miR-134-4373299	mmu-miR-376a-4373347	mmu-miR-503-4395586
mmu-miR-383-4381093	mmu-miR-136-4395641	mmu-miR-376c-4395580	mmu-miR-539-4378103
mmu-miR-467c-4395647	mmu-miR-137-4373301	mmu-miR-380-5p-4395731	mmu-miR-670-4395561
mmu-miR-496-4386771	mmu-miR-138-4395395	mmu-miR-382-4373019	mmu-miR-675-3p-4386762
mmu-miR-511-4395679	mmu-miR-215-4373316	mmu-miR-384-3p-4395733	mmu-miR-743a-4395599
mmu-miR-669a-4381091	mmu-miR-216b-4395437	mmu-miR-384-5p-4395732	mmu-miR-7a-4378130
mmu-miR-802-4395566	mmu-miR-296-5p-4373066	mmu-miR-410-4378093	mmu-miR-873-4395467
	mmu-miR-298-4395728	mmu-miR-434-3p-4395734	
	mmu-miR-325-4395640	mmu-miR-434-5p-4395711	
	mmu-miR-335-3p-4395296	mmu-miR-467b-4381084	
	mmu-miR-337-3p-4395662	mmu-miR-486-4378096	
	mmu-miR-337-5p-4395645	mmu-miR-487b-4378102	
	mmu-miR-338-3p-4395363	mmu-miR-488-4381074	

***Table 11**: miRNA up and down regulated in epithelial component of PIN in TRAMP mouse at 24 weeks.*

We also compared our previous data with miRNA expression in TRAMP mouse. We found some comparable expression trend. MiR-138, miR-31, miR-34b and miR-34c were downregulated in PCa and in LNCap cells, but were upregulated in cancer epithelium of TRAMP mouse such as in DU145 and PC3, due to the aggressiveness of cancer in this mouse model.

These preliminary results will be used to perform further analysis on epithelium-stroma interaction in prostate cancer.

4.6. CAF and NAF

Transfection efficiency was tested by GFP in CAF (cancer-associated fibroblasts) and NAF (normal associated fibroblasts) for further applications. Our results (**Figure 31)** show a good rate of transfected cells. In CAF, more than was the confluence less was the transfection efficiency (**Figure 30**) and in NAF we observed a stable rate of transfection during 96 hours of culture. The rate of transfected cells was higher in CAF than in NAF. We assessed this preliminary experiment in order to modulate miRNA expression in CAFs and NAFs.

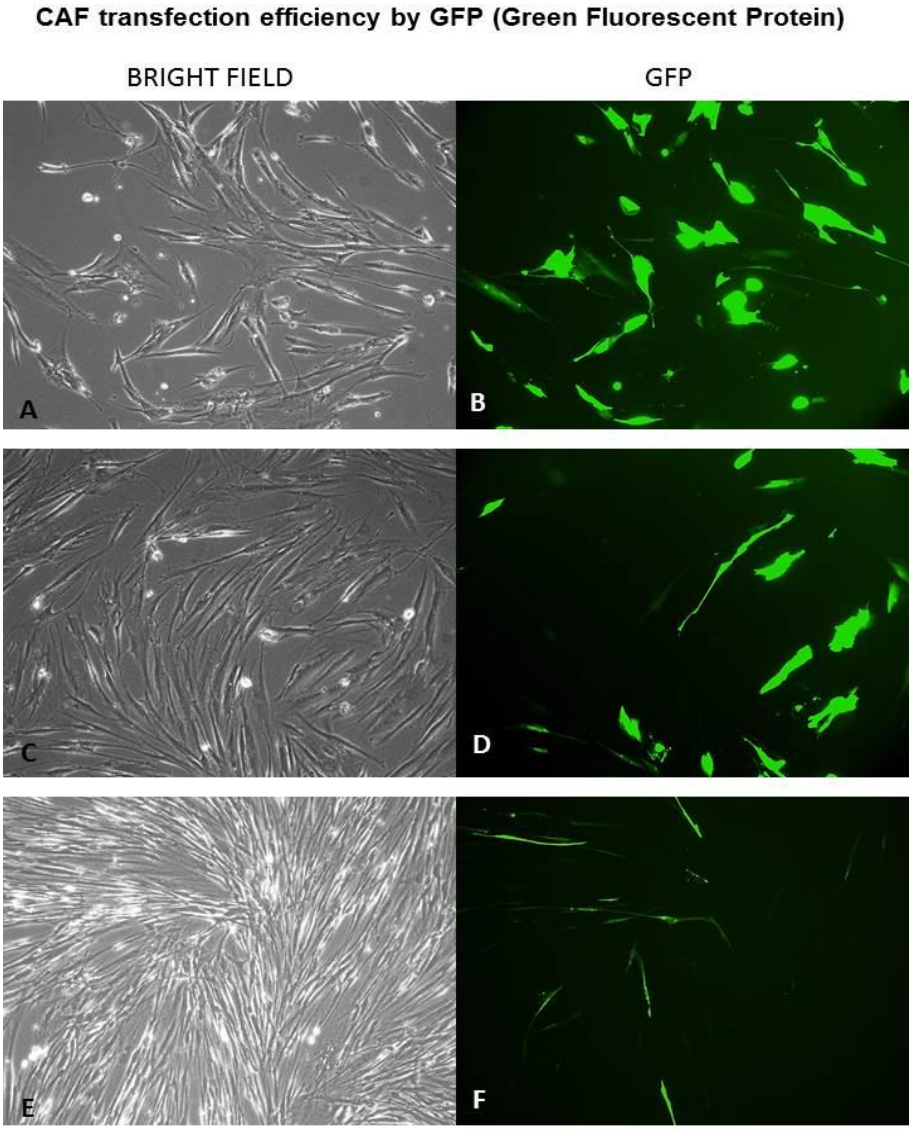

***Figure 30**: Transfection efficiency test by GFP in CAF (Cancer associate Fibroblasts). Panel A, C and E show bright field at 10x magnification of CAF cell line at 3 different rate of confluence. More than was the confluence less was the transfection efficiency (Panel B, D, F)*

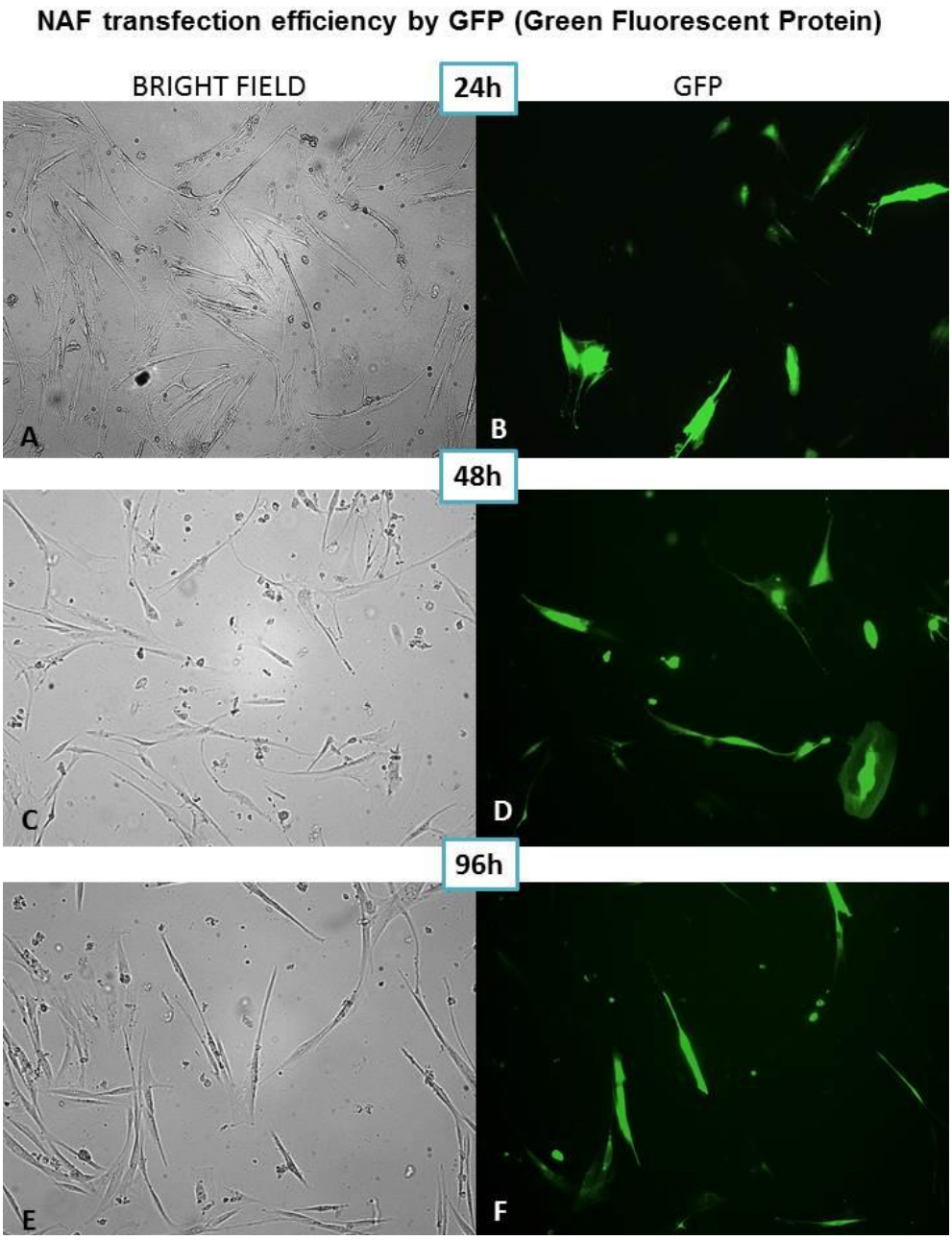

Figure 31: *Transfection efficiency test by GFP in NAF (Normal associate Fibroblasts). Panel A, C and E show bright field at 10x magnification of NAF cell line at 24-48-96h of culture. Transfection efficiency (Panel B, D, F) was stable.*

CONCLUSION

Different experimental approaches used in our research, highlight that, unlike cell lines, human prostate cancer express lower miRNA levels, compared with normal prostatic parenchyma. These data imply that, depending on the molecule under examination, the cell lines represent a reliable or not model of in vivo tumorigenesis Indeed, 3 miRNA that we expected to find upregulated, were downregulated in human PCa. We found in all 13 miRNA downregulated in cancer and 9 miRNA (hsa-miR-135b, -193a-5p, -205, -224, -22, -34b, -34c-5p, -452, -886-3p), with a p value statistically significant, downregulated in cancer progression (normal, PIN and cancer, progressively). As several studies reported, we observed a synergetic loss of miRNA with onco-suppressive role (miR-205, -224, -218) and simultaneous increase of Runx2 protein. This finding has important implications for disease progression, which will be evaluated in next steps of research project.

Our analysis indicates that miR-205 is a putative marker of disease aggressiveness and especially identifies 9 miRNAs prematurely lost in early precancerous lesions (PIN), compared to normal parenchyma.

Validation of this data with independent case studies will confirm these molecules as novel biomarkers of cancer, in order to support the PSA assessment.

Furthermore, the study of miRNAs stromal showed a profound deregulation of these molecules in the tumor microenvironment than in non-neoplastic. This result emphasizes the molecular point of view of the importance of the stroma in supporting the survival and tumor growth and provides a possible alternative therapeutic strategy, targeted to epithelial stromal cells instead, in order to induce regression of the disease. Further studies are needed to evaluate the role of miRNAs in the interaction between tumor epithelium and surrounding stroma.

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