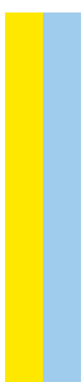
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL



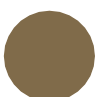
Daniela Cristina Barros Silva

MicroRNA-27a-5p Regulation by Promoter Methylation *MYC* Signaling in Prostate Carcinogenesis

U.PORTO

Daniela Cristina Barros Silva

MicroRNA-27a-5p Regulation by Promoter Methylation *MYC* Signaling in Prostate Carcinogenesis



M.ICBAS **2017**

MESTRADO EM ONCOLOGIA

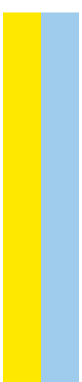
ESPECIALIZAÇÃO EM ONCOLOGIA MOLECULAR

MicroRNA-27a-5p Regulation by Promoter Methylation and *MYC* Signaling in Prostate Carcinogenesis

Daniela Cristina Barros Silva

M

2017



U.PORTO

MESTRADO EM ONCOLOGIA

ESPECIALIZAÇÃO EM ONCOLOGIA MOLECULAR

MicroRNA-27a-5p Regulation by Promoter Methylation and *MYC* Signaling in Prostate Carcinogenesis

Daniela Cristina Barros Silva

M

2017

DANIELA CRISTINA BARROS SILVA

MicroRNA-27a-5p Regulation by Promoter Methylation and *MYC* Signaling in Prostate Carcinogenesis

Dissertação de candidatura ao grau de **Mestre em Oncologia** submetida ao Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto

ORIENTADORA
Professora Doutora Carmen de Lurdes Fonseca Jerónimo
Professora Associada convidada com Agregação
Departamento de Patologia e Imunologia Molecular
Instituto de Ciências Biomédicas Abel Salazar Universidade do Porto
Investigadora Auxiliar e Coordenadora do Grupo de Epigenética e Biologia do Cancro
Centro de Investigação
Instituto Português de Oncologia do Porto Francisco Gentil, E.P.E.

CO-ORIENTADOR
Professor Doutor Rui Manuel Ferreira Henrique
Professor Catedrático Convidado
Departamento de Patologia e Imunologia Molecular
Instituto de Ciências Biomédicas Abel Salazar Universidade do Porto
Diretor do Serviço de Anatomia Patológica
Investigador Sénior do Grupo de Epigenética e Biologia do Cancro
Centro de Investigação
Instituto Português de Oncologia do Porto Francisco Gentil, E.P.E.

“Eles não sabem, nem sonham,
que o sonho comanda a vida,
que sempre que um homem sonha
o mundo pula e avança
como bola colorida
entre as mãos de uma criança.”

Pedra Filosofal,

António Gedeão

AGRADECIMENTOS

A realização da dissertação aqui apresentada só foi possível devido à contribuição de várias pessoas a quem quero expressar a minha gratidão.

À minha orientadora, a Professora Doutora Carmen Jerónimo, um agradecimento especial por me ter acolhido no Grupo de Epigenética e Biologia do Cancro. Muito obrigada pelo apoio científico e orientação ao longo do desenvolvimento de todo projeto bem como por toda a confiança depositada no meu trabalho.

Ao meu co-orientador, o Professor Doutor Rui Henrique, pelo rigor científico, dúvidas pertinentes e sugestões fundamentais que ajudaram na construção de todo o trabalho.

Ao Professor Doutor Manuel Teixeira, Diretor do Centro de Investigação do Instituto Português de Oncologia do Porto, por ter permitido o desenvolvimento deste trabalho no Centro que dirige.

Ao Pedro, pela importante ajuda e incentivo na fase inicial deste trabalho e pela disponibilidade e paciência que teve para me ensinar novas técnicas essenciais para o desenvolvimento da minha formação a nível científico.

À Catarina, pela partilha de ideias e experiências que me permitiu aprender tanto. Este trabalho não teria sido possível sem o seu apoio, otimismo e espírito crítico. Muito obrigada, pela amizade, pelas longas conversas científicas e não só e por toda a ajuda disponibilizada.

À Inês e à Ana Lameirinhas, pela importante ajuda na fase final deste trabalho, pela troca de ideias e ajuda na execução de algumas técnicas novas que permitiram tornar este trabalho mais completo.

A todos os membros do Grupo de Epigenética e Biologia do Cancro, por me terem recebido de forma tão acolhedora e rápida. Em particular aos meus colegas de mestrado, Ana Laura, Ângela, Maria, David e também à Bárbara por partilharem comigo a dura tarefa de desenvolver um projeto científico e por todo o apoio dispensado nos bons e nos maus momentos.

Finalmente e acima de tudo aos meus Pais e porque sem eles nada disto teria sido possível. Por terem sido sempre os primeiros a acreditarem em mim e nas minhas capacidades e por me incentivarem sempre não a ser a melhor, mas a dar o meu melhor em tudo o que ia fazendo. Muito obrigada pelo apoio, compreensão e carinho, por estarem sempre presentes mesmo quando as preocupações e a má-disposição reinavam.

RESUMO

A desregulação do gene *MYC* e dos miRNAs são eventos comuns no Cancro da Próstata (CaP). A sobreativação do *MYC* pode causar a desregulação dos miRNAs através de mecanismos transcricionais e pós-transcricionais. Podendo ainda esta desregulação ser devida a modificações epigenéticas. Assim, o objetivo deste estudo é clarificar a relação regulatória entre o *MYC* e a expressão de miRNAs na carcinogénese prostática.

Dados preliminares mostraram uma sobreexpressão do *MYC* em tumores primários da próstata e em lesões precursoras. Mais ainda, amostras de CaP com níveis opostos de *MYC* foram analisadas num array específico para miRNAs, no qual foram identificados miRNAs sobreexpressos nos casos contendo elevado conteúdo de *MYC*. Por outro lado, a validação do miR-27a-5p em tecidos de tumores primários mostrou uma subregulação da expressão deste miRNA, que por sua vez se correlaciona com a hipermetilação do seu promotor. Numa série de tumores prostáticos resistentes à castração, verificou-se uma sobreexpressão do miR-27a-5p com concomitante hipometilação. Os níveis de expressão do *MYC* e do miR-27a-5p obtidos para as linhas celulares LNCaP e PC3 corroboram os resultados observados nos tumores primários naíve e tumores resistentes à castração, respectivamente. A análise por imunoprecipitação da cromatina mostrou que a expressão do miR-27a-5p é somente regulada pelo c-Myc na ausência de metilação do promotor. Para além disso, quando se expressou ectopicamente o miR-27a-5p na linha celular PC3 silenciada para o *MYC* verificou-se uma atenuação do fenótipo maligno, o que sugere um papel supressor tumoral para este miRNA. Curiosamente, a sobreregulação do miR-27a-5p diminui a via de sinalização do EGFR/Akt1/mTOR.

Concluíndo, o miR-27a-5p é um alvo regulado positivamente pelo *MYC*, mas a sua expressão é silenciada pela metilação do seu promotor nas etapas iniciais da carcinogénese prostática. Contudo, ao longo da progressão tumoral, o promotor do miRNA perde metilação permitindo ao c-Myc desempenhar a sua actividade regulatória. Porém, o contexto celular alterado evita que o miR-27a-5p recupere a sua função onco-supressora neste estadío mais avançado da doença.

SUMMARY

Upregulation of *MYC* and miRNAs deregulation are common in prostate cancer (PCa). Overactive *MYC* may cause miRNAs' expression deregulation through transcriptional and post-transcriptional mechanisms and epigenetic alterations are also involved in miRNAs dysregulation. Herein, we aimed to elucidate the role of regulatory network between *MYC* and miRNAs in prostate carcinogenesis.

Preliminary data showed *MYC* overexpression in PCa and precursor lesions and microRNA's microarray analysis of PCa samples with opposed *MYC* levels identified miRNAs significantly overexpressed in high-*MYC* PCa. However, validation of miR-27a-5p in primary prostate cancer tissues disclosed downregulation in PCa, instead, correlating with aberrant promoter methylation. In a series of castration-resistant PCa (CRPC) cases, miR-27a-5p was upregulated, along with promoter hypomethylation. *MYC* and miR-27a-5p expression levels in LNCaP and PC3 cells mirrored those observed in primary PCa and CRPC, respectively. ChIP analysis showed that miR-27a-5p expression is only regulated by c-Myc in the absence of aberrant promoter methylation. Moreover, forced miR-27a-5p expression in stable *MYC* knockdown PC3 cells attenuated malignant phenotype suggesting a tumor suppressive role for this miRNA. Furthermore, miR-27a-5p upregulation decreased EGFR/Akt1/mTOR signaling.

We concluded that miR-27a-5p is a positively regulated target of *MYC*, and its silencing due to aberrant promoter methylation occurs early in prostate carcinogenesis, concomitantly with loss of c-Myc regulatory activity. Our results further suggest that along PCa progression, miR-27a-5p promoter becomes hypomethylated, allowing for c-Myc to resume its regulatory activity. However, the altered cellular context averts miR-27a-5p from successfully accomplishing its tumor suppressive function at this stage of disease.

TABLE OF CONTENTS

I. INTRODUCTION ... 1

1. Prostate Cancer ... 2

1.1 Epidemiology ... 2

1.2 Risk Factors ... 4

1.3 Screening and Diagnosis ... 4

1.4 Grading System: Gleason Score ... 6

1.5 Clinical and Pathological Staging ... 7

1.6 Therapeutic Approaches ... 9

2. Epigenetics ... 10

2.1 DNA Methylation ... 11

2.2 Histone Post-translation Modifications ... 12

2.3 Histone Variants ... 13

2.4 Non-coding RNAs ... 13

2.4.1 MicroRNAs Biogenesis ... 14

2.4.2 Epigenetic Regulation of MicroRNAs ... 15

2.4.3 MicroRNAs Deregulation in Prostate Carcinogenesis ... 16

2.4.4 MicroRNAs in Human Cancer: Possible New Biomarkers and Treatment Targets ... 17

3. Overview of c-Myc Role in Prostate Cancer ... 19

3.1 MicroRNA Regulation by c-Myc ... 19

II. PRELIMINARY DATA ... 21

1. Upregulation of *MYC* in Clinically Localized Prostate Cancer Tissue Samples (Cohort #1) ... 22

2. Regulatory network between c-MYC and microRNAs ... 24

III. AIMS OF THE STUDY ... 25

IV. MATERIAL AND METHODS ... 27

1. Clinical Samples ... 28

1.1 Patients and Samples Collection ... 28

1.2 Total RNA Extraction 28

1.3 MicroRNA Expression Assay 28

1.4 DNA Extraction and Bisulfite Modification 29

1.5 Quantitative Methylation-specific PCR 29

2. Prostate Cancer Cell Lines Studies.... 30

2.1 Cell Lines Treatment with Epigenetic-modulating Drugs 31

2.2 Total RNA and DNA Extraction from Cell Lines 31

2.3 *MYC* Expression 31

2.4 *In Silico* Analysis 32

2.5 Chromatin Immunoprecipitation for Transcription Factors 32

2.6 MicroRNA Transfection 32

2.7 Cell Viability Assay 33

2.8 Apoptosis Assay 33

2.9 Protein Extraction and Quantification 34

2.10 Western Blot 34

2.11 Statistical Analysis 35

V. RESULTS.... 36

1. Prostate Cancer Tissue Samples 37

1.1 MicroRNA-27a-5p Status in Clinically Localized Prostate Cancer (Cohort #1) ...37

1.2 Interplay between *MYC* and miR-27a-5p in Castration-Resistant Prostate Cancer (Cohort #2) 38

1.3 Association of miR-27a-5p with Clinicopathological Features 39

2. In Vitro Studies: PCa Cell Lines 40

2.1 *MYC* Characterization 40

2.2 MicroRNA-27a-5p Status: Expression vs. Methylation 41

2.3 Impact of 5-aza-2'deoxycytidine (5-Aza-CdR) Treatment 41

2.4 MicroRNA-27a-5p Regulation by *MYC* Signaling 42

2.5 Phenotypic Impact of *MYC* Silencing and miR-27a-5p Forced Expression in PC3 Cell Line 43

2.6 Putative miR-27a-5p Targets 45

VI. DISCUSSION 47

VII. CONCLUSION 52

VIII. REFERENCES 54

IX. SUPPLEMENTARY DATA 64

FIGURE INDEX

Figure 1 Estimated age-standardized incidence and mortality rates for cancer in men in 2012 [4].. 3

Figure 2 Estimated cancer incidence and mortality in portuguese men in 2012 [6]........... 3

Figure 3 Epigenetic mechanisms. M-5- methy-cytosine; the symbols (hexagon and triangle) represent different post-translational modifications in N-terminal tails of histone proteins (CBEG, IPO Porto). 10

Figure 4 MicroRNAs biogenesis (Kindly provided by Ramalho-Carvalho from [63]). 15

Figure 5 Epigenetic regulatory mechanisms crosstalk...16

Figure 6 Strategies to target the interplay between *MYC* and miRNA in cancer. In neoplasms with *MYC* deregulation, anti-miRNAs could be directed against *MYC*-induced oncogenic miRNAs (1) or miRNA mimics could be used for reintroduction of *MYC*-repressed miRNAs (2) or to target *MYC* itself (3)..20

Figure 7 *MYC* transcript levels (A) and distribution of c-Myc immunostaining (B) in cohort #1; *MYC* transcript levels in cohort #1, grouped according to c-Myc immunostaining(C); Representative images of c-Myc immunostaining in cohort #1 (D). 23

Figure 8 Expression levels of miRNA-27a-5p (A) and methylation levels of miR-27a-5p promoter (B) in cohort #1. 38

Figure 9 *MYC* transcript levels(A), expression levels of miRNA-27a-5p (B) and methylation levels of miR-27a-5p promoter (C) in cohort #2...39

Figure 10 Expression of *MYC* in PCa cell lines. Results are displayed after normalization to PNT2. 40

Figure 11 Expression (A) and methylation (B) of miR-27a-5p in PCa cell lines. Results are displayed after normalization to PNT2. 41

Figure 12 Methylation and expression levels of miR-27a-5p in LNCaP (A) and in PC3 (B) upon 5-Aza-CdR treatment. Results are displayed after normalization to mock. 42

Figure 13 Real-time PCR analysis for c-Myc chromatin immunoprecipitated of miR-27a-5p promoter region in LNCaP and PC3 cell lines. 43

Figure 14 *MYC* expression (A), c-Myc protein levels (B) and miR-27a-5p expression (C) after *MYC* knockdown in PC3 cell lines; Assessment of pre-miR-27a-5p transfection efficiency in PC3 (D); Phenotypic impact (viability and apoptosis) of *MYC* knockdown (E), pre-miR transfection (F) and both (G) in PC3 cell line. 44

Figure 15 Identification of predicted miRNA response element (MRE) using in silico screening methods (A); Protein levels in LNCaP mock and 5-Aza-CdR tretated cell lines by Western Blot analysis (B): EGFR 134 KDa, Akt1 62 KDa, mTOR 289 KDa and β-Actin 42 KDa. 46

Figure 16 Proposed model for miR-27a-5p regulation in Prostate Cancer. 51

TABLE INDEX

Table 1 Clinical and pathological staging of PCa according with AJCC/IUCC in 2010. 8

Table 2 Immunohistochemical expression of c-Myc in cohort #1. 23

Table 3 Clinical and pathological data of fresh-frozen tissues included in this study (cohort #1). 24

Table 4 TaqMan probe and primer sequence, amplicons, locations and annealing temperatures for qMSP. 30

Table 5 All antibodies used in Western blot. 34

Table 6 miR-27a-5p expression and promoter methylation levels in cohort #1. 37

Table 7 *MYC* and miR-27a-5p status in cohort #2. 38

Table 8 Clinical and pathological data of cohort #2 included in the study.........................39

Table 9 8q24 copy number in PCa cell lines. 40

Table 10 miRNA pre-computed static target predictions based on the RNA22 tool [114]. 45

LIST OF ABREVIATIONS

5-Aza-CdR	5-aza-2'deoxycytidine
β-Actin	Beta-actin
ADT	Androgen deprivation therapy
Ago	Argonaute
AJCC	American Joint Committe on Cancer
Akt1	AKT serine/threonine kinase 1
AR	Androgen receptor
ARE	Androgen-responsive element
ATCC	American Type Culture Collection
BCL2	B-cell CLL/lymphoma 2
BCR	Biochemical recurrence
bp	Base pair
BPH	Benign prostatic hyperplasia
cDNA	Complementary DNA
CHECK2	Checkpoint kinase 2
ChIP	Chromatin immunoprecipitation
CpG	Cytosine-phosphate-Guanine
CRPC	Castration-resistant prostate cancer
DGCR8	Di George Syndrome critical region gene 8
DNA	Deoxyribonucleic acid
DNMT	DNA (cytosine-5)-methyltransferase
dNTPs	Deoxynucleotide triphosphates
DRE	Digital rectal exam
dTTP	2'-deoxythymidine 5'-triphosphate
E2F3	E2F transcription factor 3
EAU	European association of urology

EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ERG	EST transcription factor
EZH2	Enhancer of the zeste homolog 2
FBS	Fetal bovine serum
FDA	Food and Drugs administration
GS	Gleason score
GSTP1	Glutathione S-transferase pi 1
GUSβ	Beta-glucuronidase
HAT	Histone acetylase
HDAC	Histone deacetylase
HDM	Histone demethylase
HMT	Histone methylase
HOXB13	Homeobox B13
IUCC	International union for cancer control
IgG	Immunoglobulin G
Let-7	Lethal-7
LHRH	Luteinizing hormone-releasing hormone
LNA	Locked nucleic acid
lncRNA	Long non-coding RNA
IgG	Immunoglobulin G
IQR	Interquartil range
MGB	Minor groove binder
miRNA	MicroRNA
MNPT	Morphologically normal prostate tissue
MRE	MicroRNA response element
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin

MYC	v-Myc avian myelocytomatosis viral oncogene homolog
n.a.	Not applicable
ncRNA	Non-coding RNA
Oncomir	Oncogenic microRNA
PBS	Phosphate-buffered saline
PCa	Prostate cancer
PCA3	Prostate cancer antigen 3
PC-8	Phenol-chlorophorm at pH8
PIN	Prostatic intraepithelial neoplasia
phi	Prostate Health index
PRC2	Polycomb repressor complex 2
pre-miRNAs	Precursor microRNAs
pri-miRNAs	Primary microRNAs
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
qMSP	Quantitative methylation-specific PCR
RAS	Rat sarcoma
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RT-qPCR	Quantitative real-time polymerase chain reaction
SDS	Sodium dodecyl sulfate
TMPRSS2	Transmembrane preotease serine 2
TNM	Tumor - Lymph Node - Metastasis
TRUS	Transrectal ultrasound
UNG	Uracil-DNA glycosylase
UTR	Untranslated region
XPO5	Exportin 5

I.INTRODUCTION

1. PROSTATE CANCER

Prostate gland is part of the male reproductive system responsible for producing important components of seminal fluid and its pathology is a major problem among older men worldwide. Prostate cancer (PCa) is considered multifocal and heterogeneous ranging from clinical indolent to more agressive disease. In last few years, PCa incidence has been rising mainly because of population aging, increased awareness and widespread introduction of PSA test [1]. Therefore, efforts have been made to understand the biology of prostate cancer in order to improve diagnostic tools and therapeutic approaches [2]. Neverthless, there are important challenges regarding PCa management that need to be solved. Specifically, new patients' stratification methods and the discovery (and validation) of novel sreening, prognostic and predictive biomarkers are still required [3].

1.1 Epidemiology

In 2012, according to GLOBOCAN, prostate cancer was the second most common cancer and the fifth leading cause of death from cancer in men (Figure 1). Geographically, PCa incidence and mortality rates have a highly heterogeneous distribution. Specifically, incidence rates vary more than 25-fold worldwide with the highest rates being observed in the most developed countries. Conversely, lesser variation in mortality rates (aproximately 10-fold worldwide) are found with a larger number of prostate cancer deaths in less developed regions and predominantly african descendents populations [4]. This widely variation between incidence and mortality rates may be related with the quality of cancer registry, genetic susceptibility, exposure to risk factors (eg. Sedentary lifestyle), variations in the access to screening methods (eg. PSA test) and treatment management [5].

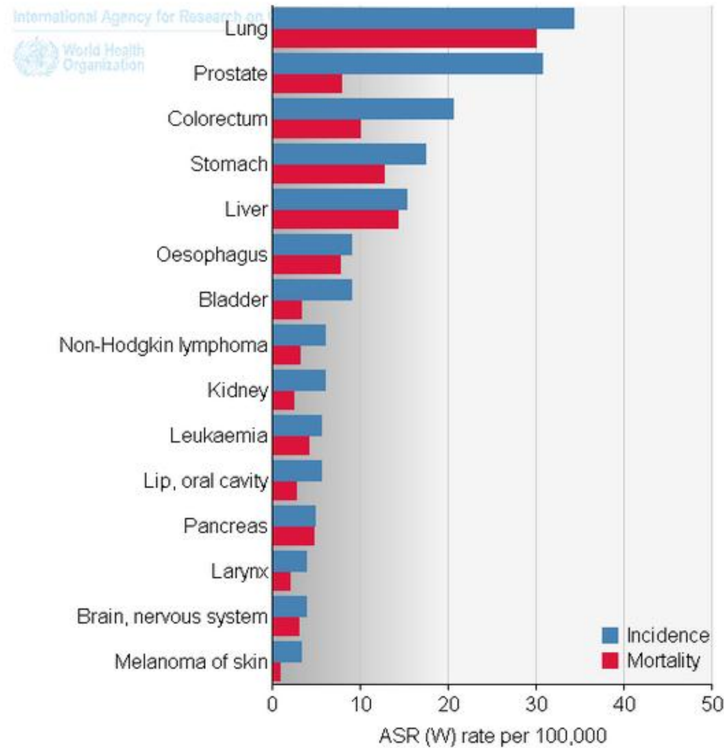


Figure 1 Estimated age-standardized incidence and mortality rates for cancer in men in 2012 [4].

In Portugal, prostate cancer was the most frequently diagnosed cancer in men and the third leading cause of cancer death in men, with an estimated 6,622 new cases and 1,582 deaths caused by PCa in 2012 (Figure 2) [6].

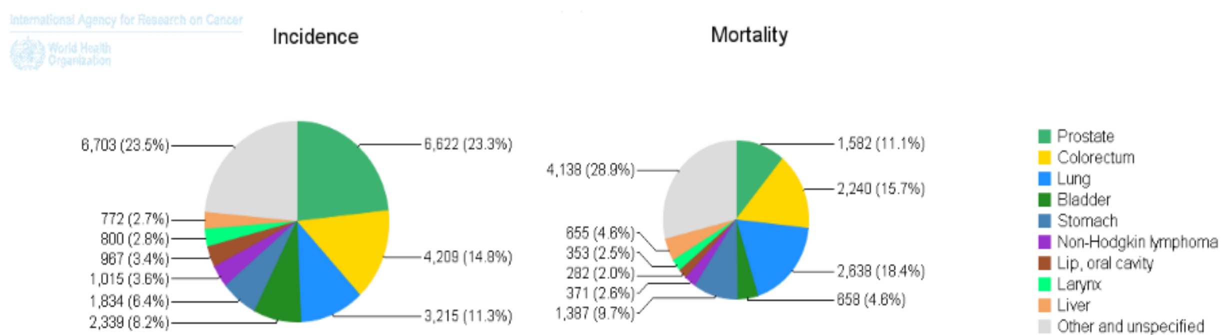


Figure 2 Estimated cancer incidence and mortality in portuguese men in 2012 [6].

1.2 Risk Factors

Although numerous behavioural factors, like smoking and obesity had been associated to prostate cancer, only three non-modifiable risk factors were identified for this disease: age, race and family history [7].

Prostate cancer is a disease of older men, a reason by which age is so many times considered the most important risk factor. Incidence of this pathology increases considerably with age and approximately 85% of the cases are diagnosed after 65 years of age [8]. In the past few years, the median age at diagnosis has substancially dropped, in particular due to early detection methods, such as measurement of serum PSA [9].

Race is another well-estabilished risk factor in prostate illness. Prostate cancer incidence varies widely between different etnic groups, being higher among African-american men and lowest in Asian men [8]. These etnic differences may result from the interaction of epidemiological and genetic factors, as well as variations in care delivery and treatment selection. Recent findings suggest unique single-nucleotide-polymorphism patterns, epigenetics changes and variations in fusion gene products may explain the higher prevalence of this disease among African origin men [10].

Prostate cancer is strongly associated with family history and genetic predisposition. It is estimated that men with one first-degree relative affected with PCa have higher risk of developping PCa and that increase is proportional to the number of relatives affected [8]. About 5-10% of PCa are familial and normally manifest at a younger age (6-7 years earlier) compared to sporadic disease [9]. Hereditary prostate cancer is a subset of familial prostate cancer and it is defined as a pattern of cancer distribution consistent with Mendelian inheritance. Numerous studies have already identified several susceptibility genes with a dominant mode of inheritance related with PCa, such as *BRCA1*, *BRCA2*, *HOXB13* and *CHEK2* [8, 9].

Notwithstanding the inevitability of all these three risk factors, further investigation into possible novel risk factors with impact in prostate carcinogenesis may be useful to determine prevention stratagies.

1.3 Screening and Diagnosis

PCa is described as a clinically silent disease until extra-prostatic invasion occurs, thus the development of screening methods to detect PCa in earlier stages, while it is still organ-confined and therefore potentially curable is mandatory. However, frequently PCa screening

has been associated to overdiagnosis and overtreatment. Hence, men considering the screening of prostate cancer should be awared about the potential benefits and harms. According to European Association of Urology (EAU) guidelines, screening should be started in men with more than 50 years of age and not recommended in men with life expectancy less than 15 years, but can start earlier depending on family history of PCa and race [11].

Currently, the main tools to screen and diagnose PCa are digital rectal examination (DRE), serum concentration of prostate-specific antigen (PSA) and transrectal ultrasound (TRUS)-guided biopsy [12]. However, both DRE and PSA test have demonstrated to be fallible. DRE was the primary screening test for PCa during many years, but the interexaminer variability and the fact of the majority of cancers detected were already at an advanced stage conditioned its use [13]. PSA is the most widely used biomarker in prostate cancer. This serine protease is responsible for semen liquefaction and secreted into the seminal plasma [14]. In normal conditions, only low levels of PSA are detected in blood, however the increase of serum PSA can represent abnormalities in prostate gland architecture but not always cancer, since prostatitis and benign prostate hyperplasia (BPH) are also associated to increased PSA values [15]. Therefore, although a strong correlation between high serum PSA levels and PCa incidence was reported, this molecular biomarker is not cancer-specific nor allows predict aggressive disease [16]. Notwithstanding the lack of specificity and low positive predictive value, specially in the "gray zone" (2-10 ng/mL), the combination of serum PSA levels ≥4.0 ng/mL with DRE was demonstrated to improve detection of prostate cancer. Taking into account the PSA limitations and its high false-positive rate, new parameters were developed to increase PSA specificity like PSA density, velocity, doubling-time and age/race-specific reference ranges [15]. Besides PCa screening and detection, PSA is also used for staging and monitoring both the patients already diagnosed and the recurrence after curative therapy [16].

Transrectal ultrasound (TRUS)-guided biopsy is the standard way to obtain material for histopathologic examination and, thus, a more detailed diagnostic. Normally, the need for prostate biopsy is determined using PSA threshold of 4ng/mL, suspicious DRE, patient's age and comorbidities [12]. However the likelihood of identification of prostate cancer on biopsy based on those parameters is only about 21%, which means that we are overtesting and overdiagnosis cancers that probably have remained undetectable [3]. Hence, it is important to realise that prostate cancer diagnosis is not always active treatment synonym because that is the only way to decrease overtreatment and, at the same time, maintain the prostate cancer screening potential benefits.

In last few years, researchers focus on the discovery and characterization of emerging biomarkers assays for prostate cancer and the urinary prostate cancer antigen (*PCA3*) and

genomic rearrangements, such as *TMPRSS2:ERG*, look promising. *PCA3* is a mRNA that is normally overexpressed in PCa. Progensa^{TM} PCA3 is a non-PSA-based diagnostic test approved by FDA in 2012 for use in conjunction with other patient information to aid in the decision for repeat prostate biopsy in men ≥ 50 years [17]. Although *PCA3* cannot replace PSA test in clinical practice, since an appropriate cutoff level is not defined yet, the addition of *PCA3* to risk assessment tools promotes an increase in predictive capability [16]. *TMPRSS2:ERG* fusion is the most common genetic abnormality found in prostate cancer and can even be detected in precursor lesions. This fusion transcript appears to be another promising urinary RNA marker for cancer detection and prognosis and, in combination with *PCA3*, improved the performance of the multivariate Prostate Cancer Prevention Trial risk calculator in predicting cancer on biopsy [18].

Actually, there is an urgent need of new biomarkers to improve disease risk stratification at the screening time and to clear the suitable approach to each case. Multivariate risk stratification appears to improve prostate cancer screening and diagnosis, classifying and monitoring the disease. Individual stratification according to risk can maximize benefits, in terms of morbidity and mortality, and decrease overdiagnosis and overtreatment costs [19]. Moreover, genomic disease signatures as well as epigenetic patterns seem to be helpful to cancer cells identification in clinical samples. These alterations might also be used to assess disease extent and prognosis, enabling the identification of more aggressive tumors and a better definition of therapeutic stratagies [20, 21].

1.4 Grading System: Gleason Score

Prostate cancer is a very heterogeneous neoplasia, as already mentioned above, whereby ascertain its potential clinical aggressiveness based on tumor grading is imperative. Gleason score, developed by Donald Gleason, is currently the major tool to graduate PCa according to architectural patterns of prostate gland [22]. This grading system assigns histological features 1 through 5 and results from adding the most common and second most common patterns, generating a combined Gleason score which ranges from 2 (1+1) to 10 (5+5) [23].

The contemporary application of Gleason score is significantly different from the original version, being the most recent update from 2014 [24]. Nowadays, a five-grade group system is proposed for PCa grading: grade group 1 (Gleason score ≤6), grade group 2 (Gleason score 3+4=7), grade group 3 (Gleason score 4+3=7), grade group 4 (Gleason score 8) and grade group 5 (Gleason score 9-10). This new methodology has potential benefits not only in reducing low-grade PCa overtreatment but also in Gleason score 7

differentiation, 3+4 and 4+3, which are prognostically very different [23]. Thus, with the current Gleason score grading system, score 2-5 are no longer assigned, being Gleason score 6 now considered the lowest PCa grade. In addition, score 6 is now graded as 7, whereby actual score 6 offers better cancer prognosis than before [22].

The Gleason score is one of the most powerful prognostic predictors in prostate cancer. Despite the significant revisions that this grading system has undergone in last few years, there are still some limitations that might have impact in patient care, such as the interobserver reproducibility and the frequent biopsies undergrading compared with the corresponding radical prostatectomy specimens [24].

1.5 Clinical and Pathological Staging

An accurate disease staging is crucial to obtain a prognosis assessment and treatment planning for prostate cancer. Nevertheless, the pathological complexity and the ambiguous clinical course of prostate carcinogenesis make difficult to ascertain a clear staging classification system for personalized medical care [25].

Depending on the moment at which tumor staging is performed, cancer staging can be divided into clinical and pathological (Table 1). The clinical staging of PCa is based on data obtained prior to the first definitive treatment and is evaluated by the combination of prostate biopsy assessment with DRE and PSA and may be more accurately if bone scanning and other image techniques are included [11]. Pathologic staging requires tumor extention histological identification and is obtained after radical prostatectomy (RP). This staging system is centered in surgical specimen’s macro and microscopic examination and dissected regional lymph nodes. Agreement between clinical and pathological stage enable better prognosis evaluation and appropriate treatment [25].

The staging of PCa is performed according to the TNM classification system, the staging tool most widely used for solid tumors. This tool is divided into three main areas: T-staging concerning to the extension of primary tumor, N-staging that evaluate the presence and extension of involved lymph nodes and M-staging that assess the existence of distant mestastasis [26].

Table 1 Clinical and pathological staging of PCa according with AJCC/IUCC in 2010.

PRIMARY TUMOR (T)	
Clinical	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor neither palpable nor visible by imaging
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy (*e.g.*, because of elevated PSA)
T2	Tumor confined within prostate
T2a	Tumor involves one-half of one lobe or less
T2b	Tumor involves more than one-half of one lobe but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through the prostate capsule
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Pathologic (pT)	
pT2	Organ confined
pT2a	Unilateral, one-half of one side or less
pT2b	Unilateral, involving more than one-half of side but not both sides
pT2c	Bilateral disease
pT3	Extraprostatic extension
pT3a	Extraprostatic extension or microscopic invasion of bladder neck
pT3b	Seminal vesicle invasion
pT4	Invasion of rectum, levator muscles, and /or pelvic wall
REGIONAL LYMPH NODES (N)	
Clinical	
Nx	Regional lymph nodes were not assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
Pathologic (pN)	
pNx	Regional nodes not sampled
pN0	No positive regional nodes
pN1	Metastases in regional node(s)
DISTANT MESTASTASIS (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Nonregional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

1.6 Therapeutic Approaches

Prostate cancer's clinical and molecular heterogeneity hinders adequate risk stratification and management. Age, life expectancy, comorbidities and patients' life quality are taken in consideration to select the better treatment approach [27]. Management decisions should be discussed within a multidisciplinary team and balance the benefits and side effects of each therapy modality is mandatory [1].

For patient with clinically localized disease the standard approaches, depending on clinical and pathological staging, are active surveillance, watchful waiting, radical prostatectomy, external radiotherapy and brachytherapy [1]. Active surveillance and watchful waiting are two distinct strategies for conservative management that aim to reduce overtreatment. Active surveillance is recommended for indolent tumors in which more aggressive treatments should be deferred to avoid life quality injury. Watchful waiting is more appropriate for men with comorbidity and limited life expectancy or slow-growing tumors [11]. On the other hand, radical prostatectomy is more suitable for more aggressive disease, although still organ-confined, and external radiotherapy for locally advanced tumors [12]. Relapse after local therapy is considered when PSA levels rise above 0.2 ng/mL after radical prostatectomy or above 2 ng/mL following radiation therapy [28].

Androgen deprivation therapy (ADT) is highly recommended for advanced and metastatic prostate cancer as well as for patients displaying biochemical recurrence (BCR) after local treatment [28]. Currently, chemical castration is more frequently used than surgical castration to achieve androgen deprivation either by suppressing the secretion of testicular androgens (LHRH agonists) or by inhibiting the action of circulating androgens at the receptor level using competitive molecules (LHRH antagonists) [29]. In men with prostate cancer metastasis at first presentation, cytotoxic chemotherapy using docetaxel in combination with ADT is chosen, unless the patient was not fit enough to receive the drug [28].

Usually prostate tumors remain hormone-sensitive at initial presentation, however, a considerable percentage of them eventually develop resistence to ADT and becomes castration-resistant prostate cancer (CRPC). The treatment of metastatic CRPC (mCRPC) includes abiraterone acetate plus prednisone (AA/P) in first-line and enzalutamide for second-line therapy [30].

2. EPIGENETICS

Epigenetics is defined as heritable and reversible mechanisms that regulate gene expression dynamic without altering the DNA sequence [31]. The majority of these heritable changes are estabilished during differentiation and are stably maintained through several cell cycles, enabling that cells containing the same genetic information present different phenotypes [32].

Epigenetic mechanisms are based in a complex network of chemical reactions involving not only DNA and histone post-translational modifications but also numerous context-specific DNA, RNA and protein interactions. All this machinery work together to regulate the genome structure, organization and function in the cellular environment and it appears significantly contribute to transformation [33]. In addition to genetic alterations, epigenetic deregulation also play crucial and complementary roles in cancer initiation and progression [34]. Moreover, since epigenetic abnormalities are potentially reversible and can be restored to their normal state, epigenetic therapies appear to be a promising field in cancer management. [32]

The key epigenetic processes involved in gene expression control are DNA methylation, histone post-translational modifications, structural and functional histone variants and non-coding RNAs [31] (Figure 3). These mechanisms work together to regulate genome functioning and their interplay constitute the "epigenetic landscape" that is involved in many cellular processes including malignant cellular transformation [32].

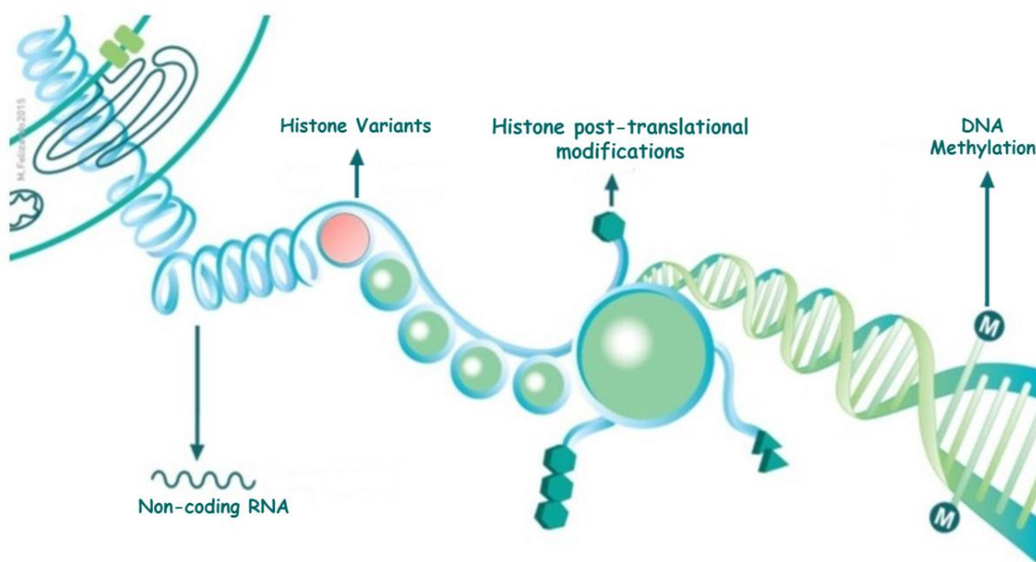


Figure 3 Epigenetic mechanisms. M-5- methy-cytosine; the symbols (hexagon and triangle) represent different post-translational modifications in N-terminal tails of histone proteins (CBEG, IPO Porto).

2.1 DNA Methylation

DNA methylation patterns' change have been widely correlated with cancer development. More specifically, there are evidences indicating that such alterations could represent early events of prostate carcinogenesis, being the most recurrent epigenetic phenomenon in both localized and metastatic prostate tumors [35, 36].

This epigenetic mechanism involves the addition by DNA-methyltransferases (DNMTs) of a methyl group in the fifth carbon of the cytosine (C), forming 5-methyl-cytosine (5mC) [37], which establish and maintain these patterns through cell division. DNMT1 is the major maintenance methylase, whereas DNMT3A and DNMT3B are two *de novo* DNA methyltransferases involved in several mechanisms during development, such as parental imprinting on the DNA, methylation of constitutive heterochromatin and host defense against foreign DNA integration and expression [38].

Despite cancer genomes are globally hypomethylated, some genome regions' are commonly hypermethylation, namely promoter regions. DNA methylation occurs almost exclusivelly in CpG dinucleotides, which occupy aproximatly 60% of human gene promoters [37]. Indeed, tumors from distinct sites display different CpG methylation profiles as well as different pathways of carcinogenesis, therefore this could be useful for diagnosis [39]. When a CpG island, a region with high frequency of CpG dinucleotides, becomes aberrantly hypermethylated, it can cause the silencing of the associated gene, either by directly interacting with transcription factors or by recruiting methyl-binding proteins that consequently interacts with histone-modifying enzymes and change chromatin to a repressive state [40]. Wereby hight levels of DNA methylation at gene promoter region correlates with low gene expression. Tumor suppressor genes are normally silenced by methylation during tumorigenesis and this could potentially influence mechanisms such as DNA repair and apoptosis. Regarding prostate cancer, *GSTP1* is the most widely studied and it is hypermethylated in 70-80% of PCa cases [36].

However, DNA methylation can also be reverted by epigenetic-modulating drugs, such as 5-azacytidine (5-aza-C) and 5-aza-2'-deoxicytidine (5-aza-CdR). These drugs are incorporated into the genome during cell division and DNA replication. Once there, they irreversiblly bind to DNMTs enabling their degradation and thus inhibiting DNA methylation [41].

2.2 Histone Post-translation Modifications

The nucleosome is composed of double-helical DNA wrapped around a histone octamer (one pair of each H2A, H2B, H3 and H4) which, depending on the packaging level, may establish different chromatin conformations. Chromatin condensed states, also known as heterochromatin, repress transcriptional activity while relaxed states, called euchromatin, facilitate the transcription [38]. Histone modifications play a major role in the development and progression of cancer and it seems to regulate key cellular processes, such as transcription, replication and repair [32]. The N-terminal tails of histones can undergo a variety of post-translation covalent modifications (PTMs), including methylation, acetylation, ubiquitylation and phosphorylation on specific residues, thus constituting the histone code [42]. Histone PTMs can regulate chromatin accessibility and/or recruit specific binding proteins (readers) like transcription factors, chromatin remodelers or chromatin structure proteins [43]. These PTMs may change histone electrostatic charge resulting in a stuctural alteration in their DNA binding [44].

Histone mdifications can lead to either activation or repression, depending on which residue is modified as well as the type of modification involved [32]. Histone methylation mainly occurs on the side chains of lysines and arginines and has been associated with both transcriptional activation and repression, based on the specific residue methylated. In contrast, histone acethylation is strongly associated with transcriptional activation, since this alteration induce positive charged histones' neutralization decreasing the interaction with negatively charge DNA [45]. Histone PTMs are dynamically regulated by enzymes responsible of adding or removing covalent modifications to histone proteins, such as histone methyltransferase (HMTs) and histone acetyltransferases (HATs) or histone demethylases (HDMs) and histone deacetylases (HDACs), respectively [46, 47].

Histone marks alterations can be associated with a variety of human cancers, including prostate cancer, and changes in global histone PTMs levels can be used as predictor of prostate cancer recurrence outcomes [48]. Besides their individual role, both histone modifications and DNA methylation may interact to regulate gene expression, chromatin organization and cellular phenotype [44].

2.3 Histone Variants

Nucleossomal histones (except H4) have different histone variants and in the last few years, these proteins were introduced as major actors in epigenetic field. Indeed, recent studies refer histone variants as key players in transcriptional regulation, cell cycle progression and DNA repair [49]. In contrast to canonical histone genes, histone variants genes are non-allelic being mainly expressed in an independent replication manner during cell cycle. Despite these differences, histone variants may also undergo post-translational modifications, which determine their nuclear localization and function [50]. Moreover, replacement of canonical histone proteins by specialized histone variants can introduce further diversification of chromatin status, enabling distinct PTMs as well as recruitment of different interactors [51]. Notwithstanding, the histone variants' temporal and tissue-specific expression, their incorporation into the nucleosome requires the intervention of histone chaperones [52]. Tacking into account the histone variants' importance to nucleosome structure and consequently gene expression regulation, it has been suggested that they may, ultimately, contribute to disease development like cancer [49].

2.4 Non-coding RNAs

Despite the majority of the genome is transcribed into RNAs, only a small percentage codes for proteins (1-2%). Thus, RNAs can be categorized in two classes: the one that has the ability to code for proteins and the non-coding RNAs (ncRNAs) without coding potential [53]. In the past few years, ncRNAs have been shown to be major gene expression regulators with critical roles both in molecular mechanisms and biological processes [54]. NcRNAs are also classified into different groups according to several criteria, being the most commonly used the length. Generally, ncRNAs smaller than 200 nucleotides are designated small non-coding RNAs, whereas the ones longer than 200 nucleotides are called long non-coding RNAs (lncRNAs) [55]. Among small ncRNAs, microRNAs (≈20 nucleotides) are the most well studied group.

2.4.1 MicroRNAs Biogenesis

MicroRNAs are small non-coding RNAs with length between 18-25 nucleotides which are encoded within intergenic regions or within the introns or exons of protein-coding genes. Currently, they are extensively studied because of their role as gene regulators during development and disease, like cancer [56]. MiRNAs are transcribed in the nucleous by RNA polymerase II or III into a primary transcript designated pri-miRNA (Figure 4). Then, Drosha/DGCR8 process the pri-miRNA producing a precursor miRNA (pre-miRNA). Subsequently, Exportin 5 (*XPO5*) and Ran-GTP export the pre-miRNA from the nucleous to the cytoplasm where it will be transformed in a mature miRNA duplex by RNase III-type enzyme Dicer. This duplex consists in a guide and a complementary passanger strand, which are also called miR-3p and miR-5p, according to the direction of the sequence. Finally, the double-strand miRNA complex binds to the RNA-induced silencing complex (RISC), which includes the transactivation-responsive RNA-binding (TRBP) and Argonaute2 (Ago2). After, a single-stranded miRNA is obtained which binds its complementary sites at 3'UTR of target mRNA regulating gene expression [57-60]. Depending on the matching degree miRNA-mRNA, there are two different transcriptional repression mechanisms. When miRNA has a near-perfect match to the mRNA target a site-specific cleavage occurs, whereas an imperfect match only abolishes target mRNA translation. However, some miRNA can bind to gene targets promoter region and increase its expression at a transcription level [57, 60]. With the advent of next-generation sequencing (NGS) technologies multiple length or sequence variants, also called isomiRs, have been identified for many miRNAs. Such variants are frequently found in several tissue types, including prostate, and they can also have a relevant biological function [61]. IsomiRs derive from the same precursor miRNA and are the result of imprecise processing by endoribonucleases (Dicer and Drosha) or enzymatic post-transcriptional modifications [62].

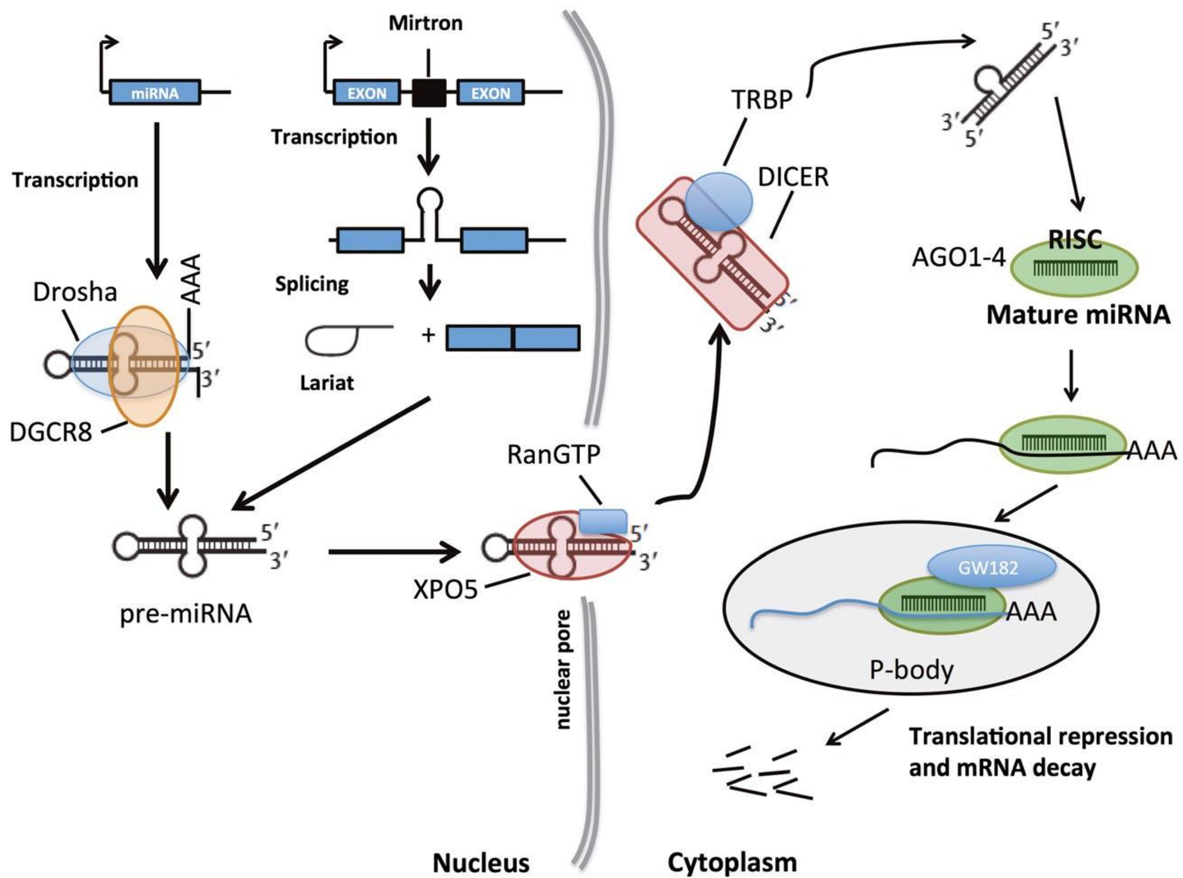


Figure 4 MicroRNAs biogenesis (Kindly provided by Ramalho-Carvalho from [63]).

2.4.2 Epigenetic Regulation of MicroRNAs

Recent information regarding epigenetic processes has pointed out miRNAs as putative targets of epigenetic modulation in both physiological and pathological conditions, such as cancer. Indeed, miRNAs genes were also found to be susceptible to epigenetic regulation (Figure 5), as DNA methylation and histone modifications [64]. One of the first evidence that microRNAs' expression is epigenetically changed was obtained by using chromatin modifying drugs to reactivate its expression at the transcription level [65] and since then several reports showed similar findings [66]. Furthermore, considering miRNAs have a huge impact in neoplastic transformation, understand how epigenetic machinery could influence its expression is crucial, particularly because epigenetic control is miRNA specific and epigenetic effector specific [67]. Generally, DNA methylation leads to miRNA silencing while histone modification, depending on the alteration type and target amino acic residue, can either trigger or suppress miRNAs [64]. Recently, 155 out of 332 human miRNA promoter genes were associated with CpG islands, that are targets of DNA methylation machinery [68].

In addition to epigenetic regulation, certain miRNAs have the ability to translocate back to the nucleus and activate or repress transcriptional activity through the recruitment of chromatin-modifying factors to the target region [69]. A subset of miRNAs, called epi-miRNAs, can also directly or indirectly target epigenetic machinery key effectors, such as DNA methyltransferases (DNMTs), chromatin remodeling enzymes and polycomb group genes [70]. MiR-101 was shown to directly target EZH2, the catalytic subunit of polycomb repressor complex 2 (PRC2) in prostate cancer, thus being suggested as an epi-miRNA, [71]. Recently, miR-34b was found to target DNMT1, HDAC1, HDAC2 and HDAC4 in PCa cell lines. Interestingly, the same study also showed that miR-34b was aberrantly methylated in the same cell lines, providing an insight into the interplay between epigenetic modulation and miRNAs [72]. Thus, miRNAs can function either as modulators of other epigenetic regulators or as specific genes' expression regulators.

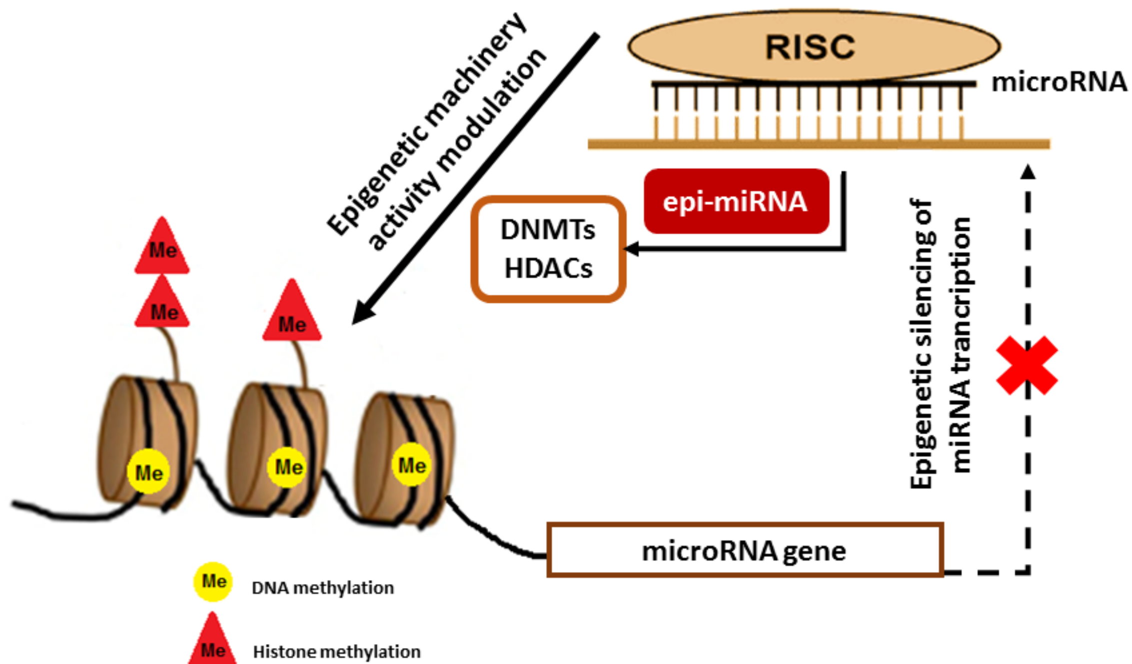


Figure 5 Epigenetic regulatory mechanisms crosstalk.

2.4.3 MicroRNAs Deregulation in Prostate Carcinogenesis

MiRNAs are involved in multiple biological processes and numerous studies already document miRNA deregulation contribution to cancer initiation, progression, metastasis and drug resistance. MiRNA profiling changes were found in several cancers and PCa is not an exception [73].

Usually, miRNAs deregulated in cancer are divided in two major classes: oncogenic miRs, which are normally overexpressed during carcinogenesis and act by repressing tumor-supressor genes, and miRs with tumor suppressive functions that negatively regulate oncogenes and are frequently underexpressed in cancer [57]. MicroRNA-21 is an example of an oncogenic miRNA whose target is the tumor suppressor gene *PTEN* [74], whereas let-7 which targets the oncogene *RAS* typify a tumor suppressive miRNA [75]. However, depending on the context, a single miRNA can act either as oncogene or as tumor suppressor. MiR-375 is an example of that in PCa cell lines, in which 22Rv1 cells displayed higher expression levels, whereas PC3 cells disclosed significantly lower levels. Interestingly, both anti-miR-375 transfection in 22Rv1 cells and forced miR-375 expression in PC3 cells attenuated the malignant phenotype. Thus, while in 22Rv1 cells (hormone-sensitive PCa cell line) an oncogenic role for miR-375 is suggested, a tumor-suppressive function is implied for PC3 cells (hormone-independent PCa cell line) [76].

Furthermore, regulation of miRNA expression is also correlated with the presence or absence of androgens in PCa, whereby their action may be androgen-dependent. In general, AR-inducible miRNAs present conserved androgen response elements (ARE) responsible for transcripton in their promoter regions [73]. MiR-32 and miR-148a are two androgen-regulated miRNAs overexpressed in castration-resistant prostate cancer (CRPC) [77]. Other miRNAs are also potential modulators of AR-mediated signalling, such as miR-488 whose upregulation represses transcriptional AR activity [78], or miR-146a loss of function that is frequent in hormone-refractory prostate cancer [79]. Despite their role in AR signaling, several miRNAs that target proto-oncogenes, such as *RAS*, *BCL2* and *E2F3*, are also frequently downregulated during prostate carcinogenesis [80].

In last years, multiple researchers focus their studies in discovery miRNAs function in PCa and their usage as tools to understand the molecular mechanisms behind oncogenesis. Hence several clinical applications were already demonstrated for miRNAs in PCa, thus providing new opportunities for stratagies in cancer diagnosis and treatment [81, 82].

2.4.4 MicroRNAs in Human Cancer: Possible New Biomarkers and Treatment Targets

As mentioned above, miRNAs are key molecules implicated in cancer development. Indeed, miRNAs are warning signs because their deregulation patterns sometimes precede phenotype changes [83]. Thus, miRNAs are attractive targets for biomarker discovery once they have short length and they are relatively resistant to RNase degradation improving

their longevity in clinical samples [84]. Furthermore, miRNAs show great potential as earlier diagnosis tools because they are the final gene product, and thus biologically relevant levels are measured, reproducible extracted from a wide range of biological samples (plasma and/or urine) and resistant to various storage conditions. The fact of miRNAs could be easily detected and accurately quantified by numerous techniques, such qRT-PCR and microarray, are also miRNAs' advantages as attractive molecular biomarkers [85]. A study concerning circulating miRNAs in prostate cancer revealed clinical relevance of several miRNAs as pronounced markers for high-risk tumors, once again in line with the potential of miRNAs as diagnostic biomarkers in cancer [86]. Recently, also exossomal miRNA, such as miR-1290 and miR-375, were found to be promising prognostic markers for PCa [87].

Aberrantly expressed miRNAs observed in cancer are very important concerning new approaches development for future therapies. MicroRNAs own several advantages which allows them to play a crucial role both as therapeutic agent or as molecular target [88]. MiRNA manipulation usually involves direct silencing or tumor promoting activity reduction. Generally, *in vivo* approaches include genetically engeneered animals and miRNA vector systems such as viral vectors, nanoparticle-based delivery, mimics and anti-miRs [89]. They have relative simple structures and predictable mechanisms whereby the design of miRNA-based therapeutic molecules seems easier than conventional chemical drugs. However, the use of miRNAs as therapeutical tools is also ambiguous because miRNAs can target multiple protein-coding genes at once and their fuctions are fine-tuned and context-dependent [90]. In other words, the manipulation of a given miRNA may change the protein products in several signal transduction pathways, causing unwanted side effects. MiRNA-based therapeutics involve both miRNA mimics and inhibitors (antimiRs). MiRNA mimics are functionally contructed to replenish the lost of the corresponding miRNA expression in disease. Inversely, antimiRs inhibit and block the function of the miRNA target by strongly binding to it. In last few years, significant improvements have been made to increase the binding affinity and stability of these RNA-based therapeutic stratagies mainly through chemical modifications to the nucleotide backbone, such as methylation or LNAs [91].

Although miRNAs have already proven to be important mediators of tumorigenesis and disease progression, there are still major challenges regarding their application in clinical daily practice. Results' discrepancy in different studies constitute a huge limitation that persists among research community, making difficult to reach a consensus about miRNAs function. The usage of distinct profiling platforms and the reduced number of samples may contribute to this disparate results [59, 92].

3. OVERVIEW OF C-MYC ROLE IN PROSTATE CANCER

MYC is a proto-oncogene which encodes c-Myc transcription factor and it was firstly reported as cellular homolog of v-*myc*, an oncogenic retrovirus that causes tumorigenesis in birds [93]. In humans, *MYC* gene is located on chromosome 8q24 and deregulations of its expression, such as, amplifications, chromosome translocations or loss of upstream repressor, have already been identified in several cancers [94]. The c-Myc oncogene encodes a conserved basic helix-loop-helix leucine zipper transcription factor that binds to approximately 10-15% of genome as well as regulate genes that codify both proteins and non-coding RNAs [95, 96]. This transcription factor regulates many genes involved in a variety of cellular processes, namely cell growth and proliferation, cell-cycle progression, transcription, differentiation, apoptosis and cell motility [97].

Regarding prostate cancer, amplification at 8q24 stated as one of the most common chromosomal abnormality with potential prognostic value and also predictor of biochemical recurrence for this neoplasia [98]. A few years ago, c-Myc was also reported to associate with higher pathological stages and Gleason's scores as well as earlier disease progression [99]. Recently, *MYC* was found to be activated in PIN lesions, a pre-maliganant lesion that is frequently regarded as prostatic adenocarcinoma's precursor [100].

3.1 MicroRNA Regulation by c-Myc

Besides genetic and epigenetic regulation, miRNAs expression can also be altered by aberrant transcription factors activity in tumor cells and recent evidence has confirmed that there is, indeed, a crosstalk between c-Myc and miRNAs. Moreover, c-Myc activation is responsible for miRNAs' expression profile reprogramming in cancer cells [101-103].

Once c-Myc acts as an oncogenic transcription factor, normally it regulates miRNA expression binding to its promoter region and promoting a widespread downregulation of miRNAs [104]. Previous observations have already reported globally reduced miRNA levels during carcinogenesis which could be explained not only by miRNA biogenesis blockade, but also by c-Myc repression of miRNA transcription [105]. Curiously, the majority of *MYC*-downregulated miRNAs have anti-tumorigenic properties suggesting a tumor suppressive role, like miR-15a and miR-16-1 [106]. Notwithstanding the extensive miRNA repression by c-Myc, there is also studies reporting that this transcription factor upregulates the pro-tumorigenic miR-17-92 cluster [107, 108].

Remarkably, transcription factors are frequently targets for repression by the miRNAs they regulate creating complex feedback circuits (example: c-Myc/let-7 miRNA regulatoty

network). One the one hand, c-Myc is described as suppressing let-7 miRNA expression [109]; on the other hand, let-7 miRNA is itself a negative regulator of c-Myc [110].

As mentioned above, several *MYC*-regulated miRNAs are already classified as oncogenic or tumor suppressor miRNAs, according with the functions they display, therefore they may represent useful anti-cancer therapies. MiRNA-based therapies in tumors with *MYC* hyperactivity could consist essencially in two types of mechanisms: the first one is the c-Myc-induced oncogenic miRNAs' inhibition; the second one is the *MYC*-downregulated tumor supressor miRNAs' reintroduction [111] (Figure 6).

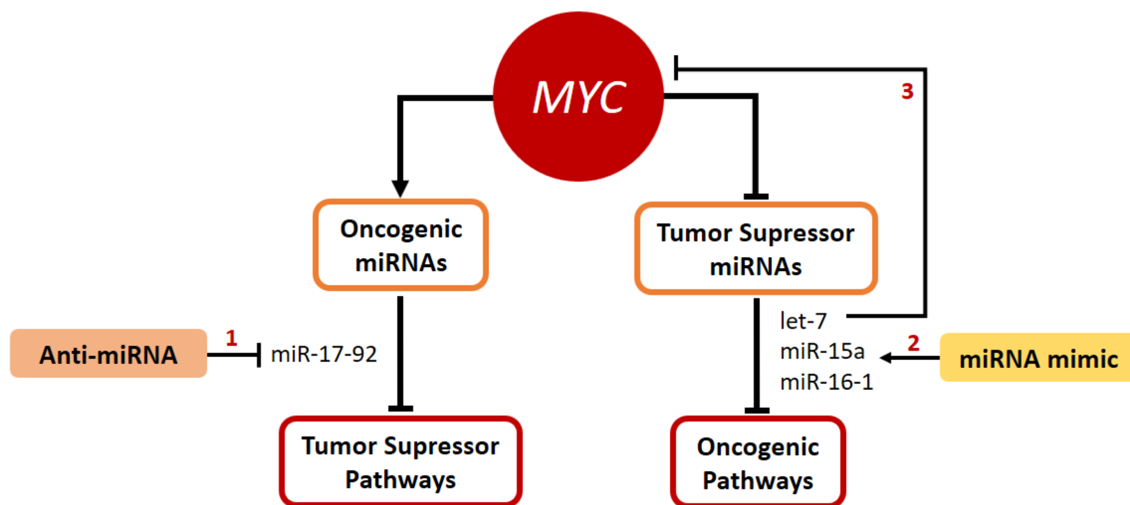


Figure 6 Strategies to target the interplay between *MYC* and miRNA in cancer. In neoplasms with *MYC* deregulation, anti-miRNAs could be directed against *MYC*-induced oncogenic miRNAs (1) or miRNA mimics could be used for reintroduction of *MYC*-repressed miRNAs (2) or to target *MYC* itself (3).

II.PRELIMINARY DATA

This study is integrated in a larger project developed in the Cancer Biology and Epigenetics Group (CI-IPO-Porto), whose major goal is to understand the regulatory network between c-Myc and specific miRNAs involved in prostate tumorigenesis.

Thus, the results gathered until the beginning of this project are:

1. UPREGULATION OF *MYC* IN CLINICALLY LOCALIZED PROSTATE CANCER TISSUE SAMPLES (COHORT #1)

MYC transcript and protein levels were assessed in 198 PCa (Primary prostate cancer) cases, 37 PIN lesions and 10 MNPT (morphologically normal prostate tissue) (Figure 7A). PCa and PIN depicted significantly higher *MYC* transcript levels compared with MNPT ($P < 0.001$). Moreover, a significant increase of c-Myc protein levels was also apparent from MNPT to PCa samples (Table 2, Figure 7B and Figure 7D). Statistically significant differences were observed concerning *MYC* transcript and respective protein levels across the three groups of immunostaining scores ($P < 0.001$), and, overall, c-Myc protein levels followed the same trend (Figure 7C). In pairwise comparisons, however, statistical significance was observed for +1 *vs.* +2 ($P < 0.001$) and +1 *vs.* +3 score groups ($P < 0.001$), but not for +2 *vs.* +3 scores.

Clinicopathological data from all clinical samples tested in this study are depicted in Table 3. No statistically significant differences between the two groups of patients were found for age. Furthermore, a statistically significant association was disclosed between c-Myc protein levels and some clinicopathological parameters. Somer's D coefficient test revealed that higher c-Myc protein levels associated with higher serum PSA (Somer's D = 0.157; $P = 0.011$) and higher GS (Somer's D = 0.131; $P = 0.044$) in PCa.

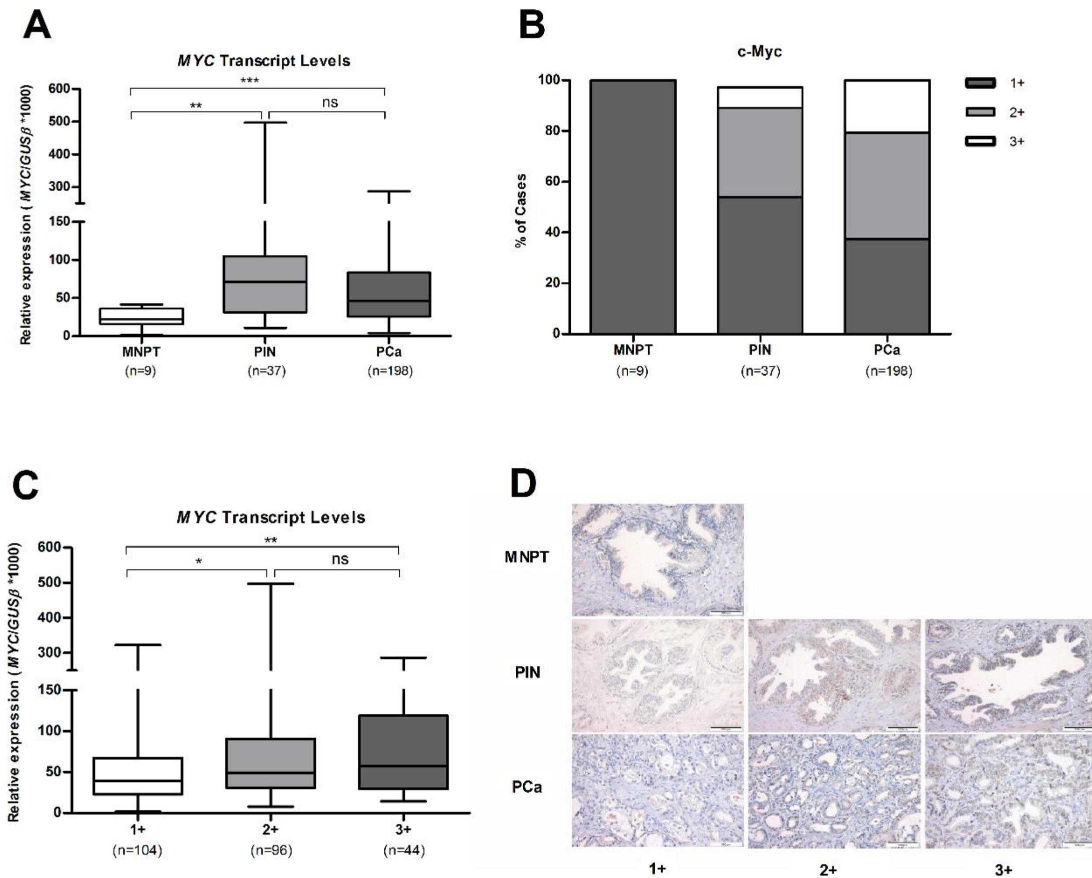


Figure 7 *MYC* transcript levels **(A)** and distribution of c-Myc immunostaining **(B)** in cohort #1; *MYC* transcript levels in cohort #1, grouped according to c-Myc immunostaining**(C)**; Representative images of c-Myc immunostaining in cohort #1 **(D)**.

Table 2 Immunohistochemical expression of c-Myc in cohort #1.

Clinical Sample Group (cohort #1)	Negative	Positive	
	1+ (≤ 10%) N (%)	2+ (10%< - ≤ 50%) N (%)	3+ (>50 %) N (%)
MNPT	9 (100 %)	-	-
PIN	20 (54 %)	13 (35.1 %)	4 (8.1 %)
PCa	74 (37.4 %)	83 (41.9 %)	41 (20.7 %)

Table 3 Clinical and pathological data of fresh-frozen tissues included in this study (cohort #1).

Clinicopathological Data Cohort #1	PCa n = 198	PIN n = 37 matched with a PCa	MNPT n = 9
Age (years), Median (range)	64 (49 - 75)	65 (51 - 75)	58 (45 - 79)
PSA (ng/mL), Median (range)	8.10 (2.66 - 35.50)	n.a.	n.a.
Pathological Stage, N (%)			
pT2	110 (55.6 %)	n.a.	n.a.
pT3a	65 (32.8 %)	n.a.	n.a.
pT3b	23 (11.6 %)	n.a.	n.a.
Gleason score, N (%)			
< 7	67 (33.8 %)	n.a.	n.a.
= 7	115 (58.1 %)	n.a.	n.a.
> 7	16 (8.1 %)	n.a.	n.a.

2. REGULATORY NETWORK BETWEEN c-MYC AND microRNAS

Three PCa cases with low *MYC* expression and four with high *MYC* expression were chosen for microarray analysis. The resulting heatmap shows only miRNAs that achieved statistical significance and revealed 78 miRNAs were overexpressed in samples with high *MYC* content, representing possible targets of c-Myc regulation (Supplementary Figure 1). From these, a panel of 3 miRNAs (miR-27a-5p, miR-570 and miR-1292) were selected for validation in a larger and independent dataset. Selection of miR-27a-5p, miR-570 and miRNA-1292 was based on a critical review of published studies so that miRNAs without prior documented implication in PCa were considered for further analysis. Further validation was only accomplished for miR-27a-5p, since very low expression levels of miR-570 and miR-1292 in the clinical samples impaired the amplification reaction.

III.AIMS OF THE STUDY

The main goal of this project is to characterize the role of a novel miRNA in prostate carcinogenesis and to further analyze its possible link to *MYC* signaling and to other epigenetic mechanisms.

Specifically the following tasks were set:

1. Evaluate miR-27a-5p expression and methylation levels in a larger and independent set of clinically localized prostate cancer tissue samples (cohort #1);
2. Assess *MYC* and miR-27a-5p expression and methylation status in a series of castration-resistant prostate cancer tissue samples (cohort #2);
3. Compare the molecular findings with clinical and pathological features of prostate cancer;
4. Characterize *MYC* and miR-27a-5p in prostate cancer cell lines;
5. Confirm the specific binding of *MYC* at miR-27a-5p promoter region in PCa cell lines;
6. Identify miR-27a-5p's targets and assess its relevance in prostate carcinogenesis.

IV.MATERIAL AND METHODS

1. CLINICAL SAMPLES

1.1 Patients and Samples Collection

Primary tumors from 198 patients harboring clinically localized prostate carcinoma (PCa) were prospectively collected after diagnosis and primary treatment with radical prostatectomy, at Portuguese Oncology Institute of Porto (IPO-Porto). In 37 cases, PIN lesions were available and included in this study. A set of 24 castration resistant prostate tumors collected from patient that accomplish transurethral resection of prostate were also included. 10 morphologically normal prostate tissues (MNPT) were collected from prostatic peripheral zone of bladder cancer patients submitted to cystoprostatectomy and used as control samples. Histological slides from formalin-fixed paraffin-embedded tissue fragments were obtained from the surgical specimens and assessed for Gleason score and TNM stage. Relevant clinical data was collected from clinical charts. Informed consent was obtained from all participants, according to institutional regulations. This study was approved by the institutional review board [Comissão de Ética para a Saúde-(CES-IPOFG-EPE 205/2013)] of IPO Porto.

1.2 Total RNA Extraction

Total RNA from clinical samples was obtained by suspension in TRIzol® reagent (Invitrogen, USA) and total RNA was purified from the aqueous phase of TRIzol® extract using PureLink™ RNA Mini Kit (Invitrogen, USA) following manufacturer recommendations. RNA concentration, purity and integrity of samples were determined on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) and electrophoresis.

1.3 MicroRNA Expression Assay

Reverse transcription (RT) was performed to a total of 350 ng using TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT human pool B v3.0 (Applied Biosystems®, USA) according to manufacturer's instructions.

Quantitative Real-Time PCR (RT-qPCR) was performed using TaqMan Small RNA Assays for miR-27a-5p (assay ID: 0004501) and TaqMan Universal PCR Master Mix II no

UNG (2x) in a 7500 Real-Time PCR system (Applied Biosystems®, USA), according to the recommended protocol. For each sample, the mean quantity of each miRNA was normalized to the mean quantity for the endogenous control RNU48 (assay ID: 001006), according to the following formula: miRNA expression = (target miRNA expression mean quantity)/(RNU48 mean quantity). Results were then multiplied by 1000 for easier tabulation. Each plate included multiple non-template controls and 5 cDNA serial dilutions (10x) obtained from human prostate RNA (Ambion, USA) were used to construct a standard curve for each plate. All experiments were run in triplicate.

1.4 DNA Extraction and Bisulfite Modification

One thousand ng of DNA was extracted from all clinical samples using phenol-chloroform method. The bisulfite modification was accomplished using EZ DNA Methylation-Gold™ Kit (Zymo Research, USA), that integrates DNA denaturation and bisulfite conversion processes into one-step, according to the recommended protocol.

1.5 Quantitative Methylation-specific PCR

Quantitative Methylation-specific PCR (qMSP) assay was performed using AmpliTaq Gold® DNA Polymerase, in a 7500 Real-Time PCR system (Applied Biosystems®, USA), according to the recommended protocol. Sequence-specific primers and a TaqMan probe used in this study were synthesized by Sigma-Aldrich (USA) (Table 4). In each sample, the mean quantity of miRNA-27a-5p DNA methylation status was normalized to the mean quantity for the endogenous control *β-Actin*, according to the following formula: miRNA DNA methylation status = (miRNA methylation mean quantity)/(*β-Actin* mean quantity). Results were then multiplied by 1000 for easier tabulation. CpGenome™ Universal Methylated DNA (Merck Millipore, Germany) underwent bisulfite conversion was used to construct a standard curve in five serial dilutions (5x factor dilution), in order to allow relative quantification and ascertain PCR efficiency.

Table 4 TaqMan probe and primer sequence, amplicons, locations and annealing temperatures for qMSP.

MiR-27a-5p (Family Name: *MIR27*)	
Forward Sequence	5'TGTATTTTAGTCGTGGCGATA3'
Probe Sequence	5'(6-FAM)AGAGATGGGGTATTGTCGTATTGC(BHQ-1)3'
Reverse Sequence	5'ATAACGACTCACGCCTATAATC3'
Amplicon Size (bp)	172
Location (bp upstream TSS)	1222
Annealing Temp (ºC)	60
β-Actin [112]	
Forward Sequence	5'TGGTGATGGAGGAGGTTTAGTAAGT3'
Probe Sequence	5'(6-FAM)ACCACCACCCAACACACAATAACAAACACA(TAMRA)3'
Reverse Sequence	5'ACCAATAAAACCTACTCCTCCCTTAA3'
Amplicon Size (bp)	132
Location (bp upstream TSS)	1599
Annealing Temp (ºC)	60

2. PROSTATE CANCER CELL LINES STUDIES

Three prostate cancer cell lines were selected for subsequent studies to explore the importance of epigenetic mechanisms on miR-27a-5p's regulation. The selected cell lines were PNT2, a normal prostatic epithelium cell line (Sigma-Aldrich, USA), LNCaP and PC3 (ATCC – American Type Culture Collection, USA). Regarding metastatic cell lines, LNCaP is hormone-sensitive, while PC3 is castration-resistant. PNT2 and LNCaP cells were grown in RPMI 1640 and PC3 cells were grown in 50% RPMI-50% F-12 medium (GIBCO, Invitrogen, USA). All culture media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO, Invitrogen, USA). Cells were maintained in an incubator at 37ºC with 5% CO_2. All PCa cell lines were routinely tested for *Mycoplasma* spp. contamination (PCR Mycoplasma Detection Set, Clontech Laboratories). To harvest the cells for subculture, TrypLE™ Express (GIBCO, Invitrogen, USA) dissociation reagent was used.

2.1 Cell Lines Treatment with Epigenetic-modulating Drugs

To reverse DNA methylation effect in the cell lines, we used 1 μM of the DNA methyltransferases inhibitor 5-aza-2-deoxycytidine (5-Aza-CdR; Sigma-Aldrich, USA).

The cell lines were grown until 20 to 30% of confluence was reached in T25cm^3 cell culture flasks and, then, medium containing the drug was added. On day 4, the cells were harvested by trypsinization and centrifuged. All the treatments were done in triplicate. Pellets were washed in PBS and stored for DNA and RNA extraction at -80ºC.

2.2 Total RNA and DNA Extraction from Cell Lines

Cell culture flasks (25 cm^3) with PCa cell lines were harvested with a dissociation reagent, TrypLE™ Express (GIBCO, Invitrogen, USA) and centrifuged for 5 minutes at 1,200 rpm. Cell pellets were ressuspended in 1 mL of PBS (GIBCO, Invitrogen, USA), divided for 2 eppendorfs (for RNA and DNA extraction) and centrifuged for another 5 minutes at 1,200 rpm. The supernatant was discarded and cell pellets were stored at -80ºC.

Total RNA from cell lines was obtained by suspension in TRIzol® reagent (Invitrogen, USA) and total RNA was purified from the aqueous phase of TRIzol® extract using PureLink™ RNA Mini Kit (Invitrogen, USA) following manufacturer recommendations. MicroRNA reverse transcription (RT) and Quantitative Real-Time PCR (RT-qPCR) for miR-27a-5p were performed in cell lines as for clinical samples.

One thousand ng of DNA was extracted from all cell lines using phenol-chloroform method. The bisulfite modification was accomplished using EZ DNA Methylation-Gold™ Kit (Zymo Research, USA), that integrates DNA denaturation and bisulfite conversion processes into one-step, according to the recommended protocol. Quantitative Methylation-specific PCR (qMSP) assay for miR-27a-5p was performed in cell lines as for clinical samples.

2.3 *MYC* Expression

For each sample, first strand synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®, USA). *MYC* Expression levels were quantified by RT-qPCR using TaqMan Universal PCR Master Mix and *MYC* TaqMan Gene Expression Assay (Hs00153408_m1) (Applied Biosystems®, USA) and *GUSβ* (Hs99999908_m1) was used as a reference gene for normalization, according to the

formula: relative expression = (target gene mean quantity/reference gene mean quantity). Ratios were then multiplied by 1000 for easier tabulation. Each plate included multiple non-template controls and cDNA serial dilutions (10x) obtained from human prostate RNA (Ambion, USA) were used to construct a standard curve for each plate. All experiments were run in triplicate.

2.4 *In Silico* Analysis

In silico analysis was performed to calculate the probability of c-Myc to bind to the promoter of gene where validated miRNA are inserted, based on a recently reported c-Myc binding sequence and its respective binding matrix [113]. MicroRNA promoter sequence were obtained from *Genome Browser* database (MIR27A: 19p13.12) and the number of transcription factor binding sites was retrieved with the help of *ConSite* web-based tool, after the alignment between miRNA promoter sequence and c-Myc binding sequence. Additionally, RNA22 tool [114] was used to predict miRNA target sites in the mRNA sequence of EGFR, Akt1 and mTOR.

2.5 Chromatin Immunoprecipitation for Transcription Factors

Cells were crosslinked with formaldehyde (37%) and chromatin was immunoprecipitated using the iDeal Chip-seq Kit for Transcription Factors (Diagenode, Belgium), according to the recommended protocol. Rabbit monoclonal antibody specific for c-Myc (Abcam®, United Kingdom) was used to immunoprecipitate chromatin fragments and rabbit IgG antibody was used as negative control. Real-time PCR was performed using 7500 Real-Time PCR system (Applied Biosystems®, USA) with NZYSpeedy qPCR Green Master Mix (NZYTech, Portugal). Sequences of primers used to amplify ChIP samples were: primer forward 5'TGCTTGGCCTGAAATTCTTAG3' and primer reverse 5'ACCAGGGCAAGATACAGGA3'. To analyze the results the percentage input method was used.

2.6 MicroRNA Transfection

A previous *MYC* gene silencing in PC3 cell line was achieved through the use of particles carrying the pGIPZ lentiviral vector containing a shRNA sequence targeting *MYC* (Thermo Scientific, USA) that functions as a small interfering RNA (siRNA). As a negative

control, one scrambled siRNA (sh-scramble RNA) sequence was used, with the same nucleotide composition as the three shRNAs randomly ordered. MicroRNA-27a-5p was transiently transfected in PC3 sh-scramble and PC3 sh-MYC with Pre-miR^{TM} miRNA Precursor (has-miR-27a-5p, AM17100, Ambion, USA). A miRNA negative control was used as control in all experiments (miR-NC, AM17010, Ambion, USA). Cells were seeded under standard conditions in six-well and 96-well plates for 24 h before transfection, reaching 30% to 50% confluence. In these experiments, pre-miR-27a-5p and miR-NC concentration was 50nM. Oligofectamine™ reagent (Invitrogen, USA) was used under conditions indicated by the manufacturer. Cells were then incubated at 37°C and 5% CO_2 in a humidified chamber for 72 h upon transfection. At 72 h, forced expression of miR-27a-5p were confirmed by RT-qPCR.

2.7 Cell Viability Assay

To evaluate the impact of *in vitro* transfection of miR-27a-5p in PC3, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma-Aldrich, USA) assay was performed in 96-well plates. Briefly, cells were incubated with 10% MTT at 5 mg/mL in a humidified chamber for 24, 48, and 72 h after transfection. Reaction was stopped by removal of MTT and addition of 100 µL DMSO (Sigma-Aldrich, USA) per well. Absorbance levels were measured using a microplate reader (Fluostar Omega, BMG Labtech) at 540 nm with background deduction at 630 nm. Number of viable cells was obtained using the following formula: (OD experiment × Mean number of cells at 0 h)/Mean OD at 0 h. Three biologically independent experiments were performed, comprising methodological triplicates for each experiment.

2.8 Apoptosis Assay

Apoptosis was assessed using the APOPercentage™ kit (Biocolor Ltd., UK). Cell lines were seeded under the same conditions as described for MTT assay and, after 72 h incubation, apoptosis assay was performed according to the manufacturer's instructions. Quantification of apoptosis was achieved by measuring the optical density of the released dye at 550 nm with background deduction at 620 nm using a FLUOstar Omega microplate reader. To normalize the OD obtained for the apoptosis assays relatively to cell number, OD of cell viability assay at 72 h was used. Results were expressed as ratio of transfected cells OD to miR-NC OD (set as 100%).

2.9 Protein Extraction and Quantification

Nuclear protein was extracted from PC3 cells using performed using Nuclear Extract Kit (Active Motif) and total protein was extracted from LNCaP cell lysates using the radioimmuno precipitation assay (RIPA) (Santa Cruz Biotechnology Inc., USA) and subsequently quantified using a Pierce BCA Protein Assay Kit (Applied Biosystems, USA), according to manufacturer instructions.

2.10 Western Blot

Briefly, 30 μg of protein from each sample were separated using 10% sodium dodecyl sulfate polyacrylamide gel, for further electrophoresis (SDS-PAGE) at 120 V and subsequently blotted onto 0.2 μm nitrocellulose membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). After that, membranes were blocked with a 5% non fat dry milk solution in TBS-T and then incubated with antibody. To ascertain equal loading of protein, the membranes were incubated with an endogenous control antibody. All antibodies used are listed in Table 5. Protein band intensities were determined using ImageJ (Wayne Rasband software from National Institute of Health), by comparing the protein band intensity with the loading control (LMNB1 in nuclear protein extract and β-Actin in total protein extract).

Table 5 All antibodies used in Western blot.

Antibodies	Vendor	Catalog number
Anti-c-Myc antibody	Abcam, Cambridge, MA, USA	ab32072
Anti-LMNB1 antibody	Cell Signaling Technology, Danvers, MA, USA	12586S
Anti-EGFR antibody	Kinexus Inc., Vancouver, Canada	AB-NK052-4
Anti-EGFR phospholylated Y1172 antibody	ProSci Inc., Poway, CA, USA	XBP-4085
Anti-Akt1antibody	Santa Cruz Biotechnology, Santa Cruz, CA, USA	sc-5298
Anti-Akt1 phosphorylated S473 antibody	EMD Millipore, Temecula, CA, USA	05-1003
Anti-mTOR antibody	Cell Signaling Technology, Danvers, MA, USA	2972
Anti-β-Actin antibody	Sigma-Aldrich, CO., St Louis, MO, USA	A5316

2.11 Statistical Analysis

Unless otherwise stated, experiments were performed in triplicates. The Shapiro-Wilk's W test allowed for the examination of the appropriateness of a normal distribution assumption for each of the parameters (data not shown). Comparisons between two groups were then performed using non-parametric Mann–Whitney U-test. *P*-values were considered statistically significant if lower than 0.05. Correlation between miRNAs' expression and methylation were measured by the Spearman correlation coefficient (r) test. Data are presented as median ± interquartile range for tissue analysis and mean ± SD for cell line analysis. Significance is shown versus the respective control and depicted as follows: *$P < 0$ .05, **$P < 0.01$, ***$P < 0.001$ and ns – non-significant.

Statistical analysis was performed using SPSS 20.0 for Mac (IBM-SPSS Inc., Chicago, IL, USA) and graphs were built using GraphPad Prism 5.0 software for Mac (GraphPad Software Inc., La Jolla, CA, USA).

V.RESULTS

1. PROSTATE CANCER TISSUE SAMPLES

1.1 MicroRNA-27a-5p Status in Clinically Localized Prostate Cancer (Cohort #1)

Contrarily to our expectations, in the validation series (cohort #1), microRNA-27a-5p expression levels were significantly downregulated in PCa ($P < 0.001$) and PIN lesions ($P < 0.01$), compared with MNPT (Table 6, Figure 8A). In an attempt to explain the previous result and since a CpG island was found at miR-27a-5p promoter region, promoter methylation status was assessed in the same cohort #1. PCa samples depicted significant higher methylation levels than PIN lesions ($P < 0.001$) and MNPT ($P < 0.001$) (Table 6, Figure 8B). Moreover, there was a significant inverse correlation between miR-27a-5p promoter's methylation and expression levels in PCa (Spearman's rho = -0.263; $P < 0.05$). Nonetheless, *MYC*-overexpressing PCa correlated positively with miR-27a-5p expression levels (Spearman's rho = 0.333; $P < 0.05$) in cases without miRNA promoter methylation (18/198 cases) (Supplementary Figure 2).

Table 6 miR-27a-5p expression and promoter methylation levels in cohort #1.

Cohort #1	MNPT	PIN	PCa
miR-27a-5p expression Median (IQR)	11.34 (7.18 – 17.00)	2.88 (1.42 – 5.18)	1.68 (0.80 – 3.18)
miR-27a-5p methylation Median (IQR)	868.86 (741.32 – 960.97)	960.29 (949.32 – 971.81)	1136.28 (926.00 – 1448.47)

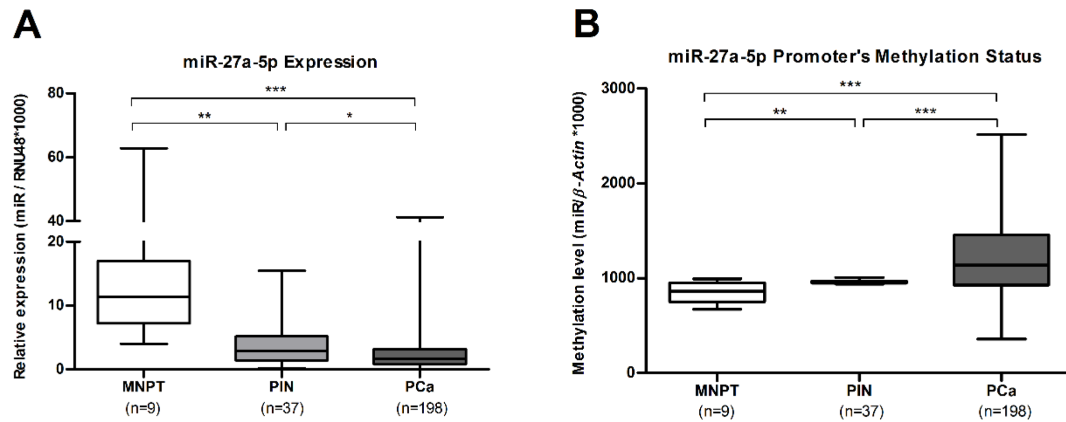


Figure 8 Expression levels of miRNA-27a-5p **(A)** and methylation levels of miR-27a-5p promoter **(B)** in cohort #1.

1.2 Interplay between *MYC* and miR-27a-5p in Castration-Resistant Prostate Cancer (Cohort #2)

MYC and miR-27a-5p status were also assessed in a second cohort of FFPE tissues consisting of castration-resistant PCa (CRPC). Clinicopathological data from cohort #2 are depicted in Table 8. No statistically significant difference between PCa and MNPT samples were found for age. A significant increase in both *MYC* ($P < 0.01$) (Table 7, Figure 9A) and miR-27a-5p ($P < 0.001$) (Table 7, Figure 9B) expression levels was found in CRPC compared to MNPT. Contrarily, miR-27a-5p promoter methylation levels in CRPC were significantly lower than those found in MNPT ($P < 0.001$) (Table 7, Figure 9C). Moreover, *MYC*-overexpressing CRPC correlated positively with miR-27a-5p expression levels (Spearman's rho = 0.274; $P < 0.05$), whereas a significant inverse correlation between miR-27a-5p promoter's methylation and expression levels in CRPC was disclosed (Spearman's rho = -0.434; $P < 0.05$).

Table 7 MYC and miR-27a-5p status in cohort #2.

Cohort #2	MNPT	CRPC
***MYC* expression** **Median (IQR)**	18.33 (14.09 – 34.89)	83.25 (28.49 – 160.52)
miR-27a-5p expression **Median (IQR)**	10.68 (7.10 – 13.16)	645.87 (293.75 – 847.82)
miR-27a-5p methylation **Median (IQR)**	852.35 (806.93 – 889.56)	495.46 (413.78 – 573.54)

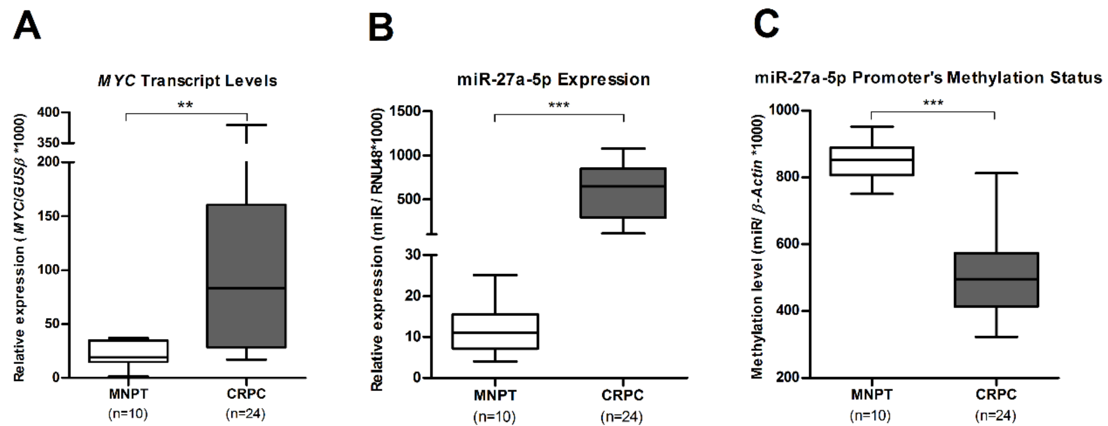


Figure 9 *MYC* transcript levels**(A)**, expression levels of miRNA-27a-5p **(B)** and methylation levels of miR-27a-5p promoter **(C)** in cohort #2.

Table 8 Clinical and pathological data of cohort #2 included in the study.

Clinicopathological Data Cohort #2	CRPC n = 24	MNPT n = 10
Age (years), Median (range)	66 (55 – 82)	58 (45 - 79)
PSA (ng/mL), Median (range)	60.88 (1.20 – 360)	n.a.
Gleason Score, N (%)		
<7	5 (20.8 %)	n.a.
=7	11 (45.8 %)	n.a.
>7	8 (33.3 %)	n.a.

1.3 Association of miR-27a-5p with Clinicopathological Features

No significant association was found between miR-27a-5p expression as well as promoter’s methylation levels and any of the clinicopathologic parameters both in PCa (cohort #1) and CRPC (cohort #2).

2. *IN VITRO* STUDIES: PCa CELL LINES

2.1 *MYC* Characterization

PCa cell lines, LNCaP and PC3, displayed higher *MYC* expression levels in comparison with PNT2 ($P < 0.001$). In PC3 cells, *MYC* expression levels were 15 times higher than in PNT2 cells (Figure 10). We also evaluated the number of copies of chromosome 8q24, in which *MYC* is mapped, and both PC3 and LNCaP displayed copy number gains (Table 9). Moreover, an association between copy number gain and *MYC* expression levels was disclosed.

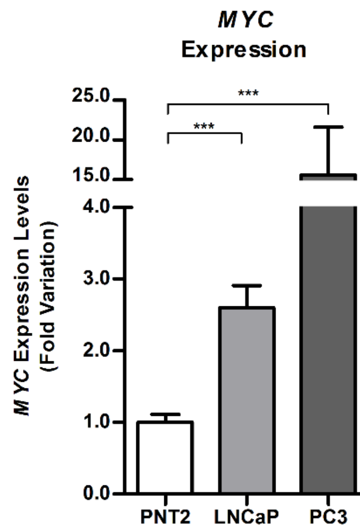


Figure 10 Expression of *MYC* in PCa cell lines. Results are displayed after normalization to PNT2.

Table 9 8q24 copy number in PCa cell lines.

8q24 amplification	
Cell Line	**Number of copies**
LNCaP	4
PC3	6

2.2 MicroRNA-27a-5p Status: Expression *vs.* Methylation

Significant differences were found for miR-27a-5p expression and promoter methylation levels in LNCaP and PC3 cells compared to PNT2 (Figure 11A and B). Indeed, LNCaP cells showed lower miRNA transcript levels (P < 0.001) and higher miR-27a-5p promoter methylation levels (p=0.004), whereas in PC3 cell line, higher miR-27a-5p expression levels (P < 0.001) and lower methylation levels (P = 0.019) were apparent.

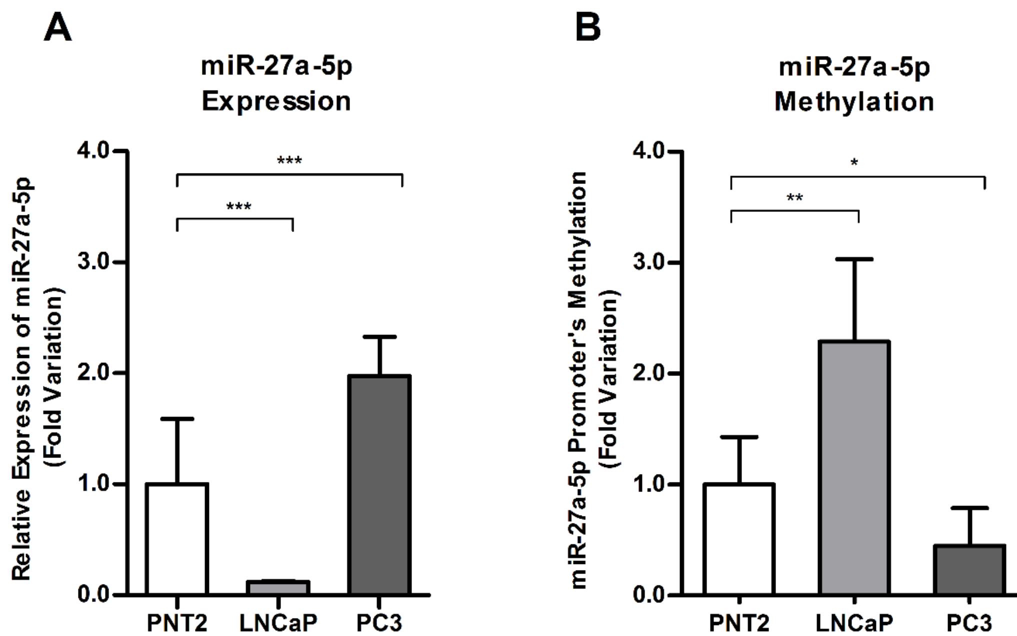


Figure 11 Expression **(A)** and methylation **(B)** of miR-27a-5p in PCa cell lines. Results are displayed after normalization to PNT2.

2.3 Impact of 5-aza-2'deoxycytidine (5-Aza-CdR) Treatment

To confirm whether miR-27a-5p expression was regulated by promoter methylation, PCa cell lines were exposed to 5-aza-2'deoxycytidine (5-Aza-CdR) (Figure 12), and a statistically significant reduction of miR-27a-5p promoter methylation levels was observed in both PCa cell lines (P < 0.05). However, significantly higher miR-27a-5p re-expression (50% increase) was only observed in LNCaP cells (P < 0.001).

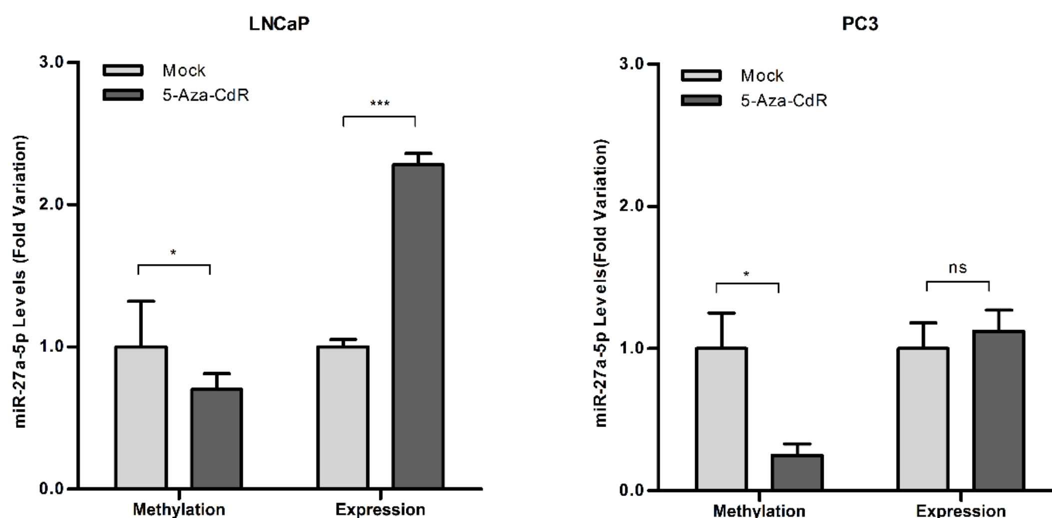


Figure 12 Methylation and expression levels of miR-27a-5p in LNCaP **(A)** and in PC3 **(B)** upon 5-Aza-CdR treatment. Results are displayed after normalization to mock.

2.4 MicroRNA-27a-5p Regulation by *MYC* Signaling

In silico analysis for putative c-Myc binding sites within miR-27a-5p promoter region was performed. c-Myc is known to bind to the canonical E-box sequence CACGTG [108] and we identified one putative binding site matching these sequence in miR-27a-5p promoter region. c-Myc regulation of miR-27a-5p expression was assessed in LNCaP cells (hypermethylated at miR-27a-5p's promoter) and PC3 (hypomethylated at miR-27a-5p's promoter) by ChIP (Chromatin immunoprecipitation) and the results are depicted in Figure 13. A significant increase ($P < 0.001$) of c-Myc binding at miR-27a-5p promoter region was apparent in LNCaP 5-Aza-CdR treated cells, whereas low c-Myc amount was found in mock cells. Conversely, in PC3, c-Myc enrichment was found at miR-27a-5p promoter both in mock and 5-Aza-CdR exposed PC3 cells. Therefore, our data suggest that miR-27a-5p is regulated by c-Myc depending on the methylation status of its promoter.

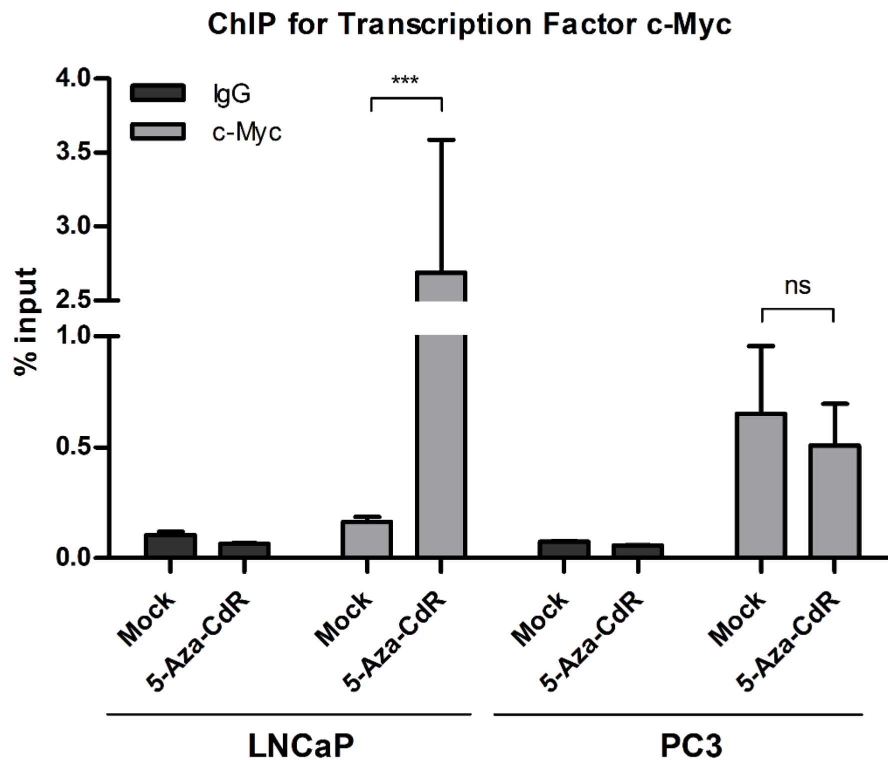


Figure 13 Real-time PCR analysis for c-Myc chromatin immunoprecipitated of miR-27a-5p promoter region in LNCaP and PC3 cell lines.

2.5 Phenotypic Impact of *MYC* Silencing and miR-27a-5p Forced Expression in PC3 Cell Line

Effective *MYC* silencing was achieved in PC3 cells, confirmed at mRNA (87%, $p < 0.001$) and protein level (50%, $P < 0.05$) (Figures 14A and 14B, respectively). Furthermore, *MYC* knockdown attenuated the malignant phenotype with a statistically significant reduction of cell viability, more evident at 48 hours (54%, $P < 0.001$), and an increase of apoptosis, at 72 hours (about 3 times, $P < 0.001$) (Figure 14E).

After *MYC* silencing we found a significant reduction in miR-27a-5p transcript levels (80%, $P < 0.01$) (Figure 14C). Mir-27a-5p mimics was transfected in sh-scramble and sh-MYC PC3 cell line and transfection efficiencies were confirmed by RT-qPCR (Figure 14D). Forced miR-27a-5p expression in sh-scramble PC-3 cells caused an inhibitory effect on cell viability more evident at 72 hours (32%; $P = 0.003$) and increased apoptosis (1.92 times; $P < 0.001$) also at 72 hours' post-transfection (Figure 14F). In sh-MYC PC-3 cells transfected with miR-27a-5p, a significant reduction in number of viable cells was found, particularly at

72 hours (40%; $P < 0.001$) and a 2.94-fold increase in apoptosis ($P < 0.001$) was also apparent at the same time point (Figure 14G).

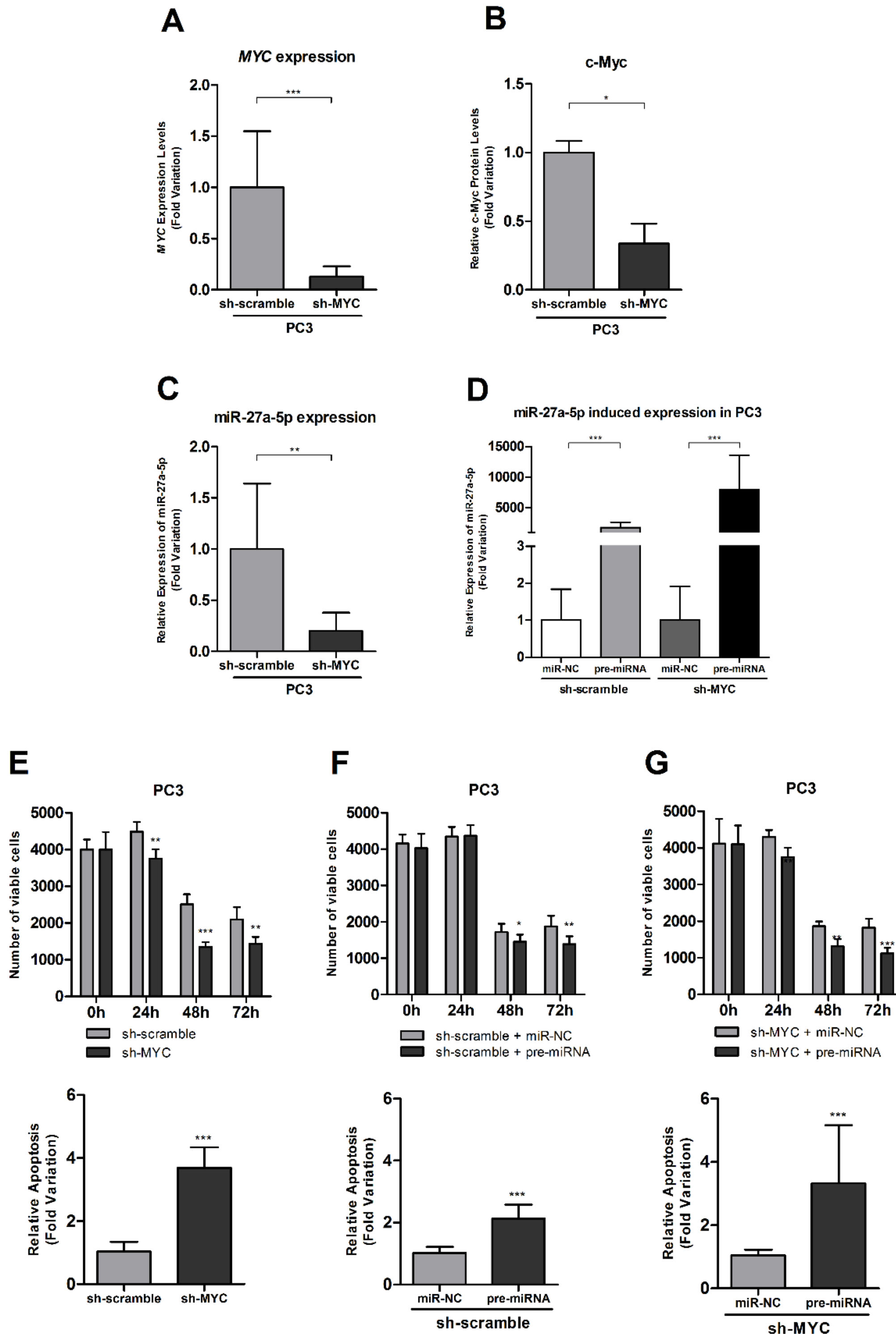


Figure 14 *MYC* expression **(A)**, c-Myc protein levels **(B)** and miR-27a-5p expression **(C)** after *MYC* knockdown in PC3 cell lines; Assessment of pre-miR-27a-5p transfection efficiency in PC3 **(D)**; Phenotypic impact (viability and apoptosis) of *MYC* knockdown **(E)**, pre-miR transfection **(F)** and both **(G)** in PC3 cell line.

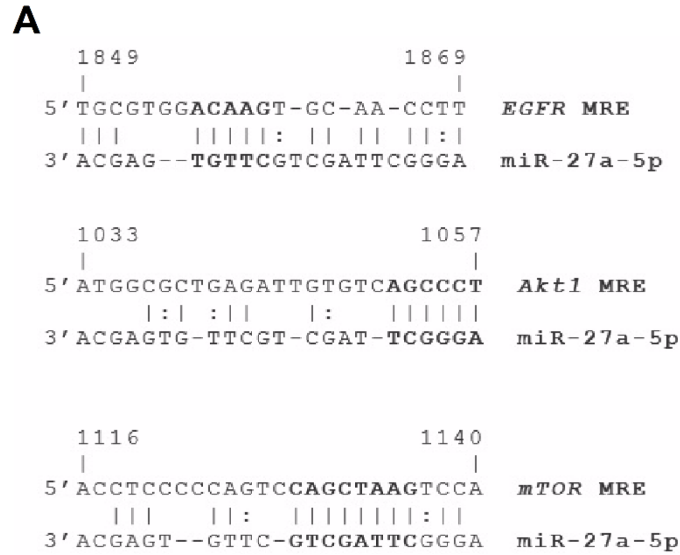
2.6 Putative miR-27a-5p Targets

In search for genes regulated by miR-27a-5p, we evaluated putative targets associated with cellular processes and pathways relevant in PCa. The epidermal growth factor receptor (EGFR) signaling pathway has been previously reported as a target of miR-27a-5p in head and neck squamous carcinoma cell lines [115] and *in silico* analysis identified one predicted miRNA response element (MRE) at CDS of EGFR. Further analysis for more potential targets of miR-27a-5p led to the identification of Akt1 (Akt serine/threonine kinase 1) and mTOR (mechanistic target of rapamycin) within the EGFR signaling axis (Figure 15A, Table 10), which are also documented as frequently deregulated in PCa [116].

Because increased miR-27a-5p transcript levels were found upon 5-Aza-CdR exposure in LNCaP cells, we looked for altered expression of some miR-27a-5p putative targets, at protein level. Thus, in the EGFR signaling axis, we found decreased expression of EGFR, EGFR phosphorylated at Y1172, Akt1 phosphorylated at S473 and mTOR, in 5-Aza-CdR exposed LNCaP cells, compared to mock (Figures 15B, C and D).

Table 10 miRNA pre-computed static target predictions based on the RNA22 tool [114].

hsa-miR-27a-5p miRNA Response Elements (MRE)				
Target	**cDNA region**	**Left most position of predicted target site**	**Folding energy (Kcal/mol)**	***P*-value**
EGFR	CDS	1849	-12.20	0.00777
AKT1	CDS	1033	-13.90	0.0135
mTOR	CDS	1116	-19.00	0.0381



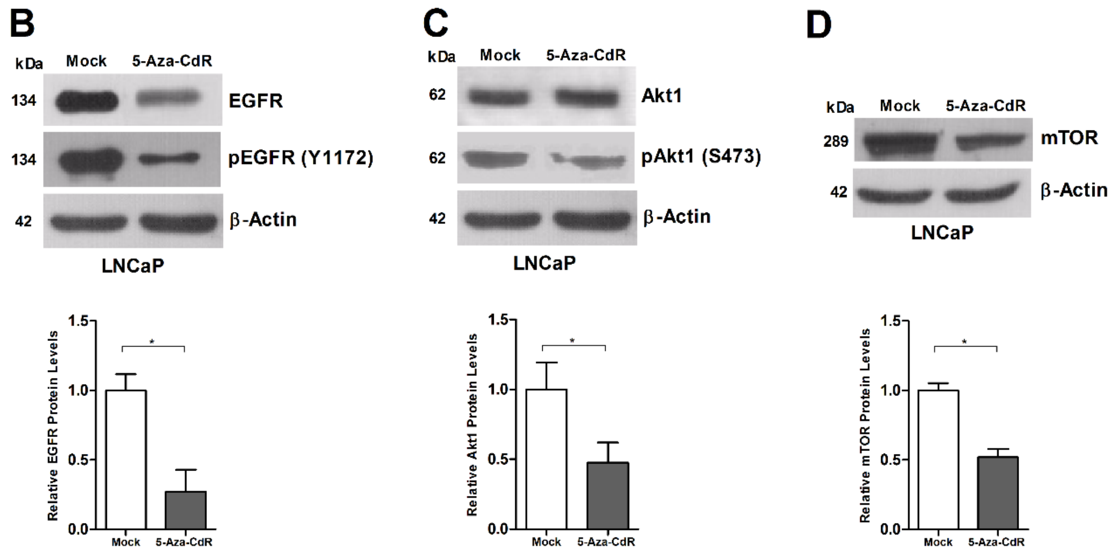


Figure 15 Identification of predicted miRNA response element (MRE) using *in silico* screening methods **(A)**; Protein levels in LNCaP mock and 5-Aza-CdR tretated cell lines by Western Blot analysis **(B)**: EGFR 134 KDa, Akt1 62 KDa, mTOR 289 KDa and β-Actin 42 KDa.

VI.DISCUSSION

Prostate cancer remains one of the major health challenges due to its incidence rate worldwide, lack of accurate biomarkers and scarce information concerning its onset and progression [2, 117, 118]. Thus, a more in-depth understanding of molecular alterations underlying prostate carcinogenesis may help improve current diagnostic and therapeutic approaches. Abnormal activation of the *MYC* oncogene may occur through several distinct mechanisms and it is currently recognized as a major event in many cellular pathways leading to the development of various types of neoplasia, including PCa [119]. Indeed, the c-Myc transcription factor, which may act as a transcription activator or repressor, greatly contributes to neoplastic transformation, by targeting genes with critical functions in cell cycle, differentiation, growth, metabolism, protein synthesis, adhesion, migration, angiogenesis and many others processes [93]. Recently, not only c-Myc was shown to regulate the transcription of several miRNA, but also several miRNAs were suggested to regulate c-Myc expression [120-122]. These data support the existence of a complex regulatory network established between *MYC* and several miRNAs, which tightly controls the expression levels of target genes in a normal cell but, once deregulated, may be critical for cancer development.

Herein, we aimed to identify novel miRNAs implicated in prostate carcinogenesis that might be regulated by c-Myc. In a set of primary PCa, PIN and MNPT samples (cohort #1), we confirmed *MYC* overexpression in PCa, in accordance with its oncogenic role [123, 124]. Additionally, in the same cohort, higher c-Myc protein levels were statistically associated with predictors of more aggressive disease (higher serum PSA and GS), again in line with previous findings [98]. Moreover, in PIN lesions, c-Myc protein levels were higher than in MNPT but lower than in PCa, which is consistent with its precursor lesion status in prostate carcinogenesis [125].

Microarray analysis identified three miRs - miR-27a-5p, miR-570 and miR-1292 - as overexpressed in *MYC* upregulated PCa samples, suggestive of possible targets of c-Myc regulation. However, miRNA candidate validation was only accomplished for miR-27a-5p, because no successful amplification was accomplished for miR-570 and miR-1292, probably due to the very low levels of those two miRNAs in prostatic tissues. This highlighs the importance of microarray validation through different techniques, as microarray and RT-qPCR methodologies have different detection sensitivities [126]. Interestingly, miR-27a-5p is part of the miR-23a-27a-24-2 cluster, previously reported to be frequently overexpressed [127] and targeted by c-Myc regulation in breast cancer [128].

Surprisingly, when miR-27a-5p expression levels were assessed in the large series of clinically localized PCa (cohort #1), for validation purposes, significant downregulation was found in PCa compared to MNPT, suggesting a tumor suppressive role for this miRNA, contrarily to the data from microarray analysis. Because we have found aberrant promoter

methylation to be associated with silencing of several miRNAs in PCa [81, 82, 92], we hypothesized that this epigenetic mechanism might be responsible for miR-27a-5p downregulation. Indeed, significantly higher methylation levels were found in PCa and PIN lesions comparatively with MNPT, and a significant inverse correlation between expression and promoter methylation levels was disclosed. This pattern suggest tumor suppressive functions for miR-27a-5p, in agreement with previous findings in a different cancer model [115]. Interestingly, miR-23a, another member of miR-23a-27a-24-2 cluster, was also found downregulated due to promoter methylation in PCa [129]. Intriguingly, high-*MYC* PCa samples that displayed higher miR-27a-5p expression levels in the array, depicted the lowest methylation levels. Hence, the discrepancy between results of microarray and the validation cohort may be due to the small number of samples used in the array, whose selection was based only in *MYC* transcript levels. Thus, it is possible that we have introduce a significant bias in miRNAs analysis, emphasizing the need to always validate array results in an independent series with a different method.

As previously stated, the oncogenic role of *MYC* in prostate cancer is well documented and its amplification is often associated with the emergence of CRPC phenotype [130, 131]. We confirmed this finding in a cohort CRPC tissues (cohort#2), which displayed *MYC* upregulation, with higher *MYC* transcript levels compared with clinically localized PCa (cohort #1, data not shown). Interestingly, in the CRPC cohort, miR-27a-5p expression and promoter methylation levels followed opposite trends compared with primary PCa (cohort #1). These results suggest that miR-27a-5p regulation might be context dependent (primary PCa *vs.* CRPC), with a predominantly epigenetic regulation in hormone-naïve tumors, whereas other mechanisms prevail in advanced, castration-resistant disease.

Remarkably, the results observed in primary PCa and CRPC tissues were paralleled by those of obtained in PCa cell lines. Although *MYC* upregulation was found both in LNCaP (androgen-sensitive) and PC3 (androgen-insensitive) cells, expression levels were much higher in PC3 cell line. Moreover, miR-27a-5p expression and promoter methylation levels disclosed the same trends found in primary PCa (LNCaP) and CRPC (PC3). Interestingly, although exposure to 5-Aza-CdR decreased promoter methylation levels in both cells lines, only in LNCaP cell significant restored expression was disclosed, reinforcing that in CRPC cells DNA methylation is not the main miR-27a-5p expression regulatory mechanism. *In silico* analysis identified putative c-Myc binding sites at miR-27a-5p promoter region and ChIP assay results strongly suggested that miR-27a-5p's regulation by c-Myc only occurs in the absence of promoter methylation. This is likely due to conformational modifications induced by DNA methylation in chromatin framework, preventing c-Myc binding at miR-27a-5p promoter region [132, 133].

We further verified that *MYC* knockdown in PC3 cells not only mitigated the malignant phenotype, as expected, but it also caused miR-27a-5p downregulation, further supporting miR-27a-5p as a putative target of c-Myc regulation. Interestingly, the phenotypic impact of *MYC* knockdown was mimicked by miR-27a-5p ectopic expression (increased apoptosis and diminished viability) in PC3 cells. Furthermore, restored miR-27a-5p expression in LNCaP cells, upon 5-Aza-CdR exposure, was associated with EGFR/Akt1/mTOR signaling pathway downregulation. EGFR signaling axis is deregulated in various solid tumors, including prostate cancer [134, 135], and during prostate carcinogenesis, *EGFR* is normally upregulated and plays an important role in fostering cancer cell growth [136]. Considering these data, we propose that miR-27a-5p role in PCa depends on the stage of disease. In normal prostate epithelial cells, miR-27a-5p promoter in not hypermethylated and its expression is regulated by c-Myc, constituting a negative feedback loop that counteracts *MYC* signaling, eventually as a similar mechanism to oncogene-induced senescence. Then, at the earliest steps of neoplastic transformation, miR-27a-5p promoter gradually acquires methylation and its expression is silenced. This abolishes the *MYC* feedback loop and stimulates cell proliferation and survival, contributing to the emergence of PIN (which consists of neoplastic cells accumulate in glands, with preserved architecture, due to excessive proliferation and impaired cell death) and, subsequently, of invasive carcinoma. As PCa evolves, locus-specific hypermethylation is accompanied by global expansion of DNA hypomethylation, causing chromosome instability [137], which promotes disease progression and metastatic spread. Thus, in CRPC, the miR-27a-5p promoter becomes hypomethylated, allowing for c-Myc to resume its regulatory role and leading to increased miR-27a-5p expression. However, at this stage, miR-27a-5p increased expression is no longer sufficient to halt PCa progression as cancer cells have acquired many genetic and epigenetic alterations, which concur to the fatal evolution of the disease. Although this is a rather speculative hypothesis (Figure 16), the data presented here fully support it and provides a framework for subsequent research in this field.

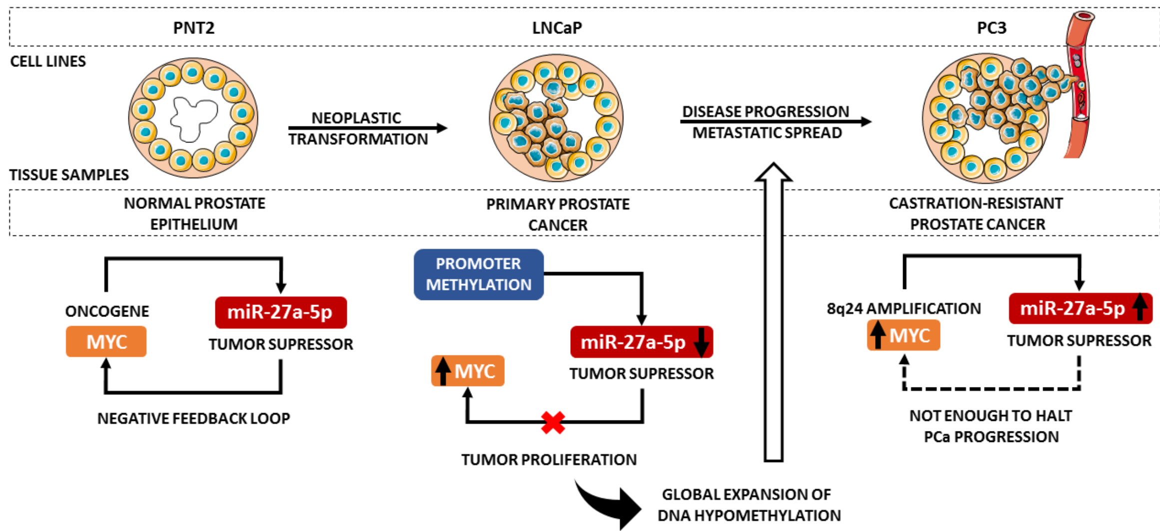


Figure 16 Proposed model for miR-27a-5p regulation in Prostate Cancer.

VII.CONCLUSION

In conclusion, our study provides further insight into miRNAs' deregulation in PCa. Specifically, this is the first study reporting the interplay between two independent mechanisms, aberrant promoter methylation and *MYC* signaling, in the regulation of miR-27a-5p in prostate cancer. Our results further emphasize that the role of miRNA deregulation in neoplastic transformation and progression is highly context-dependent, even in the same cancer model.

VIII. REFERENCES

1. Mottet, N., et al., *EAU–ESTRO–SIOG Guidelines on Prostate Cancer. Part 1: Screening, Diagnosis, and Local Treatment with Curative Intent.* European Urology, 2016.
2. Shen, M.M. and C. Abate-Shen, *Molecular genetics of prostate cancer: new prospects for old challenges.* Genes & development, 2010. **24**(18): p. 1967-2000.
3. Attard, G., et al., *Prostate cancer.* The Lancet, 2016. **387**(10013): p. 70-82.
4. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.* International journal of cancer, 2015. **136**(5): p. E359-E386.
5. Center, M.M., et al., *International variation in prostate cancer incidence and mortality rates.* European urology, 2012. **61**(6): p. 1079-1092.
6. Ferlay, J., et al., *Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012.* European journal of cancer, 2013. **49**(6): p. 1374-1403.
7. Leitzmann, M.F. and S. Rohrmann, *Risk factors for the onset of prostatic cancer: age, location, and behavioral correlates.* Clin Epidemiol, 2012. **4**: p. 1-11.
8. Patel, A.R. and E.A. Klein, *Risk factors for prostate cancer.* Nature Clinical Practice Urology, 2009. **6**(2): p. 87-95.
9. Hussein, S., S. Satturwar, and T. Van der Kwast, *Young-age prostate cancer.* Journal of clinical pathology, 2015: p. jclinpath-2015-202993.
10. McGinley, K.F., K.J. Tay, and J.W. Moul, *Prostate cancer in men of African origin.* Nature Reviews Urology, 2016. **13**(2): p. 99-107.
11. Mottet, N., et al., *Guidelines on prostate cancer. 2014*. 2015, ISBN 978-90-79754-65-6.
12. Heidenreich, A., et al., *EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and local treatment with curative intent—update 2013.* European urology, 2014. **65**(1): p. 124-137.
13. Hoffman, R.M., *Screening for prostate cancer.* New England Journal of Medicine, 2011. **365**(21): p. 2013-2019.
14. Stephan, C., B. Ralla, and K. Jung, *Prostate-specific antigen and other serum and urine markers in prostate cancer.* Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 2014. **1846**(1): p. 99-112.
15. Prensner, J.R., et al., *Beyond PSA: the next generation of prostate cancer biomarkers.* Science translational medicine, 2012. **4**(127): p. 127rv3-127rv3.
16. Stephan, C., K. Jung, and B. Ralla, *Current biomarkers for diagnosing of prostate cancer.* Future Oncology, 2015. **11**(20): p. 2743-2755.
17. Filella, X., et al., *PCA3 in the detection and management of early prostate cancer.* Tumor Biology, 2013. **34**(3): p. 1337-1347.

18. Tomlins, S.A., et al., *Urine TMPRSS2: ERG fusion transcript stratifies prostate cancer risk in men with elevated serum PSA.* Science translational medicine, 2011. **3**(94): p. 94ra72-94ra72.
19. Roobol, M.J. and S.V. Carlsson, *Risk stratification in prostate cancer screening.* Nature Reviews Urology, 2013. **10**(1): p. 38-48.
20. Carver, B.S., et al., *Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer.* Cancer cell, 2011. **19**(5): p. 575-586.
21. Jerónimo, C. and R. Henrique, *Epigenetic biomarkers in urological tumors: A systematic review.* Cancer letters, 2014. **342**(2): p. 264-274.
22. Matoso, A. and J.I. Epstein, *Grading of Prostate Cancer: Past, Present, and Future.* Current urology reports, 2016. **17**(3): p. 1-6.
23. Epstein, J.I., et al., *A contemporary prostate cancer grading system: a validated alternative to the Gleason score.* European urology, 2016. **69**(3): p. 428-435.
24. Gordetsky, J. and J. Epstein, *Grading of prostatic adenocarcinoma: current state and prognostic implications.* Diagnostic pathology, 2016. **11**(1): p. 1.
25. Cheng, L., et al., *Staging of prostate cancer.* Histopathology, 2012. **60**(1): p. 87-117.
26. Challacombe, B.J. and G. Rottenberg, *The Staging of Prostate Cancer*, in *Prostate Cancer: A Comprehensive Perspective*. 2013, Springer. p. 525-536.
27. Spahn, M., et al., *What is the need for prostatic biomarkers in prostate cancer management?* Current urology reports, 2015. **16**(10): p. 1-7.
28. Cornford, P., et al., *EAU-ESTRO-SIOG Guidelines on Prostate Cancer. Part II: Treatment of Relapsing, Metastatic, and Castration-Resistant Prostate Cancer.* European Urology, 2016.
29. Hammerer, P. and S. Madersbacher, *Landmarks in hormonal therapy for prostate cancer.* BJU international, 2012. **110**(s1): p. 23-29.
30. Oudard, S., *Progress in emerging therapies for advanced prostate cancer.* Cancer treatment reviews, 2013. **39**(3): p. 275-289.
31. Kanwal, R. and S. Gupta, *Epigenetic modifications in cancer.* Clinical genetics, 2012. **81**(4): p. 303-311.
32. Sharma, S., T.K. Kelly, and P.A. Jones, *Epigenetics in cancer.* Carcinogenesis, 2010. **31**(1): p. 27-36.
33. Yegnasubramanian, S., *Prostate cancer epigenetics and its clinical implications.* Asian journal of andrology, 2016.
34. Valdes-Mora, F. and S. Clark, *Prostate cancer epigenetic biomarkers: next-generation technologies.* Oncogene, 2015. **34**(13): p. 1609-1618.

35. Massie, C.E., I.G. Mills, and A.G. Lynch, *The Importance of DNA methylation in Prostate Cancer development.* The Journal of steroid biochemistry and molecular biology, 2016.
36. Gurioli, G., et al., *Methylation pattern analysis in prostate cancer tissue: identification of biomarkers using an MS-MLPA approach.* Journal of Translational Medicine, 2016. **14**(1): p. 249.
37. Yong, W.-S., F.-M. Hsu, and P.-Y. Chen, *Profiling genome-wide DNA methylation.* Epigenetics & Chromatin, 2016. **9**(1): p. 26.
38. Weksberg, R., et al., *Epigenetics: updated for 6th.* Emery and Rimoin's Principles and Practice of Medical Genetics, 2013. **Chapter 6**: p. 1-31.
39. Virani, S., et al., *Cancer epigenetics: a brief review.* ILAR Journal, 2012. **53**(3-4): p. 359-369.
40. Portela, A. and M. Esteller, *Epigenetic modifications and human disease.* Nature biotechnology, 2010. **28**(10): p. 1057-1068.
41. Liu, M., et al., *Understanding the epigenetic regulation of tumours and their microenvironments: opportunities and problems for epigenetic therapy.* The Journal of Pathology, 2016.
42. Ngollo, M., et al., *Epigenetic modifications in prostate cancer.* Epigenomics, 2014. **6**(4): p. 415-426.
43. Waldmann, T. and R. Schneider, *Targeting histone modifications—Epigenetics in cancer.* Current opinion in cell biology, 2013. **25**(2): p. 184-189.
44. Füllgrabe, J., E. Kavanagh, and B. Joseph, *Histone onco-modifications.* Oncogene, 2011. **30**(31): p. 3391-3403.
45. Bannister, A.J. and T. Kouzarides, *Regulation of chromatin by histone modifications.* Cell research, 2011. **21**(3): p. 381-395.
46. Haberland, M., R.L. Montgomery, and E.N. Olson, *The many roles of histone deacetylases in development and physiology: implications for disease and therapy.* Nature Reviews Genetics, 2009. **10**(1): p. 32-42.
47. Shi, Y., *Histone lysine demethylases: emerging roles in development, physiology and disease.* Nature Reviews Genetics, 2007. **8**(11): p. 829-833.
48. Dryhurst, D. and J. Ausió, *Histone H2A. Z deregulation in prostate cancer. Cause or effect?* Cancer and Metastasis Reviews, 2014. **33**(2-3): p. 429-439.
49. Zink, L.-M. and S.B. Hake, *Histone variants: nuclear function and disease.* Current opinion in genetics & development, 2016. **37**: p. 82-89.
50. Skene, P.J. and S. Henikoff, *Histone variants in pluripotency and disease.* Development, 2013. **140**(12): p. 2513-2524.

51. Filipescu, D., E. Szenker, and G. Almouzni, *Developmental roles of histone H3 variants and their chaperones.* Trends in Genetics, 2013. **29**(11): p. 630-640.
52. Vardabasso, C., et al., *Histone variants: emerging players in cancer biology.* Cellular and molecular life sciences, 2014. **71**(3): p. 379-404.
53. Beermann, J., et al., *Non-coding RNAs in Development and Disease: Background, Mechanisms, and Therapeutic Approaches.* Physiological Reviews, 2016. **96**(4): p. 1297-1325.
54. Lo, P.-K., et al., *Noncoding RNAs in breast cancer.* Briefings in functional genomics, 2016. **15**(3): p. 200-221.
55. Zhang, M. and X. Du, *Noncoding RNAs in gastric cancer: Research progress and prospects.* World Journal of Gastroenterology, 2016. **22**(29): p. 6610.
56. Trang, P., J.B. Weidhaas, and F.J. Slack, *MicroRNAs and Cancer*, in *The Molecular Basis of Human Cancer*. 2017, Springer. p. 277-286.
57. Goto, Y., et al., *Functional significance of aberrantly expressed microRNAs in prostate cancer.* International Journal of Urology, 2015. **22**(3): p. 242-252.
58. Wang, Y.-L., et al., *Role of microRNAs in prostate cancer pathogenesis.* Clinical genitourinary cancer, 2015. **13**(4): p. 261-270.
59. Gill, B.S., J.M. Alex, and S. Kumar, *Missing link between microRNA and prostate cancer.* Tumor Biology, 2016: p. 1-22.
60. Piletič, K. and T. Kunej, *MicroRNA epigenetic signatures in human disease.* Archives of Toxicology, 2016: p. 1-15.
61. Landgraf, P., et al., *A mammalian microRNA expression atlas based on small RNA library sequencing.* Cell, 2007. **129**(7): p. 1401-1414.
62. Kim, V.N., *MicroRNA biogenesis: coordinated cropping and dicing.* Nature reviews Molecular cell biology, 2005. **6**(5): p. 376-385.
63. Ramalho-Carvalho, J., et al., *Deciphering the function of non-coding RNAs in prostate cancer.* Cancer and Metastasis Reviews, 2016. **35**(2): p. 235-262.
64. Liu, X., et al., *Regulation of microRNAs by epigenetics and their interplay involved in cancer.* Journal of Experimental & Clinical Cancer Research, 2013. **32**(1): p. 1.
65. Saito, Y., et al., *Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells.* Cancer cell, 2006. **9**(6): p. 435-443.
66. Kunej, T., et al., *Epigenetic regulation of microRNAs in cancer: an integrated review of literature.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2011. **717**(1): p. 77-84.
67. Fabbri, M., et al., *Epigenetic regulation of miRNAs in cancer*, in *Epigenetic Alterations in Oncogenesis*. 2013, Springer. p. 137-148.

68. Weber, B., et al., *Methylation of human microRNA genes in normal and neoplastic cells.* Cell cycle, 2007. **6**(9): p. 1001-1005.
69. Schwarzenbach, H., et al., *Clinical relevance of circulating cell-free microRNAs in cancer.* Nature reviews Clinical oncology, 2014. **11**(3): p. 145-156.
70. Singh, P.K. and M.J. Campbell, *The interactions of microRNA and epigenetic modifications in prostate cancer.* Cancers, 2013. **5**(3): p. 998-1019.
71. Friedman, J.M., et al., *The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2.* Cancer research, 2009. **69**(6): p. 2623-2629.
72. Majid, S., et al., *miRNA-34b inhibits prostate cancer through demethylation, active chromatin modifications, and AKT pathways.* Clinical Cancer Research, 2013. **19**(1): p. 73-84.
73. Fang, Y. and W. Gao, *Roles of microRNAs during prostatic tumorigenesis and tumor progression.* Oncogene, 2014. **33**(2): p. 135-147.
74. Chan, J.A., A.M. Krichevsky, and K.S. Kosik, *MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells.* Cancer research, 2005. **65**(14): p. 6029-6033.
75. Ventura, A. and T. Jacks, *MicroRNAs and cancer: short RNAs go a long way.* Cell, 2009. **136**(4): p. 586-591.
76. Costa-Pinheiro, P., et al., *MicroRNA-375 plays a dual role in prostate carcinogenesis.* Clinical epigenetics, 2015. **7**(1): p. 1.
77. Jalava, S., et al., *Androgen-regulated miR-32 targets BTG2 and is overexpressed in castration-resistant prostate cancer.* Oncogene, 2012. **31**(41): p. 4460-4471.
78. Sikand, K., et al., *miR 488* inhibits androgen receptor expression in prostate carcinoma cells.* International journal of cancer, 2011. **129**(4): p. 810-819.
79. Lin, S.-L., et al., *Loss of mir-146a function in hormone-refractory prostate cancer.* Rna, 2008. **14**(3): p. 417-424.
80. Jerónimo, C., et al., *Epigenetics in prostate cancer: biologic and clinical relevance.* European urology, 2011. **60**(4): p. 753-766.
81. Ramalho-Carvalho, J., et al., *Downregulation of miR-130b~301b cluster is mediated by aberrant promoter methylation and impairs cellular senescence in prostate cancer.* Journal of Hematology & Oncology, 2017. **10**(1): p. 43.
82. Ramalho-Carvalho, J., et al., *Epigenetic disruption of miR-130a promotes prostate cancer by targeting SEC23B and DEPDC1.* Cancer letters, 2017. **385**: p. 150-159.
83. Iorio, M.V. and C.M. Croce, *MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review.* EMBO molecular medicine, 2012. **4**(3): p. 143-159.

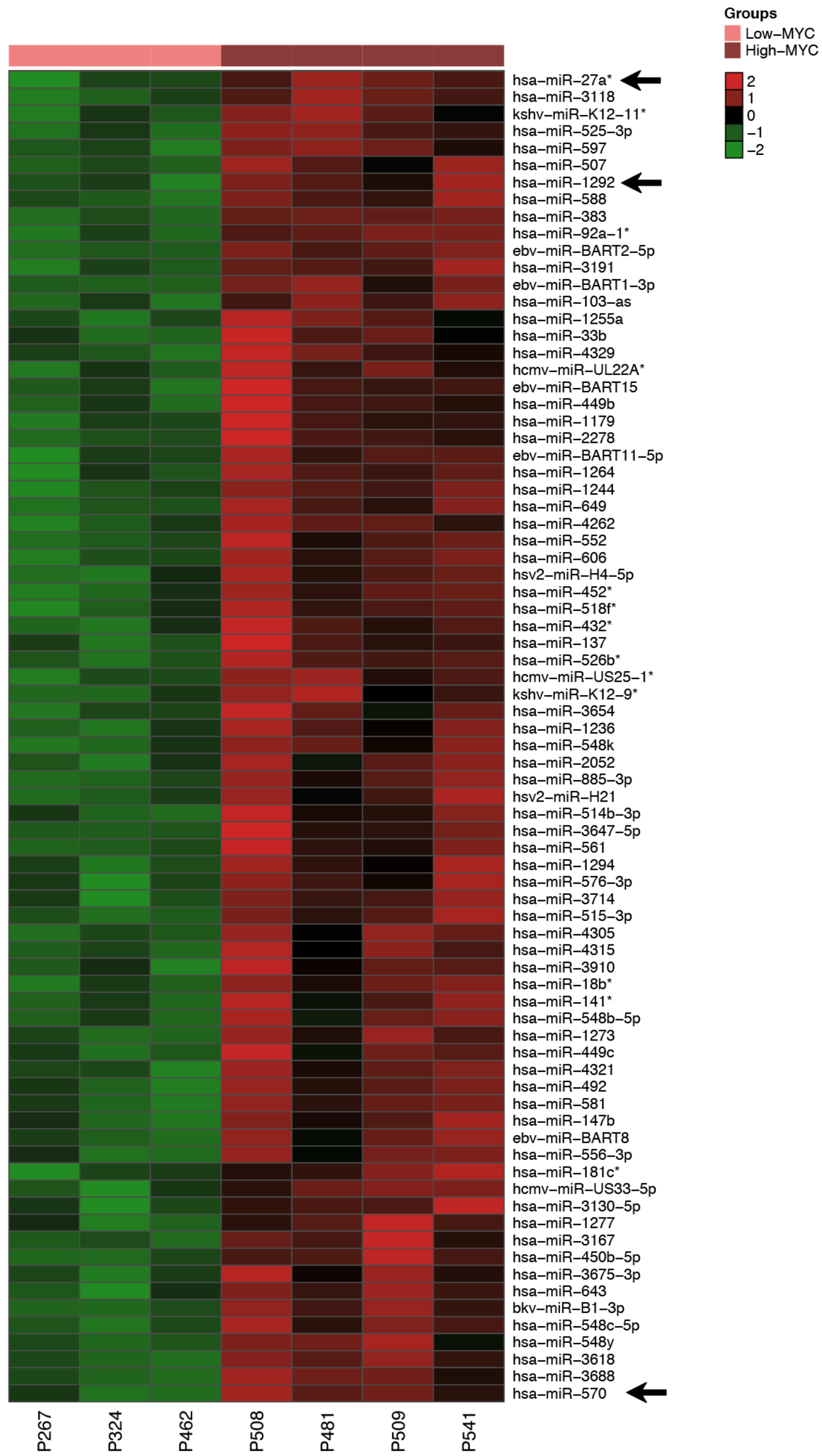
84. Jackson, B.L., A. Grabowska, and H.L. Ratan, *MicroRNA in prostate cancer: functional importance and potential as circulating biomarkers.* BMC cancer, 2014. **14**(1): p. 1.
85. Fabris, L., et al., *The Potential of MicroRNAs as Prostate Cancer Biomarkers.* European urology, 2016.
86. Brase, J.C., et al., *Circulating miRNAs are correlated with tumor progression in prostate cancer.* International journal of cancer, 2011. **128**(3): p. 608-616.
87. Huang, X., et al., *Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer.* European urology, 2015. **67**(1): p. 33-41.
88. Sita-Lumsden, A., et al., *Circulating microRNAs as potential new biomarkers for prostate cancer.* British journal of cancer, 2013. **108**(10): p. 1925-1930.
89. Yang, M. and J. Mattes, *Discovery, biology and therapeutic potential of RNA interference, microRNA and antagomirs.* Pharmacology & therapeutics, 2008. **117**(1): p. 94-104.
90. Ling, H., *Non-coding RNAs: Therapeutic Strategies and Delivery Systems*, in *Non-coding RNAs in Colorectal Cancer*, O. Slaby and A.G. Calin, Editors. 2016, Springer International Publishing: Cham. p. 229-237.
91. Rupaimoole, R. and F.J. Slack, *MicroRNA therapeutics: towards a new era for the management of cancer and other diseases.* Nature Reviews Drug Discovery, 2017.
92. Torres-Ferreira, J., et al., *MiR-193b promoter methylation accurately detects prostate cancer in urine sediments and miR-34b/c or miR-129-2 promoter methylation define subsets of clinically aggressive tumors.* Molecular Cancer, 2017. **16**(1): p. 26.
93. Dang, C.V., *MYC on the path to cancer.* Cell, 2012. **149**(1): p. 22-35.
94. Hsieh, A.L., et al. *MYC and metabolism on the path to cancer*. in *Seminars in cell & developmental biology*. 2015. Elsevier.
95. Dang, C.V., et al. *The c-Myc target gene network*. in *Seminars in cancer biology*. 2006. Elsevier.
96. Meyer, N. and L.Z. Penn, *Reflecting on 25 years with MYC.* Nature Reviews Cancer, 2008. **8**(12): p. 976-990.
97. Vita, M. and M. Henriksson. *The Myc oncoprotein as a therapeutic target for human cancer*. in *Seminars in cancer biology*. 2006. Elsevier.
98. Hawksworth, D., et al., *Overexpression of C-MYC oncogene in prostate cancer predicts biochemical recurrence.* Prostate cancer and prostatic diseases, 2010. **13**(4): p. 311-315.

99. Sato, H., et al., *Fluorescence in situ hybridization analysis of c-myc amplification in stage T3N0M0 prostate cancer in Japanese patients.* International journal of urology, 2006. **13**(6): p. 761-766.
100. Koh, C.M., et al., *MYC and prostate cancer.* Genes & cancer, 2010. **1**(6): p. 617-628.
101. Sachdeva, M., et al., *p53 represses c-Myc through induction of the tumor suppressor miR-145.* Proceedings of the National Academy of Sciences, 2009. **106**(9): p. 3207-3212.
102. Wang, H., et al., *miR-320b suppresses cell proliferation by targeting c-Myc in human colorectal cancer cells.* BMC cancer, 2015. **15**(1): p. 748.
103. Wang, H., et al., *MicoRNA-451 is a novel tumor suppressor via targeting c-myc in head and neck squamous cell carcinomas.* Journal of cancer research and therapeutics, 2015. **11**(6): p. 216.
104. Jansson, M.D. and A.H. Lund, *MicroRNA and cancer.* Molecular oncology, 2012. **6**(6): p. 590-610.
105. Lu, J., et al., *MicroRNA expression profiles classify human cancers.* nature, 2005. **435**(7043): p. 834-838.
106. Cimmino, A., et al., *miR-15 and miR-16 induce apoptosis by targeting BCL2.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(39): p. 13944-13949.
107. Dews, M., et al., *Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster.* Nature genetics, 2006. **38**(9): p. 1060-1065.
108. O'Donnell, K.A., et al., *c-Myc-regulated microRNAs modulate E2F1 expression.* Nature, 2005. **435**(7043): p. 839-843.
109. Chang, T.-C., et al., *Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation.* Proceedings of the National Academy of Sciences, 2009. **106**(9): p. 3384-3389.
110. Kim, H.H., et al., *HuR recruits let-7/RISC to repress c-Myc expression.* Genes & development, 2009. **23**(15): p. 1743-1748.
111. Frenzel, A., J. Lovén, and M.A. Henriksson, *Targeting MYC-regulated miRNAs to combat cancer.* Genes & cancer, 2010. **1**(6): p. 660-667.
112. Eads, C.A., et al., *Epigenetic patterns in the progression of esophageal adenocarcinoma.* Cancer research, 2001. **61**(8): p. 3410-3418.
113. Seitz, V., et al., *Deep sequencing of MYC DNA-binding sites in Burkitt lymphoma.* PloS one, 2011. **6**(11): p. e26837.

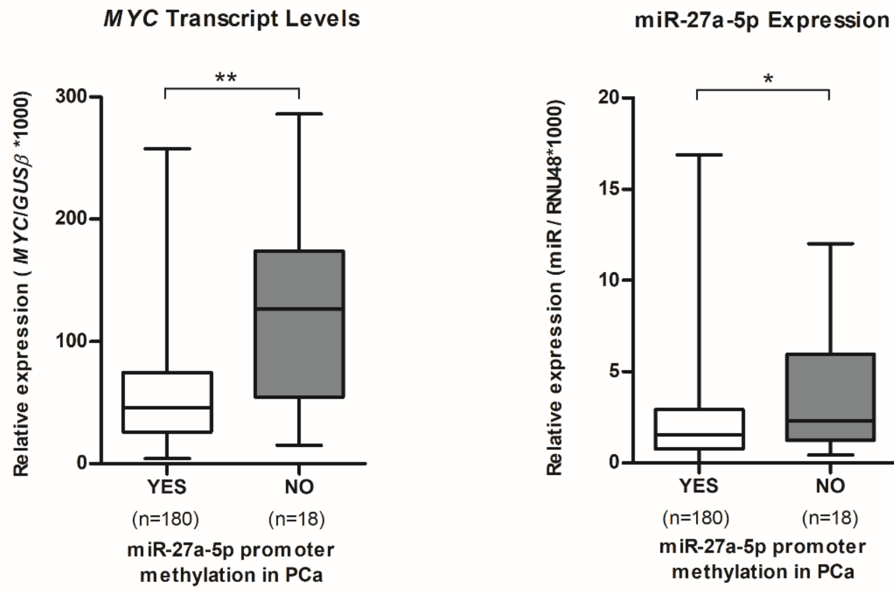
114. Miranda, K.C., et al., *A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes.* Cell, 2006. **126**(6): p. 1203-1217.
115. Wu, X., et al., *Coordinated targeting of the EGFR signaling axis by microRNA-27a*.* Oncotarget, 2013. **4**(9): p. 1388.
116. Bitting, R.L. and A.J. Armstrong, *Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer.* Endocrine-related cancer, 2013. **20**(3): p. R83-R99.
117. Felgueiras, J., J.V. Silva, and M. Fardilha, *Prostate cancer: the need for biomarkers and new therapeutic targets.* Journal of Zhejiang University Science B, 2014. **15**(1): p. 16-42.
118. Barqawi, A.B., K.J. Krughoff, and K. Eid, *Current challenges in prostate cancer management and the rationale behind targeted focal therapy.* Advances in urology, 2012. **2012**.
119. Dang, C.V., *Enigmatic MYC conducts an unfolding systems biology symphony.* Genes & cancer, 2010. **1**(6): p. 526-531.
120. Tao, J., X. Zhao, and J. Tao, *c-MYC–miRNA circuitry: a central regulator of aggressive B-cell malignancies.* Cell Cycle, 2014. **13**(2): p. 191-198.
121. Wang, X., et al., *c-Myc modulates microRNA processing via the transcriptional regulation of Drosha.* Scientific reports, 2013. **3**.
122. Gao, P., et al., *c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism.* Nature, 2009. **458**(7239): p. 762-765.
123. Pomerantz, M.M., et al., *Evaluation of the 8q24 prostate cancer risk locus and MYC expression.* Cancer research, 2009. **69**(13): p. 5568-5574.
124. Fromont, G., et al., *8q24 amplification is associated with Myc expression and prostate cancer progression and is an independent predictor of recurrence after radical prostatectomy.* Human pathology, 2013. **44**(8): p. 1617-1623.
125. Brawer, M.K., *Prostatic intraepithelial neoplasia: an overview.* Reviews in urology, 2005. **7**(Suppl 3): p. S11.
126. Chen, Y., et al., *Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis.* BMC genomics, 2009. **10**(1): p. 407.
127. Mattie, M.D., et al., *Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies.* Molecular cancer, 2006. **5**(1): p. 24.

128. Li, X., et al., *c-MYC-regulated miR-23a/24-2/27a cluster promotes mammary carcinoma cell invasion and hepatic metastasis by targeting Sprouty2.* Journal of Biological Chemistry, 2013. **288**(25): p. 18121-18133.
129. Cai, S., et al., *Downregulation of microRNA-23a suppresses prostate cancer metastasis by targeting the PAK6-LIMK1 signaling pathway.* Oncotarget, 2015. **6**(6): p. 3904.
130. Gao, L., et al., *Androgen receptor promotes ligand-independent prostate cancer progression through c-Myc upregulation.* PloS one, 2013. **8**(5): p. e63563.
131. Vander Griend, D.J., I.V. Litvinov, and J.T. Isaacs, *Conversion of androgen receptor signaling from a growth suppressor in normal prostate epithelial cells to an oncogene in prostate cancer cells involves a gain of function in c-Myc regulation.* Int J Biol Sci, 2014. **10**(6): p. 627-42.
132. Chen, Y., et al., *Integration of genome and chromatin structure with gene expression profiles to predict c-MYC recognition site binding and function.* PLoS computational biology, 2007. **3**(4): p. e63.
133. Fernandez, P.C., et al., *Genomic targets of the human c-Myc protein.* Genes & development, 2003. **17**(9): p. 1115-1129.
134. Dobashi, Y., et al., *EGFR-dependent and independent activation of Akt/mTOR cascade in bone and soft tissue tumors.* Modern Pathology, 2009. **22**(10): p. 1328-1340.
135. Tolcher, A.W., *Novel therapeutic molecular targets for prostate cancer: the mTOR signaling pathway and epidermal growth factor receptor.* The Journal of urology, 2004. **171**(2): p. S41-S44.
136. Di Lorenzo, G., et al., *Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer.* Clinical Cancer Research, 2002. **8**(11): p. 3438-3444.
137. Schulz, W.A., et al., *Genomewide DNA hypomethylation is associated with alterations on chromosome 8 in prostate carcinoma.* Genes, Chromosomes and Cancer, 2002. **35**(1): p. 58-65.

IX.SUPPLEMENTARY DATA



Supplementary Figure 1 Heatmap resulting from the miRNA microarray performed with total RNA extracted from PCa cases with high and low levels of *MYC* transcript. The selected miRNAs candidates were evidenced by the black arrows.



Supplementary Figure 2 *MYC* and miRNA-27a-5p expression levels according with miR-27a-5p promoter methylation in PCa cases.